Chromatin Control of Viral Infection

Edited by

Thomas M. Kristie

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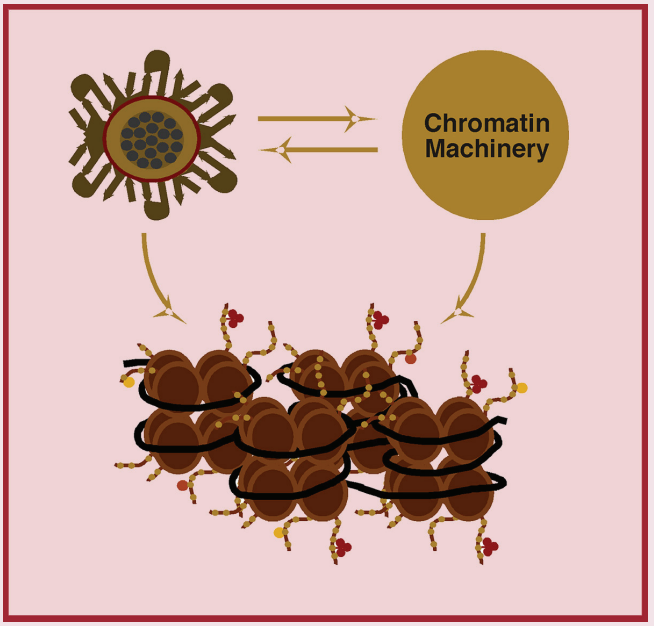

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Thomas M. Kristie (Ed.)

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Preface

It is simplistic to state that the chromatin structure regulates accessibility to the genome. Ultimately that is the case. However, the complexity of the chromatin structure and its regulation by a plethora of protein and RNA complexes maintains the stability of the genome, controls the transcriptional program, modulates DNA replication throughout the cell cycle, and determines the identity or differentiation state of the cell. In addition to the linear organization of the genome into nucleosome arrays, multi-layered chromatin interactions define domains and enhance or suppress long-range coordinate regulatory foci.

The control of cellular processes by chromatin-mediated regulatory paradigms has been intensely studied in recent years. This body of work has served to enhance recognition of the significance of the—chromatin structure and chromatin modulation machinery. It has also served to promote an appreciation for the immense complexity of chromatin regulation, a process which includes numerous families of proteins cooperating as regulatory complexes to direct nucleosome assembly/disassembly, post-transcriptional histone modification, and nucleosome recognition and positioning that ultimately controls functional access to the genome. Added to this complexity is the epigenetics of DNA modification that is often intertwined with nucleosome modulation. The field is rich with new insights into control of cellular processes from basic replication to differentiation and development. Arising from the discoveries of the enzymology of chromatin modulation, are novel translational-therapeutic approaches based on inhibition of histone and DNA modification and/or recognition proteins. This promising new field, while most evident in the oncology arena, has the potential to be applied to a wide range of diseases.

In contrast to the investigation of chromatin regulation in cellular processes, the impacts of chromatin and/or chromatin modulation machinery on viral infection has been less well defined. Early studies focused on small DNA viruses that are packaged as nucleosomal genomes, or those that integrate into host cell chromatin, have recognized the importance of chromatin-based regulation as a determinant of infection. More recently, there has been a breadth of studies on chromatin-mediated control of viral infection, latency, and persistence in many systems. In addition, viruses represent unique models as many encode factors with functions that impact host cell epigenetics. The emerging picture is one of complex dynamics of pathogen and host cell mediated chromatin impacts that can ultimately determine the course of viral infection and/or the modulation of the cell state. The rapid development and use of pharmaceuticals targeting chromatin modulation machinery in oncology strongly suggests that understanding the components and the dynamic interplay of virus and host cell chromatin could also point to new directions in antiviral therapies.

The collection of reviews included here, represents a selection of research at the forefront of chromatin regulation during viral infection. The papers cover a number of viral families and illustrate concepts including: (i) the initial dynamics of virus–host cell chromatin interactions; (ii) host cell

chromatin machinery that serves to enhance or repress viral infection; (iii) viral factors that utilize or circumvent this machinery; and (iv) the significance of chromatin modulation in determining viral latency and persistence.

It is important to recognize that the research of many laboratories have led to a greater understanding and appreciation of the varied "host–viral chromatin landscapes" in different viral families. The papers presented here represent a small subset of those important contributions. I am, however, most grateful to those who have taken the time and effort to provide the reviews that are included in this collection.

Thomas M. Kristie
Guest Editor

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Review

The Dynamics of HCF-1 Modulation of Herpes Simplex Virus Chromatin during Initiation of Infection

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Abstract: Successful infection of herpes simplex virus is dependent upon chromatin modulation by the cellular coactivator host cell factor-1 (HCF-1). This review focuses on the multiple chromatin modulation components associated with HCF-1 and the chromatin-related dynamics mediated by this coactivator that lead to the initiation of herpes simplex virus (HSV) immediate early gene expression.

Keywords: herpes simplex virus; chromatin; HCF-1; Setd1A; latency; histone demethylase

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Abbreviations

HCF-1: host cell factor-1
GABP: GA-binding protein
VZV: varicella zoster virus
PCAF: histone acetyltransferase KAT2B
ISWI: Imitation SWI
Med: mediator
LSD1: lysine-specific demethylase 1
PHF8: PHD finger protein 8
NURF: nucleosome remodeling factor
THAP: thanatos-associated domain-containing apoptosis-associated protein
PGC: peroxisome proliferator-activated receptor gamma coactivator
PRC: peroxisome proliferator-activated receptor gamma coactivator PGC-1-related coactivator
FHL2: four and a half limb domain protein-2
Oct-1: octamer binding protein-1
ASF1: anti-silencing function protein 1
SWI/SNF: switch/sucrose non-fermentable
TBP: tata-binding protein
MLL: histone-lysine N-methyltransferase MLL1
JMJD2: Jumonji domain-containing protein 2
CHD8: chromodomain helicase DNA binding protein 8

1. Introduction

Infection of a cell with herpes simplex virus (HSV) results in a complex and dynamic interplay between host and pathogen on numerous levels. The progression of successful infection leading to the production of progeny virus is dependent upon both circumventing host suppression mechanisms and utilization of host machinery to express viral immediate early (IE) genes and thus establish the initiation of infection.

This review focuses on the viral and cellular factors that govern the expression of IE genes and specifically, the more recently recognized impact of chromatin and chromatin modulation machinery in determining initial events leading to IE gene expression.

2. Viral and Cellular Factors that Govern IE Gene Expression

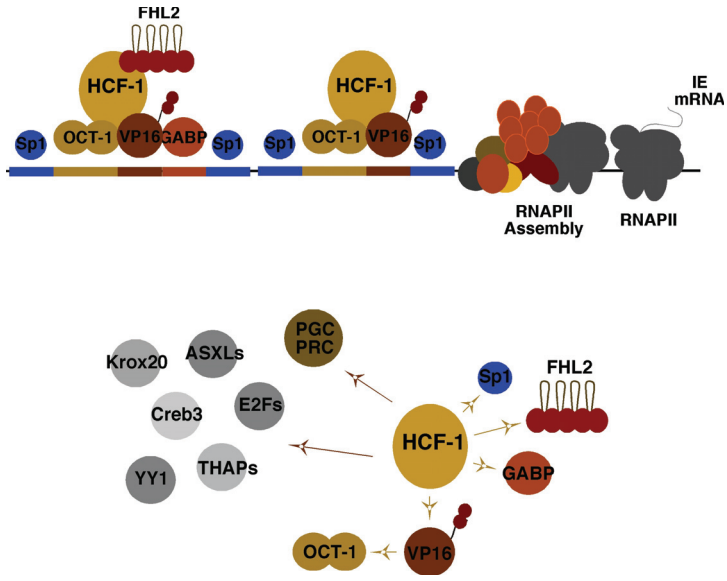
The historical perspective of HSV viral IE gene expression is defined by the identification of IE regulatory elements and a complex set of transcription factors that cooperatively promote the expression of these genes (Figure 1, Top). One of the most critical elements is a reiterated sequence that functions as a classical enhancer module. This enhancer core element (ATGCTAATGARAT) is recognized by the cooperative assembly of the cellular Oct-1 POU domain factor (ATGCTAAT) and the viral encoded IE gene activator VP16 (α-TIF; GARAT) [1,2]. However, stable assembly of the enhancer core complex requires a third component, the cellular HCF-1 protein, which is now functionally recognized as a critical cellular coactivator/corepressor [3–6].

In addition to the enhancer core complex, the regulatory domains of each IE gene contains binding sites for the ETS protein GABP and Kruppel/Sp1 factors that synergistically amplify viral IE gene expression [7,8]. However, while expression of viral IE genes is enhanced by the VP16 enhancer complex, it is not entirely dependent upon this factor or upon the formation of the enhancer core complex. GABP and the coactivator FHL2 are examples of factors that have the potential to (i) stimulate IE reporter gene expression in the absence of VP16 and are (ii) regulated by multiple signaling pathways [9,10]. Thus, it is likely that many, as yet unidentified, activators and repressors regulate viral IE genes in distinct cell types and states.

3. The Essential HCF-1 Coactivator

While initially identified as a cellular component required for the stable assembly of the viral enhancer core complex and a determinant of VP16-mediated IE gene activation, the coactivator HCF-1 is an essential factor for IE gene expression. Depletion of HCF-1 results in abrogation of IE gene expression in contrast to the impacts of depleting other factors such as the enhancer core protein Oct-1 [11,12]. One rationale for this critical dependence is the striking direct interactions of HCF-1 with each of the identified primary transcription factors/coactivators (VP16, Sp1, GABP, FHL2) [5,9,10,13] and the requirement of this coactivator for mediating the activation potential of these components (Figure 1, Bottom).

Figure 1. Elements and factors mediating herpes simplex virus (HSV) immediate early (IE) gene expression. (Top Panel) Viral IE promoter domains contain multiple reiterations of an enhancer core element that assembles the core complex (Oct-1, VP16, HCF-1), as well as sites for factors such as GABP and Sp1. FHL2 functions as a coactivator with HCF-1 for stimulation of IE gene transcription. (Bottom Panel) The essential coactivator HCF-1 interacts directly with transcription factors and coactivators that mediate IE gene expression (yellow arrows). A selection of other cellular factors that have been demonstrated to interact and require HCF-1 are illustrated (brown arrows).



In addition to those that have been demonstrated to regulate viral IE gene expression, the critical nature of HCF-1 in the regulation of cellular programs has been demonstrated by its interaction and regulation of multiple DNA binding factors and coactivators [14–19] including: (i) cell cycle progression via modulation of the activities of the E2F family [20–22]; (ii) embryonic stem cell pluripotency and metabolism via E2F and THAP proteins [23–25]; (iii) metabolism and nuclear respiratory control via GABP and the coactivators PGC and PRC [26,27].

Importantly, the dependence of IE gene expression on HCF-1 suggested that the protein must mediate critical rate-limiting stage(s) in transcription.

4. HCF-1 and Multiple Chromatin Modulation Complexes

Studies that led to the elucidation of the role(s) of HCF-1 in governing chromatin modulation of alpha-herpesvirus (HSV and VZV) IE genes came from two intersecting directions; (i) the demonstration of the impacts of host-cell assembled chromatin on infecting viral genomes and (ii) the association of HCF-1 with multiple chromatin modulation components.

4.1. Chromatin Modulation of HSV Gene Expression

The first important clue to the regulation of HSV by chromatin was the observation that a virus encoding a mutant VP16 that lacked the protein's activation domain exhibited accumulation of nucleosomes on the viral IE gene promoters [28]. This was consistent with a plethora of studies in general transcription biology using the VP16 activation domain that linked the activator to recruitment of basal transcription factors, mediator components (TBP, TFIIB, Med25), and chromatin modulation complexes (SAGA, SWI/SNF) [1,28–34]. However, the impact of VP16 on viral chromatin went largely ignored, due to the lack of substantial evidence that the infecting viral genome was subject to significant chromatin assembly and regulation during the lytic replication cycle.

Subsequently, however, it was clearly demonstrated that the viral genome was rapidly assembled into nucleosomal arrays mimicking host cell chromatin [35–37] (see Schang *et al.*, this issue). In addition, (i) histone chaperones were required for efficient HSV IE gene expression (HIRA) [38] and subsequent DNA replication (ASF1b) [39]; and (ii) nucleosomes bearing marks characteristically associated with active chromatin were detected at viral gene promoters at appropriate times post infection [40–42]. With respect to chromatin remodeling complexes, while the viral activator VP16 can recruit the BAF (SWI/SNF) complex remodelers BRG/BRM, these proteins had no apparent impact on viral IE gene expression [43]. Rather, the ISWI component SNF2H was recruited to viral IE promoters and was important for IE gene expression [44].

Studies to identify acetyltransferases that might regulate viral chromatin concluded that p300, CBP, PCAF and GCN5 did not appear to play a significant role [45]. However, the circadian acetyltransferase CLOCK was shown to localize to sites associated with the infecting virus, was stabilized by infection, and was required for efficient viral gene expression [46,47] (see Roizman *et al.*, this issue). These data suggested that CLOCK might be important for acetylation of nucleosomes associated with the viral genome, although direct histone acetylation remains to be demonstrated.

In addition to the investigation of host cell acetyltransferases that may regulate viral gene expression, the viral IE protein ICP0 has been implicated in promoting histone acetylation and preventing deacetylation, leading to a decreased level of stable nucleosomes on the viral genome [40,48–50]. Thus, while the acetyltransferase complexes that impact viral chromatin remain undefined to date, it is clear that both viral and cellular factors will contribute to the regulation of acetylation levels.

Most importantly, the combined studies clearly pointed to the requirement for various histone/chromatin modulation machinery for efficient viral IE gene expression and progression of infection. With respect to the characteristics of the chromatin associated with the viral genome at the onset of infection, several important studies demonstrated that marks characteristic of repressive chromatin (*i.e.*, histone H3K9-methylation) were prevalent, likely as a result of cellular responses to the invading pathogen [51–53]. As described below, this represents a critical dynamic interplay between host and pathogen, leading to either (i) suppression or (ii) circumvention of repression and progression of infection.

4.2. HCF-1 Couples Removal of Repressive Histone H3K9-Methylation with Deposition of Activating Histone H3K4-Methylation

Concomitant with the developing hypothesis that the infecting HSV genome was subject to the regulatory impacts of assembled chromatin, the critical IE transcriptional coactivator HCF-1 was identified as a component of the Setd1A and MLL histone H3K4 methyltransferase complexes [54–58]. As H3K4-methylation is the canonical activating mark recognized by complexes that promote transcription, these studies were the first indication that the role(s) of HCF-1 in mediating gene expression, and presumably IE gene expression, were via chromatin modification/modulation.

Subsequently, two studies indicated that the HCF-1-associated methyltransferases were important for regulation of viral gene expression. In these studies: (i) depletion of Setd1A resulted in impaired HSV gene expression [41]; (ii) Setd1A/MLL1 were both recruited to the related alpha-herpesvirus Varicella Zoster Virus (VZV) IE gene promoter in an HCF-1-dependent manner [52]; and (iii) the resulting H3K4-methylation was correlated with viral activator-mediated IE induction [41,52]. Interestingly, as noted above, in very early stages of infection or in HCF-1 depleted cells, nucleosomes bearing the dominant repressive H3K9-methylation mark were readily detected on the viral genome and specifically on the promoter domains of the viral IE genes [51,53]. This led to the hypothesis that initial infection triggered cell-mediated deposition of repressive chromatin on the viral genome that required removal by specific H3K9 demethylases in order to promote viral gene expression.

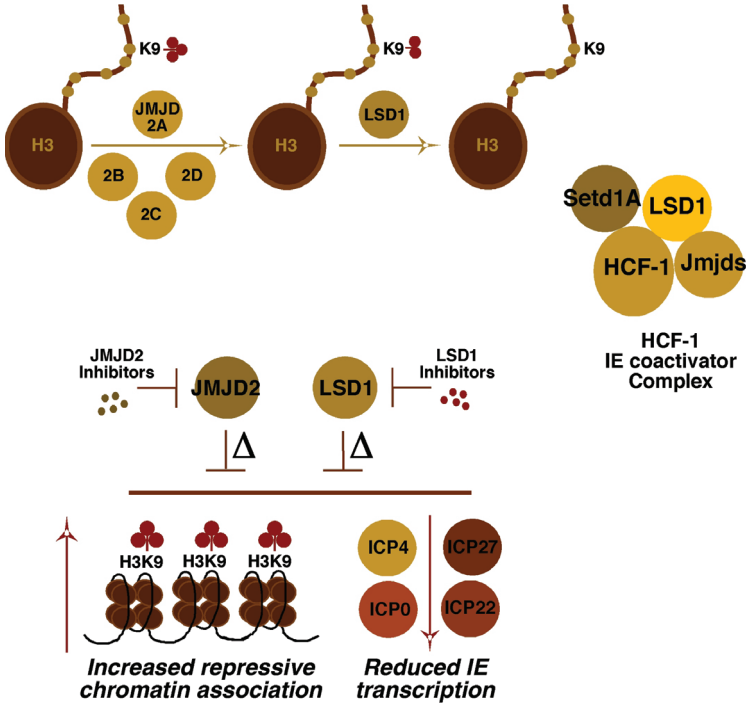
Investigations into the components that might play roles in reversing repressive chromatin marks associated with the viral genome revealed two sets of interdependent H3K9 demethylases (Figure 2). Strikingly, both the family of JMJD2 proteins [59] and LSD1 [60] were shown to be important to limit the accumulation of repressive H3K9-methylation on the viral genome [51,61,62]. Either depletion of these proteins or inhibition of their catalytic activities with small molecule inhibitors blocked IE expression and resulted in enhanced histone and H3K9-methylation associated with the viral IE promoter domains. With respect to the role of HCF-1 in this conversion from repressive to activating methylation, an HCF-1 complex was identified that contained both the required demethylases (JMJD2/LSD1) and methyltransferase (Setd1A/MLL1) activities [51,62]. Thus, the recruitment of HCF-1 complex(es) by the enhancer core factors or via factors regulating IE genes suggested a combined potential to circumvent cell-mediated repression and promote IE gene transcription.

4.3. Multiple Chromatin Modulation Components/Complexes Associated with the HCF-1 Coactivator

In addition to the Setd1A/MLL1 H3K4 methyltransferases and the H3K9 demethylases LSD1/JMJD2(s), HCF-1 has been identified as a component of or has been associated with multiple chromatin modulation components. Whether these complexes/components are involved in the regulation of alpha-herpesviral chromatin has not yet been determined. It should be noted that the global state of HSV chromatin during various stages of infection is relatively uncharacterized and therefore, future studies may ultimately provide linkages between multiple HCF-1 complexes and modulation of HSV chromatin. Given the important nature of this coactivator for viral expression and

the interplay of histone modification, recognition, and remodeling components that must occur during IE gene transcription, there is the potential that insights into control of viral chromatin may come from the studies of HCF-1 chromatin complexes. Therefore, relationships between HCF-1 and various chromatin components are shown in Figure 3 and detailed below.

Figure 2. Cooperating activities of the histone demethylases, LSD1 and JMJD2(s), for derepression of HSV IE genes. The JMJD2 family of histone demethylases can remove histone H3K9-me3 but require the cooperating activity of a second demethylase (*i.e.*, LSD1) to remove H3K9-me2/1. The HCF-1 coactivator complex identified contains both a histone H3K4-methyltransferase (Setd1A) and H3K9-demethylases (JMJD2/LSD1) to promote viral IE transcription. Depletion (Δ) or inhibition of the catalytic activity of either LSD1 or the JMJD2 family results in reduced viral IE gene transcription and increased assembly of repressive chromatin on IE gene promoter domains.



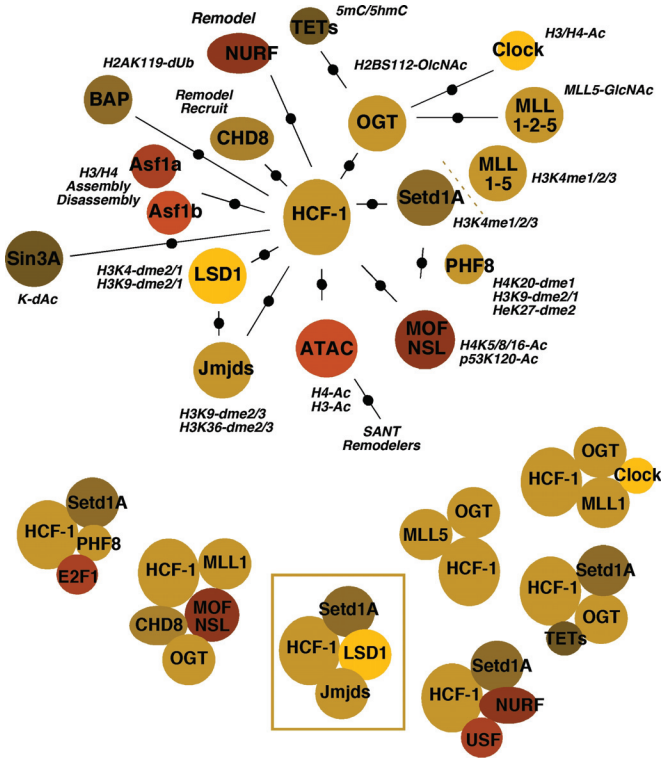
4.3.1. Histone Methyltransferases and Demethylases

In addition to the demonstrated roles of JMJD2s/LSD1 in removing repressive H3K9-methylation coupled with Setd1A/MLL1 in promoting H3K4-methylation of IE promoter-associated nucleosomes, HCF-1 is a component of complexes containing multiple members of the MLL family (MLL1, MLL2, MLL5) [55,56,58,63,64]. Several lines of evidence indicate that, while the bulk of cellular

H3K4-methylation is due to Setd1A, the MLLs are responsible for the control of some developmental (MLL1/2), cell cycle (MLL1) and signal-induced transcription (MLL3/4).

With respect to cell cycle, HCF-1 interacts with E2F1 in conjunction with either MLL1 or Setd1A and the demethylase PHF8, resulting in H4K20-demethylation, H3K4-methylation, and cell-cycle-dependent transcription (G1/S transition) [21,22].

Figure 3. Multiple associations and complexes of the coactivator HCF-1 with chromatin modulation components. The multiple interactions or associations of chromatin modulation machinery are shown relative to HCF-1. Refer to the text for appropriate description and references. (Top Panel) The activities of components/complexes are noted (Ac, acetylation; GlcNAc, O-linked N-acetylglucosamine; dme, demethylation; dUB, deubiquination; dAc, deacetylation). (Bottom Panel) HCF-1 chromatin modulation complexes that have been clearly defined or identified in specific contexts are represented. The HCF-1 complex whose components are critical for viral IE gene expression is boxed.



4.3.2. Acetyltransferases and Deacetylases

Three sets of acetyltransferases have been associated with HCF-1. The MOF/NSL complex cofractionates with the HCF-1/MLL1 complex and the chromatin remodeling factor chromodomain

helicase DNA binding protein 8 (CHD8) [65–68]. This complex thus couples the HMT with acetyltransferase activity targeting histone H4K5-8-16, marks characteristic of euchromatin. In addition, the NSL complex also modifies the activity of other targets including p53. The second complex, ATAC, contains the histone acetyltransferases GCN5/ATAC2 and appears to target histone H3/H4 for acetylation [69–73]. Interestingly, this complex also contains a stress-activated kinase (TAK1/MAP3K7), suggesting that the complex activity may be subject to specific induced signaling. Finally, the third complex is of specific interest as it is composed of HCF-1, MLL1, and the H3/H4 acetyltransferase CLOCK [74,75], a protein implicated in the regulation of HSV gene expression [46].

In addition to multiple acetyltransferase complexes, HCF-1 is also a component of the repressive Sin3A complex containing the histone deacetylases HDAC1/2 [57]. This complex, targeted via interactions with THAP transcription factors, mediates repression of differentiation-linked gene expression in embryonic stem cells [23,76]. In a parallel, but contrasting manner, an HCF-1/Setd1A complex is also recruited by THAP factors and is involved in stimulation of genes, leading to the induction of cell cycle and increased cell mass [24], also contributing to the maintenance of embryonic stem (ES) cell pluripotency.

4.3.3. Chaperones

HCF-1 directly interacts with the histone H3/H4 chaperones Asf1a and Asf1b [39]. Although both chaperones associate with HCF-1, the functional significance may be distinct. Asf1a is a constitutively expressed chaperone that is involved in non-replicative histone assembly/disassembly. In contrast, Asf1b levels increase during the S phase of the cell cycle and the protein appears to preferentially function during DNA replication [77,78].

For HSV, HCF-1/Asf1b complexes are linked to viral replication factors, providing a unique mechanism for nucleosome reorganization coupled to viral DNA synthesis [39]. In contrast, one function of Asf1a appears to be non-replicative assembly of chromatin on the viral genome that may play a role in initial repression of IE gene expression. However, the role(s) of Asf1a in the regulation of viral gene expression throughout the lytic replication cycle is less clear, as depletion of Asf1a increased expression of viral IE genes but reduced viral replication and progeny [79].

4.3.4. Remodelers

Two HCF-1-associated remodeling activities are components of multifunctional complexes. As noted above, the HCF-1/MLL1/MOF-NSL methyltransferase/acetyltransferase complex also contains CHD8, which recognizes histone H3K4-me2/3 [66]. While CHD8 has been characterized as a transcriptional repressor via remodeling and recruitment of histone H1 [80], it also plays a critical activating role in stimulating U6 RNAPIII transcription [81].

Interestingly, CHD8 is also a component of a second HCF-1 associated remodeling complex, NURF, which is recruited by the upstream binding factor (USF) along with HCF-1/Setd1A as components of chromatin boundary elements that prevent the spread of heterochromatin [82,83]. In

addition to CHD8, NURF contains the important remodeler SNF2L that is characteristically involved in remodeling to create nucleosome-free regions in promoter domains.

4.3.5. OGT

O-linked N-acetylglucosamine transferase, the sole enzyme responsible for O-GlcNAc modification of proteins at S/T residues, is a component of multiple HCF-1 chromatin-related complexes. Its activity is required in the HCF-1/MLL5 complex where O-GlcNAc modification of MLL5 significantly enhances H3K4-methyltransferase activity [63]. Similarly, the protein modulates the stability of the histone acetyltransferase CLOCK [84].

More recently, OGT has been recognized as a contributor to the histone code by modification of histones including histone H2BS112, thus promoting H2BK120-monoubiquination and enhanced H3K4-methylation [85,86].

OGT, in complex with HCF-1/Setd1A, is also targeted to CpG islands by the recently identified ten-eleven translocation (TET) proteins [87–90]. Occupancy of these regions correlates with the lack of detectable cytosine modifications. Interestingly, as the TET proteins are involved in active DNA-demethylation (conversion of 5-methylcytosine to 5-hyroxymethylcytosine), these complexes may represent a coupling of both DNA and histone modifications that promote transcriptional activation.

4.3.6. BAP1

BRCA1-associated protein-1 is a deubiquitinase (DUB) with multiple targets including histone H2AK119-Ub, a repressive mark linked with histone H3K27-methylation, DNA-methylation, and histone H1 association [91,92]. Interestingly, the role of BAP1 and H2AK119-Ub remains unclear and may be dependent upon the particular context. As a component of the polycomb repressive complex PR-DUB, BAP1 appears to be required to balance the levels of H2AK119/H2AK119-Ub for effective PRC-mediated repression [93,94]. In contrast, deubiquination of H2A is correlated with gene activation and increased histone H3K4-methylation [92].

The roles of this protein and its DUB activity in association with HCF-1 may be significantly more complex. In addition to modulating the repressive H2A-Ub, BAP1 has multiple protein substrates, including HCF-1, OGT, and perhaps, YY1 [95–97]. BAP DUB activity is required for stabilization of OGT and may affect the interactions or functions of HCF-1 in cell-cycle regulation, thus having pleiotropic impacts.

4.3.7. The Potential for Multiple Roles of HCF-1 Complexes in Viral IE Gene Expression

It is now clear that epigenetic regulation of HSV infection is an important contributor to the determination of the outcome of infection and represents a supra-regulatory overlay beyond the contributions of individual DNA binding transcription factors [98–101]. Much remains to be determined with respect to the components required for modulation of chromatin associated with the viral genome during each stage of primary/lytic infection. However, the clear role(s) of the coactivator

HCF-1 in mediating chromatin modulation during viral infection suggest that additional complexes associated with this protein may also contribute. It is important to note that while HCF-1 has been ascribed or linked to many chromatin modulation factors/complexes, it is also unclear whether there are core units, such as HCF-1/Setd1A/OGT, that recruit/interact transiently and sequentially with multiple factors to enact viral gene expression. Additionally, it is unclear as to the mechanisms by which specific HCF-1 complexes may be selectively recruited. For HSV IE gene expression, the viral activator VP16 preferentially interacts with the HCF-1/Set1 complex, as opposed to complexes containing Sin3A. Whether other regulatory factors recruit distinct HCF-1 complexes that are required or contribute to viral IE gene expression remains to be determined.

5. The Potential of Epigenetic-Based Antivirals

The recognition of important elements of chromatin-mediated control of viral infection coupled with the rapidly advancing development of specific chromatin modulation machinery inhibitors [102–107] leads to new potential for novel antivirals [108]. As a striking example, monoamine oxidase inhibitors originally developed to target MAO-A and -B for the treatment of severe depression, also inhibit the histone demethylase, LSD1 [109–111]. As noted, LSD1 is critical to the initiation of alpha-herpesvirus IE gene expression and in cultured cells, MAOIs and other developed specific LSD1 inhibitors block infection by preventing reversal of the accumulated repressive chromatin on the genome.

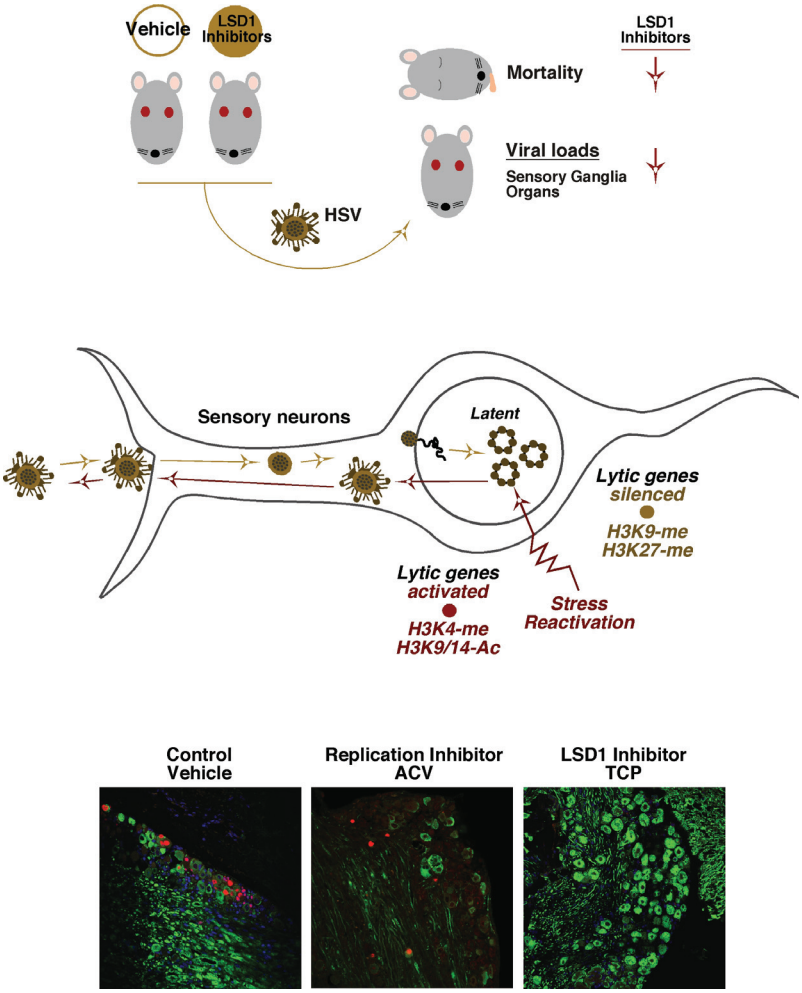
As a “proof of principle”, these compounds were tested for their ability to reduce an HSV primary infection in the mouse model system [51,61]. Treatment of mice prior to infection significantly reduced HSV mediated mortality and viral loads in sensory ganglia and other organs [61] (Figure 4, Top). While this represents an initial step in moving these compounds and related chromatin modulation inhibitors forward, it does indicate the potential for new inroads in antivirals that target the initiation stage of infection, prior to the expression of viral gene products.

6. HCF-1 Chromatin Modulation Complexes and HSV Latency-Reactivation

HCF-1 chromatin modulation complexes play a dominant role in mediating lytic viral gene expression upon infection. Importantly, several lines of evidence also suggest that HCF-1 is an important component of the regulation that mediates the initiation of viral reactivation from latency.

HSV latency is established in neurons of sensory ganglia where the virus persists in a quiescent state in the absence of detectable viral IE gene expression [2] (Figure 4, Middle). Important and insightful studies have clearly correlated characteristic repressive marks (H3K9- and H3K27-methylation) associated with the viral lytic gene promoters during latency and activating marks (H3K4-methylation, H3-acetylation) with viral IE genes during reactivation [98,112–120] (see Bloom *et al.*, this issue). These studies were fundamental in establishing the potential control of latency-reactivation cycles by epigenetic mechanisms.

Figure 4. Inhibitors of the HCF-1 associated histone demethylases reduce primary infection and block viral reactivation from latency. (Top Panel) Mice treated with either Vehicle control or LSD1 inhibitors were infected with HSV. Mortality and viral loads were assessed at defined time periods post infection. LSD1 inhibitors reduce mortality and viral loads relative to control. (Middle Panel) HSV infection of sensory neurons results in the establishment of latency in which lytic genes are repressed. Stress-mediated reactivation of viral infection results in conversion of repressive chromatin marks to activating marks on viral lytic genes. (Bottom Panel) Latently infected trigeminal ganglia were explanted into culture to induce viral reactivation in the presence of control vehicle, ACV (acycloguanosine, DNA replication inhibitor), or the LSD1 inhibitor, TCP (tranylcypromine). Ganglia were sectioned and stained for neurofilament (green) and the viral lytic replication protein UL29/ICP8 (red) to mark neurons undergoing productive reactivation.



With respect to HCF-1, the protein is uniquely concentrated in the cytoplasm of sensory neurons but rapidly is transported to the nucleus of these cells in response to signals that promote viral reactivation from latency [121–123]. In addition, upon stimulation, HCF-1 can be rapidly detected occupying IE promoters of the viral genome [124]. These initial observations led to the hypothesis that HCF-1 is a component of the switch mechanism for HSV latency-reactivation cycles. Given the role(s) of HCF-1 in chromatin modulation during lytic infection, a model was proposed in which HCF-1 would be an integral part of the chromatin dynamics that must occur during the initiation of the reactivation process. The model was supported by studies in which inhibitors of either the HCF-1-associated demethylases, LSD1 or JMJD2(s), blocked the expression of viral IE genes and the production of viral progeny in induced latently infected sensory ganglia [51,61,62] (Figure 4, Bottom).

7. Concluding Remarks

Recently recognized, the role of chromatin modulation of alpha-herpesvirus infection represents an intricate and complex regulatory overlay. Intrinsically linked to viral-host interactions, chromatin presents a dynamic of repression/subversion of infection by the host cell. This is countered by mechanisms employed by the infecting virus to interface with the cellular chromatin machinery to promote viral gene expression and replication. A key player in this dynamic is the cellular chromatin regulator HCF-1 associated with multiple chromatin modulation components that the virus recruits at an early stage to promote lytic infection. Many stages of chromatin modulation and the enzymology that mediates it remain to be delineated for a true mechanistic view of this important aspect of viral infection. Understanding this dynamic and the key components could significantly increase the avenues for the development of novel antivirals with distinct advantages over present therapies.

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Conflicts of Interest

The National Institutes of Health has the following patent applications relative to the information contained in this review: (i) US patent application No. 61/083,304; DHHS: E-275-2008/1-US-01, international application No. PCT/US2009/051557; and (ii) US patent application No. 61/366,563; DHHS: E-184-2010/0-US-01. The authors declare no additional conflict of interest.

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Review

The Role of the CoREST/REST Repressor Complex in Herpes Simplex Virus 1 Productive Infection and in Latency

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Abstract: REST is a key component of the HDAC1 or 2, CoREST, LSD1, REST (HCLR) repressor complex. The primary function of the HCLR complex is to silence neuronal genes in non-neuronal cells. HCLR plays a role in regulating the expression of viral genes in productive infections as a donor of LDS1 for expression of α genes and as a repressor of genes expressed later in infection. In sensory neurons the HCLR complex is involved in the silencing of viral genome in the course of establishment of latency. The thesis of this article is that (a) sensory neurons evolved a mechanism to respond to the presence and suppress the transmission of infectious agents from the periphery to the CNS and (b) HSV evolved subservience to the HCLR with at least two objectives: to maintain a level of replication consistent with maximal person-to-person spread and to enable it to take advantage of neuronal innate immune responses to survive and be available for reactivation shielded from adaptive immune responses of the host.

Keywords: HCLR; herpes viruses; productive infection; latency

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1. Background

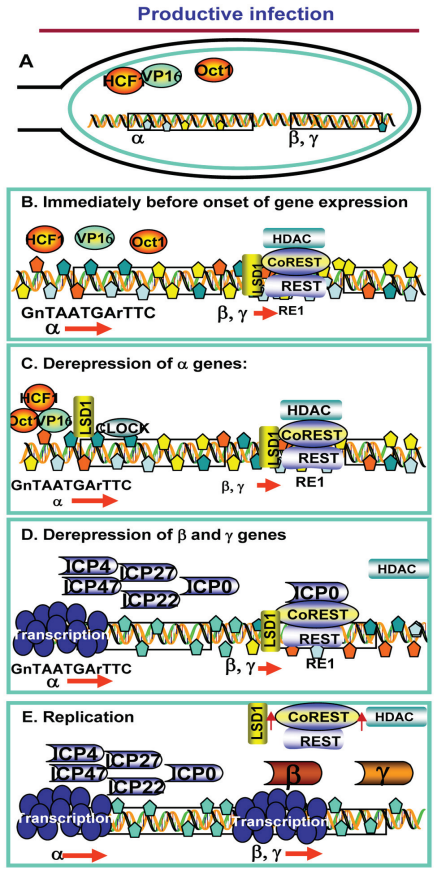
Herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) replicate and destroy cells at the portal of entry into the body (mouth, genitals), but remain latent without obvious effects in sensory ganglia. The same viruses readily replicate and destroy neurons maintained in culture [1,2]. The fundamental question posed in numerous studies over the past century is how a virus that efficiently and vigorously replicates and can cause massive tissue destruction is nevertheless silenced in sensory ganglia. In the absence of any data, two hypotheses could explain the biology of HSV. The first is that HSV evolved two sets of regulatory pathways, one for viral gene expression at the portal of entry into the body, and a totally different pathway for viral gene expression in sensory ganglia. The alternative hypothesis is based in part on the observation that HSV recruits host proteins to perform the functions it needs. In this context, the host proteins available for recruitment in cells at the portal of entry into the body and in sensory neurons could be different.

2. Productive Infection

The mantra of HSV-1 gene expression modified in recent years is that HSV genes form several groups that are coordinately and sequentially derepressed [1]. Thus, on entry into the nucleus HSV-1 DNA is immediately bound by repressive histones and cellular repressors. The α genes are derepressed first, followed by the β and γ genes [2]. The focus of the studies carried out at the time of the discovery of the role of the CoREST/REST repressor complex was on ICP0, an HSV multifunctional regulatory protein [3–5]. Thus, at low multiplicity of infection with ΔICP0 mutants α genes are expressed, but the transition from α to β gene expression does not ensue [2,6,7]. One "desperate" hypothesis we pursued is that ICP0 mimics a less efficient cellular protein since, at high multiplicity of infectionΔICP0 mutants do replicate. To our surprise, a blast analysis of human genes known at that time revealed that a stretch of approximately 70 ICP0 residues at the amino terminus of CoREST were conserved with relatively good homology. We demonstrated that (a) CoREST and ICP0 interacts, (b) the binding site of ICP0 in CoREST corresponded to the homologous sequence in the amino terminal of the protein. The binding site for CoREST in ICP0 was immediately downstream of the homologous sequence [4,8].

CoREST and REST are components of a larger complex, consisting minimally of histone deacetylase (HDAC)/corepressor element-1 silencing transcription factor (CoREST)/lysine specific demethylase1(LSD1)/RE1-silencing transcription factor (REST) repressor complex (HCLR) [9–13]. In this complex CoREST binds HDACs, LSD1 and REST [9]. The primary function of this complex is to repress neuronal genes in non-neuronal cells [14–18]. REST is usually absent or in very low amounts in neurons [19–22]. Stressed neurons, for example in Huntington's disease, may express REST and this may lead to their death [23–26]. REST binds to a somewhat degenerate response element known as RE1 [27–30]. Analyses of the HSV DNA sequence predicts numerous REST binding sites scattered through the genome (unpublished data).

Figure 1. Schematic representation of the role of the HCLR complex in productive infection. (**A**) HSV DNA entering the nucleus is bereft of bound proteins. Several viral proteins are introduced into the cell, and ultimately into the nucleus, during infection; among these VP16 is shown here; (**B**) Within a very short time frame viral DNA is coated by repressive histones and interacts with the HCLR complex. In addition (not shown here) ND10 nuclear bodies assemble at the viral DNA; (**C**) VP16 recruits HCF1, Oct1, LSD1 and other transcriptional factors to derepress and transcribe α genes. Concurrently (not shown), the ND10 bodies disassemble; (**D**) ICP0, one of the□ αproteins, binds to CoREST and dislodges HDAC1. Transcription of β and γ genes ensues; (**E**) Late in infection, after the onset of viral DNA synthesis, a fraction of LSD1, CoREST, REST and HDAC 1 is translocated to the cytoplasm.



To assess the significance of the interaction between ICP0 and CoREST, three series of experiments were done. In the first the open reading frames expressing ICP0 were replaced with those encoding a dominant negative CoREST lacking the binding sites of ICP0 and HDAC1. The mutant replicated

10 to 100 fold better than ΔICP0 mutant, indicating that a key function of ICP0 was to block the repressive action of HCLR complex [8]. In the second series of experiments the binding site of CoREST on ICP0 was mutagenized by codon substitution. In wild type infected cells ICP0 colocalizes first with ND10 bodies in the nucleus [31,32]. Within a few hours, however, key components of ND10 are degraded, the constituents of ND10 are dispersed, and ICP0 becomes dispersed in the nucleus. Between 6 and 9 h after infection ICP0 is fully localized in the cytoplasm [33,34]. ICP0 mutants lacking the CoREST binding site were largely retained in the nucleus [4]. Finally, the conclusion that ICP0 blocks the repressive functions of the HLCR complex was supported by the evidence that ICP0 displaced HDAC1 from the complex [5,8]. Late in infection at least a fraction of HDAC1, CoREST, LSD1 and REST were exported into the cytoplasm. The translocation of the HCLR components was delayed in the presence of inhibitors of viral DNA synthesis [3]. A schematic representation of this model is shown in Figure 1.

The studies described above indicate that HCLR complex was a key component in repressing the transition from β to γ gene expression. Curiously, this complex, at least indirectly, plays a role in the initiation of expression of α gene. Thus, to initiate gene expression, VP16, a virion protein introduced into the cell during infection, recruits several proteins to the promoters of α genes, especially Host Cell Factor 1 (HCF1), Octamer binding protein 1(Oct1) and LSD1 [1,2]. LSD1 is unstable in the absence CoREST [17,35,36]. Indeed, silencing of either CoREST or REST by siRNA decreased the levels of LSD1 and reduced the expression of α genes [37].

The fundamental conclusion of these studies is that on entry of viral DNA into the nucleus all viral genes are repressed and that the HCLR complex plays a key role in the expression of viral genes during productive infection. The obvious question is why it plays this role. Viruses can evolve faster than the cells they infect. In the course of its evolution, HSV could have readily escaped from the repressive effects of the HCLR complex through base substitution. Based on the effectiveness of the repressive effects of the HCLR complex, it would appear that HSV evolved to embrace the HCLR repressor rather than avoid it. In evolving ICP0, HSV has effectively nullified the repressive effects of the HCLR complex. One hypothesis to explain the interaction with HCLR is that HSV needs it to establish a latent state.

3. Establishment of Latency and Reactivation

REST is not a normal constituent of neurons [19–22]. To play a role in the establishment of latency, REST would have to be induced or recruited from satellite cells. As reported elsewhere, REST was detected in immunoblots of extracts from whole murine trigeminal ganglia, but not in extracts of an intact mouse brain [38]. One way to test whether it plays a role in establishment of latency is to insert a dominant negative (dn) REST, driven by an SV40 promoter, into the HSV genome. The dnREST we constructed contains the DNA binding domain, but lacks the N and C terminal domains required for binding the repressive components of the complex [38]. The prediction would be that if the HCLR complex plays no role in the establishment of latency, the expression of a dominant negative REST would have no effect. If REST is induced or recruited by the entry of the virus into the neuron, the

dominant negative REST would be predicted to compete with wild type REST, bind to viral DNA and preclude its silencing. In all, 3 viruses were constructed, *i.e.*, a virus encoding a dnREST, a virus encoding a wild-type rest and finally, a virus containing a series of stop codons at the site of insertion of the REST genes [38]. In these experiments, mice were inoculated by the corneal route. The trigeminal ganglia were harvested at frequent intervals during the first two weeks and at the end of the test period (28–30 days). The key findings were that the virus carrying the gene encoding the dnREST was more virulent and replicated to higher titers than either the wild type virus or the control viruses, *i.e.*, those encoding wild-type rest or carrying the string of stop codons. Specifically, we observed higher levels of HSV in mouse brains, total destruction of trigeminal ganglia, higher amounts of virus in the brain and high mortality even when the size of the inoculum was reduced. The enhanced virulence of the virus encoding dnREST was verified by inoculating mice by the intraperitoneal route [39].

The studies on the dnREST led to 2 conclusions. As construed by the experimental design, higher virulence suggests that dnREST competed with wild-type REST and blocked the HCLR complex. Confirmatory results emerged as described below in the section on virus reactivation.

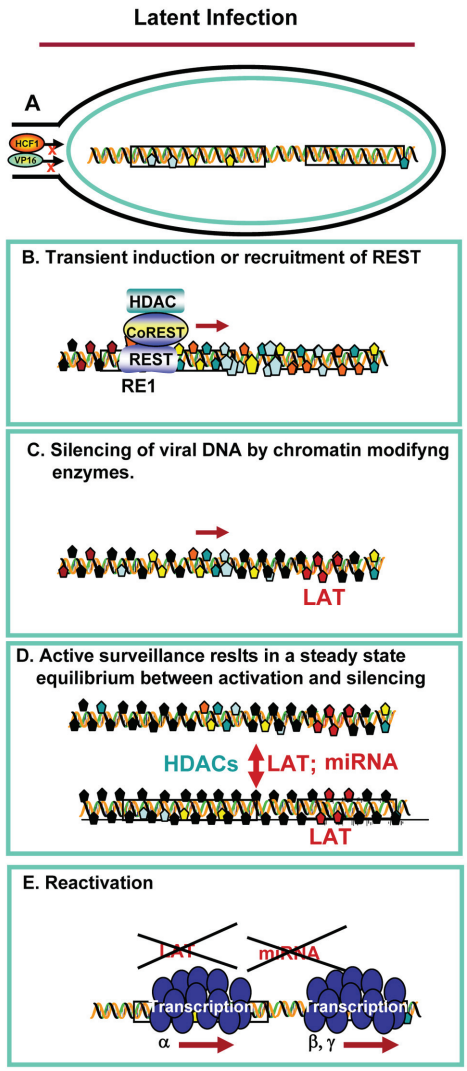
Perhaps the most significant and unexpected finding to emerge from these experiments is that the dnREST virus was more virulent than the wild-type parent [1]. Implicit in this finding is the proposition that HSV could have evolved to be far more virulent than it is. A necessary extension of this proposition is that, in the course of its evolution, HSV was constrained from replicating in every cell it infects and from efficient spread from the site of infection to target organs (e.g., brain, liver, *etc.*) that could cause the infected individual to succumb to infection. Also implicit in this proposition is that the HCLR complex plays a role in defining the virulence of the virus, in that it blocks viral replication in a significant fraction of the cells it infect *in vivo*. The failure to evolve into a more pathogenic virus is not unexpected: highly virulent viruses that kill their hosts rapidly are defective in spreading. Another way to put it is that very sick people do not efficiently spread a sexually transmitted virus.

4. Reactivation from Latency

HSV reactivates in response to environmental, hormonal and emotional stress [39–41]. On reactivation, HSV multiplies and is translocated to a site at or near the portal of entry [39,40]. Reactivation presents two problems. The first is how diverse stimuli, that at times are highly individual, cause latent virus to reactivate. The second puzzle is the mechanism of reactivation. In latently infected neurons, viral DNA forms an episome [2,42,43]. As noted earlier, the only viral gene products accumulating in neurons are a long, non-coding RNA, designated the Latency Associated Transcript (LAT) [44–50], and a set of micro RNAs (miRNAs) [51–54].

Viral DNA itself is in the form of facultative heterochromatin [42,55–58]. The preeminent question is how HSV reactivates from the silent, repressed state in the absence of VP16 and ICP0. The question also arises as to the role, if any, of the CoREST/REST complex in the reactivation from latent state.

Figure 2. Schematic representation of the role of the HCLR complex in HSV latency. (**A**) Viral DNA is released into the neuronal nucleus free of bound DNA. Available data suggest that HCF1 and VP16 do enter the nucleus; (**B**) REST is induced or recruited from satellite cells. The HCLR repressor initiates silencing of the viral genome; (**C**) HSV DNA protein complex is modified by chromatin modifying enzymes; (**D**) HSV is in a latent (silent) state. The neuron is surveyed by LAT, miRNAs. The state of repression requires the presence of active HDACs except for transcription of the LAT and miRNAs; (**E**) On reactivation all viral genes are expressed at once. The LAT and miRNAs are degraded. There is no evidence that REST is induced or recruited during reactivation.



The model we have chosen to resolve these questions are organ cultures of trigeminal ganglia harvested 30 days after corneal inoculation [59]. Incubation of ganglia in medium containing the antibody to NGF causes the virus to reactivate. Incubation in medium containing NGF and EGF delays reactivation [59]. The relevant finding obtained to date is that in medium containing anti-NGF antibody, viral mRNAs representative of all classes of viral genes exhibit an increase in amounts, beginning 5 h after excision and incubation of the ganglia. Concurrently the LAT and miRNAs decrease in amount [59]. Reactivation of all genes also takes place in the presence of cycloheximide, indicating that (a) activation does not require prior protein synthesis, and (b) the reactivation in this system is a catastrophic event that causes total derepression of the viral genome. Finally, in this system a wild-type virus carrying wild-type REST does not reactivate. Reactivation, as measured by transcription of all viral genes, does occur if the ganglia infected with the virus encoding REST are incubated in medium containing cycloheximide, either for the entire 24 h interval or for at least 5 hr interval after excision [60].

The conclusion drawn from these studies is that the CoREST/REST complex plays a role in the establishment of latency, but not in reactivation. A model of the establishment of latency is shown schematically in Figure 2.

5. Conclusions: The Role of the CoREST/REST Repressor Complex in the HSV Lifestyle

The fundamental findings summarized above indicate that the CoREST/REST complex is involved in the suppression of viral gene expression, in both productive infection and in the establishment of latent, silent infections in sensory neurons. As noted above, HSV could have readily escaped interactions with the CoREST/REST repressor complex, by base substitution. One interpretation of the data is that in fact HSV subserved itself to the repressor complex. The data posit the following:

(i) All of the data available to date indicate that no viral function is required to establish latent infections [2,42]. Silencing of viral DNA in neurons appears to be a neuronal function, most likely a defense mechanism to block transmission of viruses from the periphery to the central nervous system. Viewed from this prospective, HSV took advantage of the innate neuron defenses to enable itself to be silenced and remain as a reservoir in its human host.

(ii) One response to stress in neurons is activation of the CoREST/REST complex. This has been reported in some degenerative diseases of the CNS [23–26]. It is conceivable that transient expression of REST following entry of the virus into the CNS, is sufficient to initiate the epigenetic modifications essential to silence viral DNA, but not irreversibly damage the neuron harboring the virus in the silent state.

(iii) In cultured cells productively infected with HSV-1, the CoREST/REST repressor complex is readily overcome by displacement of HDACs from the repressor complex by ICP0, or in high multiplicity infections [8]. The studies on the HSV-1 mutant carrying dnREST [38] suggest that the interaction with the CoREST/REST repressor complex serves to maintain equilibrium between excessive replication—that would irreversibly injure the host and prevent transmission—and minimal

replication—which is insufficient to secure establishment of latent virus, and ultimately frequent transmission from infected to uninfected individuals.

Viewed in this light, the hypothesis driving these studies is that HSV entering sensory neurons triggers transient synthesis or recruitment of REST from satellite cells. The transiently assembled repressor complex initiates the silencing of viral DNA by cellular enzymes. The observation that the virus encoding wild-type REST does not reactivate, suggests that REST is not activated in neurons harboring virus and deprived of NGF [60]. Key questions—which mechanisms suppresses the continued presence of REST in neurons harboring latent virus, or mechanisms that lead to the massive derepression of viral genome during reactivation—remain unanswered.

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Conflicts of Interest

The authors declare no conflict of interest.

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Review

Chromatin Dynamics during Lytic Infection with Herpes Simplex Virus 1

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Abstract: Latent HSV-1 genomes are chromatinized with silencing marks. Since 2004, however, there has been an apparent inconsistency in the studies of the chromatinization of the HSV-1 genomes in lytically infected cells. Nuclease protection and chromatin immunoprecipitation assays suggested that the genomes were not regularly chromatinized, having only low histone occupancy. However, the chromatin modifications associated with transcribed and non-transcribed HSV-1 genes were those associated with active or repressed transcription, respectively. Moreover, the three critical HSV-1 transcriptional activators all had the capability to induce chromatin remodelling, and interacted with critical chromatin modifying enzymes. Depletion or overexpression of some, but not all, chromatin modifying proteins affected HSV-1 transcription, but often in unexpected manners. Since 2010, it has become clear that both cellular and HSV-1 chromatins are highly dynamic in infected cells. These dynamics reconcile the weak interactions between HSV-1 genomes and chromatin proteins, detected by nuclease protection and chromatin immunoprecipitation, with the proposed regulation of HSV-1 gene expression by chromatin, supported by the marks in the chromatin in the viral genomes and the abilities of the HSV-1 transcription activators to modulate chromatin. It also explains the

sometimes unexpected results of interventions to modulate chromatin remodelling activities in infected cells.

Keywords: Herpes simplex virus 1; chromatin; silencing; nucleosome; histone; fluorescence recovery after photobleaching (FRAP)

1. Introduction

Herpes simplex virus 1 (HSV-1) establishes lytic infections in most cells, but latent infections in neurons *in vivo*. During lytic infections, the structural virion protein VP16 indirectly binds to the 'TAATGARAT' sequences in the promoters of the "immediate-early" (IE) genes in a complex with cellular HCF-1 and Oct1 to activate IE gene transcription [1–6]. Two IE proteins, ICP4 and ICP0, then activate transcription of 'early' (E) and 'late' (L) genes. The mechanisms of transcription activation by ICP0 and ICP4 are not yet fully characterized, but require no binding to specific DNA sequences [7,8]. It is widely accepted that at least ICP0 activates HSV-1 transcription, mostly by counteracting not yet fully characterized cellular defenses [9–12]. ICP4 activates transcription through interaction with components of the RNA polymerase II transcription machinery, such as the TFIID and mediator complexes. E proteins replicate HSV-1 DNA, whereas the L genes encode structural and other proteins required for the assembly of infectious virions. In contrast to lytic infections, transcription (except for the LAT locus), HSV-1 DNA replication, and production of new virions, are all inhibited during latency. HSV-1 mutants in the transcription activators ICP4, ICP0, and VP16 are also transcriptionally silent (*i.e.*, 'quiescent') in fibroblasts [13–18]. Quiescent infections can also be established in certain neuronal cultures under specific conditions [19–23].

Transcription, replication, and encapsidation of the approximately 150 kbp double stranded DNA HSV-1 genome all occur in the nucleus. Physiologically, nuclear DNA is organized in chromatin, a chain of nucleosomes. Each nucleosome is composed of 165 bp of DNA wrapped 1.46 times around an octamer of core histones H2A, H2B, H3 and H4. The core nucleosome is bound by linker histone H1, for a total of 200 bp and ~2 turns. The linear chain of nucleosomes is then folded into complex higher order structures to form the chromatin fibers. Chromatin is essential to compact the long eukaryotic genomic DNA to fit within the cell nucleus. For example, the more than 1m of human genomic DNA is packaged within the human nuclei (which are typically ~7 μM in diameter). It also regulates DNA accessibility and, consequently, all processes requiring access to nuclear DNA. This regulation is mediated largely by histone post-translational modifications (PTM) and the different variants. The amino-terminal tails of the core histones are extensively post-translationally modified. These PTM provide docking sites for proteins that regulate transcription (the proposed "histone code" [24–28]) and regulate nucleosome stability [29,30]. The different histone variants also regulate DNA accessibility. One variant of histone H3, CENP-A, for example, is particularly enriched in non-transcribed centrosomes [31]. Another, H3.3, is enriched in transcribed chromatin, perhaps because it

forms unstable nucleosomes with a variant of histone H2A, H2A.Z [32–34]. Linker histones also regulate accessibility to the nucleosomal DNA [35–40]. Transcribed genes have lower H1 density than silenced ones [41–43]. Most nucleosome turnover occurs during transcription, DNA repair, and DNA replication, when the nucleosomes are disassembled to allow access to the DNA. Subsequently, nucleosomes are reassembled to maintain chromatin integrity. Nucleosome dynamics are thus important in the regulation of the processes requiring access to cellular DNA.

We will focus on HSV-1 to discuss the recent evidence strongly implicating nucleosome dynamics in the regulation of viral gene expression, and perhaps, DNA replication. The overarching model is that the infected cells attempt to silence the infecting genomes by mobilizing histones away from their chromatin to assemble silencing chromatin on the viral genomes. However, viruses have evolved proteins that counteract this silencing, to allow viral replication. These proteins therefore act as (indirect) activators of viral transcription.

2. Results and Discussion

2.1. The Chromatinization of HSV-1 Genomes in Lytically Infected Cells, an Apparent Paradox

The first evaluations of the nucleoprotein complexes containing HSV-1 DNA were performed in the context of the then current understanding that chromatin served mostly structural functions. Gibson and Roizman showed in 1971 that encapsidated HSV-1 genomes are complexed with enough spermine to neutralize 40% of the negative charges [44]. Mouttet and colleagues first described that nuclear HSV-1 genomes are far more accessible to micrococcal nuclease (MCN) than the DNA in the cellular chromatin [45], as confirmed later [18,46–50]. Muggerridge, in Fraser's group, tested the chromatinization of HSV-1 genomes in infected mice [51]. During acute infection, the viral genomes were far more accessible to MCN than the cellular chromatin [51]. However, Deshmane, also in Fraser's group, showed that the HSV-1 DNA in latently infected neurons was only as accessible as the cellular DNA [52]. Similar apparent regular chromatinization was later observed for quiescent HSV-1 genomes [53], chromatinization which was disrupted during reactivation [54]. The overall classic conclusion from all these results was that HSV-1 genomes were not regularly chromatinized in lytically infected cells, but were regularly chromatinized during latency or quiescence.

When chromatin was found to regulate gene expression, new techniques were developed to study chromatin in this context. The potential roles of chromatin in the regulation of HSV-1 gene expression were re-visited with these techniques. The pioneering work from Dr. Bloom's group showed that latent HSV-1 genomes are associated mostly with chromatin bearing PTM of silenced genes [55], except for the transcriptionally active promoter of the LAT gene [56]. In contrast, the promoter of the ICP0 gene is associated during reactivation with chromatin bearing PTM of transcribed genes [57]. Independent results from Dr. Knipe's group are mostly consistent with those from Dr. Bloom's group, although virus and mouse strain-specific differences became apparent [58,59]. All of these results were fully consistent with the classic models that latent HSV-1 genomes were regularly chromatinized.

The seminal papers published in 2004 from Triezenberg's, Fraser's and Berger's groups demonstrated that HSV-1 genomes surprisingly also co-immunoprecipitate with histones in lytically infected cells [50,60]. The histones associated with HSV-1 DNA appear to regulate viral gene expression. Highly transcribed HSV-1 genes associate with histones bearing PTM associated with transcription, whereas non-transcribed genes associate with histones bearing PTM associated with silenced genes [60–62] (although there are some exceptions [63]). The specific histone variants were also consistent with the transcription status in that histone H3 variant H3.3 associated preferentially with transcribed genes [64]. In cells with HCF-1 knocked down, HSV-1 DNA was not transcribed and predominantly in chromatin with marks associated with silencing [65]. Consistently, recruitment of histone acetylases [60], methyltransferases (Set1) [66] or demethylases (LSD1) [67] to IE promoters by VP16 appears required to activate transcription [68] (but see also [69]).

These and other results suggesting that HSV-1 transcription was regulated by chromatin were difficult to reconcile with the earlier results of MCN digestions. Regularly chromatinized cellular DNA is digested by MCN to fragments of sizes of multiples of nucleosome DNA. In contrast, the HSV-1 DNA in lytically infected cells is digested to fragments of heterogeneous sizes (for examples, see [18,45–50]). Nuclear HSV-1 DNA is also digested much faster than cellular DNA (although also much slower than naked DNA). Only much smaller percentages of HSV-1 than cellular DNA consistently co-immunoprecipitate with histones (for example, see [50,60–62,65]; for a notable exception, at a low multiplicity of infection, see [70]). A most common interpretation was that the HSV-1 DNA in lytically infected cells had low nucleosome occupancy, with long "linker" non-nucleosomal DNA of variable sizes between nucleosomes [54,63,70,71] (Figure 1a). This model could account for the variable sizes of the protected fragments and the small percentage of HSV-1 DNA protected to nucleosome sizes or co-immunoprecipitated with histones. However, it is difficult to reconcile with the models in which chromatin regulates HSV-1 transcription [9,72–74]. Low-density nucleosomes would not be expected to act as a barrier to impede transcription, neither to cooperatively recruit transcription activators. Alternatively, only a small subpopulation of the viral genomes could be regularly chromatinized, whereas the majority would not be associated with any histones (Figure 1b). The former would be protected to nucleosome sizes and co-immunoprecipitated with histones; the latter, randomly cleaved and not co-immunoprecipitated. This model is compatible with the proposed chromatin regulation of gene expression only if the specific subset of properly chromatinized HSV-1 genomes contained all the biologically relevant ones. Interestingly, only a subpopulation of all the genomes of a related alpha-herpes virus that infect a cell start successful replication cycles [75]. However, there is no evidence that the biologically active genomes are fully chromatinized and the inactive ones are not.

Mixed models, in which some genomes would have higher nucleosome density than others, have the same limitations of the two models discussed. The simplest prediction of any combination of these models is that the association of differentially transcribed HSV-1 genomes with histones bearing PTM of transcribed or non-transcribed chromatin is functionally irrelevant. In apparent support of this prediction, depletion of certain histone lysine acetyltransferases (KAT) and other chromatin modifiers,

or infections of cells lacking specific chromatin remodellers, had no obvious effects on HSV-1 transcription or replication [69]. However, the depletion of others or of the histone chaperones that assemble chromatin, affected HSV-1 transcription or replication [61,64,76].

Another line of evidence supports the models proposing that chromatin regulates HSV-1 transcription. While it was still generally accepted that chromatin played no role in HSV-1 transcription, all three major HSV-1 transcription activators, VP16, ICP0, and ICP4, were surprisingly found to be capable of (indirectly) inducing chromatin modifications. As discussed in detail later, ICP0 interacts with several histone deacetylases [77–79], destabilizes CENP-A [80] and decreases the levels of ubiquitinated H2A variants [81]. ICP0 and ICP4 together disrupt silencing of cellular facultative heterochromatin, although the biological importance of this activity remains unclear [82,83]. ICP4 functionally interacts with two chromatin architectural proteins, HMGA and B [84,85], although the biological importance of these interactions, if any, also remains unknown. VP16 also has chromatin remodelling activities. Being a classic model for activators of eukaryotic transcription, VP16 was used to study transcription regulation by chromatin [86]. In this artificial model, a large number of binding sites for the lac repressor were recombined in the cellular genome. The acidic carboxy-terminal transcription activation domain of VP16 was then expressed as a fusion protein with the DNA binding domain of the lac repressor (and an EGFP tag) in the absence of any other HSV-1 factor. Under these conditions, the VP16 activation domain promoted large-scale chromatin decondensation (spanning several megabases) [86]. Decondensation preceded the activation of transcription [87]. The relevance of these VP16 activities for HSV-1 remained unclear at the time, when chromatin was assumed to play no role in the regulation of HSV-1 gene expression.

2.2. Chromatin and Histone Dynamics Are Altered in Cells Lytically Infected with HSV-1

2.2.1. HSV-1 DNA Is in Unstable Nucleosomes during Lytic Infections

The abilities of the HSV-1 transcription activators to modulate chromatin are consistent with the models proposing that chromatin generally inhibits HSV-1 gene expression in lytic infections [9,72–74,88]. Also in support of such models, deletion of VP16, ICP0, and ICP4 results in quiescent infections [13–15], in which the HSV-1 DNA is in chromatin with silencing PTM [13,53]. Quiescent infections of cultured neurons are also characterized by silencing PTM on the viral nucleosomes, which are disrupted when VP16 is recruited to the viral genomes during reactivation [23]. However, the histone occupancy of HSV-1 genomes, as tested by ChIP or MCN accessibility assays, appears far too low for the standard mechanisms of transcription regulation by chromatin [69,89]. ChIP assays, however, do not detect unstable cellular nucleosomes [34], which are enriched in highly transcribed genes. Likewise, MCN assays are based on protection and, consequently, are not well suited to identify highly dynamic DNA-protein interactions.

As of 2010, there was an apparent inconsistency in the data regarding the chromatinization of HSV-1 genomes during lytic infections. To explore it, we analyzed the chromatin from HSV-1 infected cells by classical biophysical chromatin fractionation [47]. HSV-1 DNA forms large branched

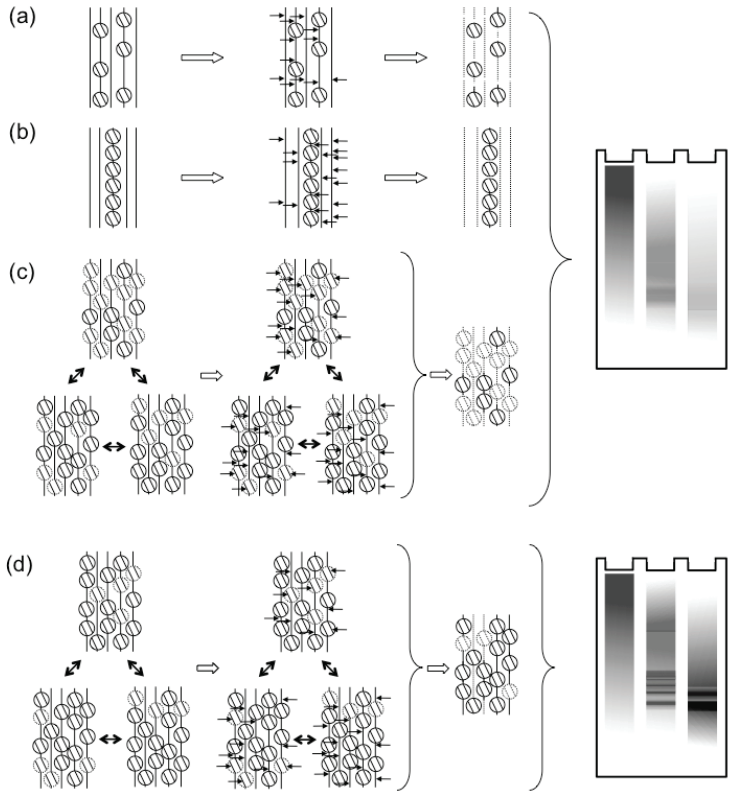
structures during replication. We therefore first restricted the nuclear DNA with BamH1, to release fragments of chromatin or naked DNA of sizes that can be resolved in sucrose gradients. Deproteinized HSV-1 DNA added to nuclei of HSV-1 infected cells before restriction fractionated to the very top fractions almost exclusively, as expected for naked DNA. Cellular DNA fractionated to lower fractions, as expected for chromatinized DNA. Most surprisingly, the nuclear HSV-1 DNA fractionated almost exclusively like regularly chromatinized cellular DNA, not like deproteinized HSV-1 DNA. Consistently with the classic results, however, only a minority of the HSV-1 DNA fragments released by standard MCN digestions fractionated as chromatinized DNA. Nonetheless, basically all HSV-1 DNA released in the "soluble chromatin" fraction (*i.e.*, the chromatin not pelleted in 20 min at 8,000 g) fractionated as cellular mono- to di-nucleosomes after multistep fractionations through differential centrifugation, sucrose gradient, and size exclusion chromatography. Most intriguingly, the percentage of HSV-1 DNA that was released in complexes migrating as mono- to di-nucleosomes did not increase with MCN digestion time. As expected, longer digestions resulted in higher percentages of cellular DNA released in complexes migrating as mono- to di-nucleosomes. Among the possibilities, the released HSV-1 DNA might have been protected in unstable complexes. These unstable complexes would allow their DNA to be further digested, essentially to nucleotides, during longer digestions. Meanwhile, new complexes of mono- to di-nucleosome size would be released from the "insoluble chromatin" fraction (*i.e.*, the chromatin pelleted in 20 minutes at 8,000 g), replenishing the pool of mono- to di-nucleosome sized complexes in the "soluble chromatin" fraction.

To test this model, we designed a variation of the standard MCN protection assays [47]. The MCN digestion was performed while the soluble and insoluble chromatins were being fractionated by centrifugation. The digestion was stopped every 5 minutes, the released soluble chromatin was collected and the MCN in it was immediately quenched to prevent further digestion of the released complexes. New MCN was added to the insoluble chromatin and the process was repeated. After nine [47] or six [46] repeats, all the soluble chromatin collected was pooled and fractionated by sucrose gradient. Under these limiting digestions, most HSV-1 DNA was released into the soluble chromatin in fragments of sizes of multiples of the nucleosome DNA (Figure 1c). In fact, the fractionation of the HSV-1 DNA complexes was indistinguishable from that of the cellular DNA complexes [47]. The fractionated mono- to di-nucleosome sized nucleoprotein complexes were next cross-linked before re-digesting them with MCN [47]. The complexes containing cellular DNA were equally protected with or without cross-linking, as expected for stable complexes. In contrast, the complexes containing HSV-1 DNA were far better protected from the MCN re-digestion after cross-linking [47], consistent with their proposed instability (Figure 1c).

Nucleosomes are disassembled during DNA replication, to allow the progression of the replication fork, and are reassembled behind it. The HSV-1 nucleoprotein complexes could very well be unstable only because of the fast rate of HSV-1 DNA replication. However, the HSV-1 nucleoprotein complexes were still far more unstable than cellular nucleosomes when HSV-1 DNA replication was inhibited with phosphonoacetic acid (PAA) [46]. The degree of instability was affected by PAA,

however, indicating that HSV-1 DNA replication does modulate the dynamics of the HSV-1 nucleosomes to some extent.

Figure 1. The dynamics of HSV-1 nucleosomes in lytic or latent infections. (**a**, **b**) Classic models proposed that most HSV-1 genomes were not properly chromatinized in lytic infections. Either all genomes were proposed to have only sporadic nucleosomes, or some genomes were proposed to be chromatinized, whereas most were proposed to not associate with nucleosomes. Both models would result in the observed protection from MCN digestion to mostly fragments of variable sizes, which show as a "smear" in Southern blots. In contrast, latent genomes were proposed to be normally chromatinized. In these classic models, the difference between lytic and latent viral genomes was qualitative (*i.e.*, non-chromatinized or chromatinized, respectively). (**c**) More recent evidence points to the HSV-1 genomes forming unstable nucleosomes, which unbind during the digestion, failing to protect the viral DNA. (**d**) In contrast, latent or quiescent HSV-1 genomes are in far more stable nucleosomes, which protect their DNA from MCN digestion, resulting in the typical "nucleosome ladder". According to the more recent evidence, the difference between lytic and latent HSV-1 genomes is quantitative (*i.e.*, different degree of nucleosome stability).



Two different patterns of HSV-1 nucleosome stability were observed at the earliest time that could be studied (1h after removing inocula). At these early times, the HSV-1 DNA was in very inaccessible nucleoprotein complexes in half of the experiments, or in the unstable complexes in the other half [46]. The highly resistant HSV-1 DNA observed at these early times (in half of the experiments) may well be HSV-1 DNA that has been mostly silenced by the cell and not yet desilenced by the virus. Alternatively, it could be DNA that is not yet decapsidated, or a mix of encapsidated and silenced DNA. However, HSV-1 DNA in naked capsids is fully resistant to MCN [90], whereas most of the so-called inaccessible nuclear HSV-1 DNA is eventually digested [47].

2.2.2. Histone Dynamics during Lytic Infections

As discussed, the interactions between histones and HSV-1 DNA are far more dynamic than those with cellular DNA. The DNA in the infecting virions amounts to only a fraction of the cellular DNA under common culture conditions. Assuming 10 infectious virions per cell and a reasonable 1:100 ratio of infectious to non-infectious virions, the total amount of HSV-1 DNA infecting a nucleus would amount to 2.5% of the cellular DNA if all genomes entered into the nucleus. Not all do, and the number of virions infecting each cell *in vivo* is estimated to be lower. However, HSV-1 DNA replication is exponential, and the total amount of viral DNA would still reach up to a significant proportion of the total nuclear DNA at later times after infection if 10 viral genomes replicated 1,000-fold each.

Most cells are not infected in S-phase when most core histone synthesis occurs, and infection inhibits histone synthesis [91–93]. There is a population of histones at any given time in the process of exchanging between different DNA binding sites (Figure 2a). These exchanging histones are momentarily not in nucleosomes and could therefore be used to chromatinize the infecting viral genomes. However, such sequestration of free histones would alter the equilibrium, indirectly promoting further release of histones from the cellular chromatin (Figure 2b). Considering the magnitude of the pool of the HSV-1 DNA at later times, the normal pool of the free histones would be insufficient to chromatinize all HSV-1 DNA. There is of course a much larger pool of histones in the cellular chromatin, but these histones would have to first be mobilized away from the cellular chromatin to be available (Figure 1c). Moreover, any histones in the particularly unstable HSV-1 chromatin would undergo chromatin exchange at a much faster rate than those in the cellular chromatin.

We therefore evaluated whether histone dynamics were altered in infected cells. Such dynamics can only be analyzed in intact nuclei. We therefore used fluorescence recovery after photobleaching (FRAP) [94], which had already been used to describe histone dynamics in non infected cells [95–97]. The highly mobile linker histone H1 was further mobilized in infected cells in a variant- and multiplicity-dependent manner [94]. The mobilization of the variant H1.2, which is widely expressed in all cells in which HSV-1 replicates, was evaluated in detail. Mobilization required the presence of transcriptionally capable HSV-1 genomes in the nucleus. It did not require VP16 or ICP0, but it was enhanced by VP16. It was surprisingly not affected by HSV-1 DNA replication, in that PAA did not affect it. At a given multiplicity of infection, H1.2 was mobilized to a larger degree in U2OS cells,

which functionally complement for defects in VP16 and ICP0 [94], than in Vero cells, which do not. U2OS appear to be defective in their ability to mobilize all histones in response to infection. This inability may facilitate the replication of mutants defective in proteins that counteract silencing.

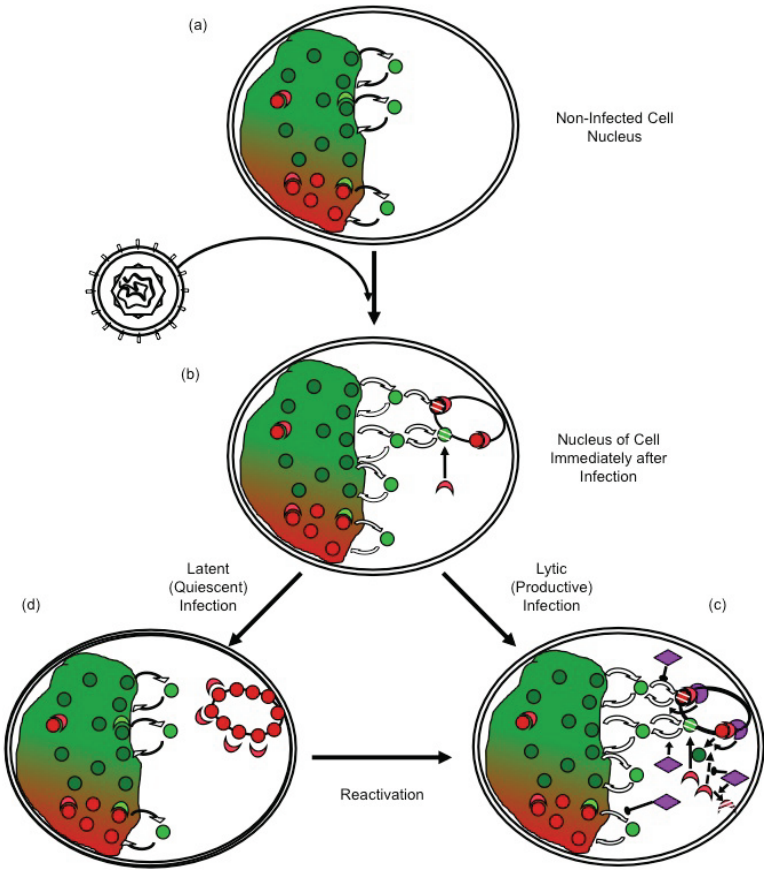
H1 mobilization may or may not reflect the faster core histone exchange predicted by the instability of the HSV-1 nucleosomes. It would not provide the core histones required to assemble HSV-1 nucleosomes, either. We therefore evaluated next the dynamics of core histones. We started with histones H2B and H4 [98], which have no variants that could be differentially mobilized. They also are components of each of the two tetramers within the nucleosome. Both H2B and H4 were mobilized in infected cells. Their free pools had already increased at four hours after infection, and continued to increase for another three hours (reaching approximately 50% over the free pools in mock infected cells).

The mobilization of H2B was analyzed in further detail. Its rate of fast chromatin exchange was slower in infected cells [98]. H2B is therefore likely mobilized away from cellular chromatin by preferentially inhibiting the re-binding of unbound H2B, not by promoting its release. H2B mobilization required no VP16 or ICP0, but both VP16 and ICP0 enhanced it [98]. Mobilization in the absence of VP16 and ICP0 in complementing U2OS cells did not decrease the rate of fast chromatin exchange [98]. Mobilization in U2OS cells in the absence of ICP0 alone even decreased the rate of fast chromatin exchange [98]. These results suggest that VP16 is the primary modulator of H2B fast chromatin exchange, at least under conditions of viral replication. Moreover, the pool of free H2B increased to a larger extent in Vero cells infected with the ICP0 null mutant n212 [98], suggesting that ICP0 induces the degradation of the mobilized histones. This potential role of ICP0 would be consistent with its many other ones inhibiting cellular defenses against viral gene expression [9].

Most surprisingly, H2B mobilization required no HSV-1 DNA replication (or late proteins) [98]. It did, however, require nuclear and transcriptionally capable HSV-1 genomes (although not their actual transcription). Mobilization was not dependent on relative histone levels, and the histones were mobilized throughout the population of infected cells [98].

The H3 variant H3.1 associates with HSV-1 genomes only after the onset of HSV-1 DNA replication, whereas the H3.3 variant associates with them, both before and after. The evidence to date suggests that histones are mobilized away from the cellular chromatin to silence HSV-1 genomes, but that HSV-1 proteins counteract this silencing by further mobilizing histones. If this model was correct, then the mobilization of histones H3.1 and H3.3 would be differentially affected by inhibition of HSV-1 DNA replication. Our ongoing experiments suggest that this is indeed the case. Inhibition of HSV-1 DNA replication by PAA drastically increases the pools of free H3.1, but not those of free H3.3 [99]. These results suggest that H3.1 is mobilized away from cellular chromatin immediately after infection, but it is assembled into viral chromatin only during HSV-1 DNA replication. Another testable prediction of the model is that specific HSV-1 proteins should induce enhanced histone mobilization, and that these proteins should be involved in the regulation of HSV-1 gene expression (Figure 1c). We are also working to test this prediction.

Figure 2. Regulation of HSV-1 gene expression by chromatin silencing and anti-silencing. (**a**) In the nuclei of non infected cells, histones (red and green circles) unbind from chromatin, diffuse through the nucleus and rebind to a different site. The diffusing histones are bound to histone chaperones in the "free pool". Histones exchange in both transcribed (green) and silenced (red) chromatin, but at different rates. Chromatin modifying enzymes (crescent shapes) modulate histone dynamics by modulating affinity for chromatin, DNA, or other proteins. (**b**) Immediately after nuclear entry, HSV-1 genomes are complexed by the cell in silencing chromatin (red), to which further silencing chromatin modifying proteins are recruited (red crescent shapes). (**c**) In productive infections, HSV-1 proteins (purple) mobilize histones away from the viral genomes, by indirectly modulating their PTM, availability, localization, or interaction with other proteins, and recruiting activating chromatin modifying proteins (green crescents). (**d**) Viral genes are then transcribed and the genomes are replicated. In latent or quiescent infections, the initial silencing chromatin is not disrupted efficiently, leading to the formation of (almost) fully silenced chromatin, which must be disrupted for reactivation to occur.



2.3. Potential Mechanisms of Regulation of Nucleosome Dynamics in HSV-1 Infected Cells

The regulation of nucleosome dynamics in infected cells is not yet fully understood. The same cellular chromatin proteins, chromatin-modifying enzymes, and chromatin-remodelling complexes regulate viral and cellular chromatin dynamics. It is consequently difficult to distinguish between cellular and viral nucleosome dynamics, or to identify the processes that regulate the turnover of the nucleosomes on the viral genome among the background turnover of the nucleosomes on the cellular genome.

The HSV-1 genomes in virions have no associated histones [62] and enter the nucleus compacted with spermine [44]. They must then be decondensed and assembled into chromatin for the cellular transcription proteins to access them. These processes may be concurrent or sequential. Meanwhile, cellular antiviral responses target repressive proteins to viral genomes [100,101], promoting the assembly of silencing heterochromatin [73]. The histones in the HSV-1 chromatin immediately after infection consequently bear PTM characteristic of heterochromatin [68,72,102–104], as do those associated with latent or quiescent genomes [13,53,55]. In lytic infections, however, these early HSV-1 nucleosomes are promptly modified with PTM characteristic of euchromatin. These PTM may cause the instability of the HSV-1 nucleosomes [46,47] and the increase in the overall histone exchange rates [94,98]. Conversely, the rapid turnover of HSV-1 nucleosomes may in itself impair the repressive PTM, and the recruitment of repressive heterochromatin proteins. Increased nucleosome dynamics also directly facilitates access to HSV-1 DNA, fostering transcription. The instability and dynamic nature of the viral chromatin may in itself inherently counteract genome silencing.

The cellular antiviral responses would thus be expected to decrease the dynamics of HSV-1 chromatin, while increasing those of cellular chromatin to make chromatin proteins available to silence the viral genomes (Figure 2b). Conversely, HSV-1 proteins would be expected to increase the dynamics of the viral nucleosomes, while inhibiting those of cellular chromatin to minimize the availability of silencing proteins (Figure 2c). If the cellular antiviral responses generally mobilize histones away from the cellular chromatin, then an initial outcome of the infection would be a generalized increase in chromatin dynamics. Indeed, histones are mobilized upon nuclear entry of HSV-1 genomes [94,98]. Mobilized cellular repressive proteins are also likely targeted to the viral genomes, in that HSV-1 proteins inhibit this targeting [9]. HSV-1 proteins also recruit activating chromatin proteins and modifying complexes (e.g., KATs or chromatin remodellers) [60,105,106]. This recruitment directly stimulates viral nucleosome turnover and facilitates transcription. Moreover, it also isolates such complexes from the cellular chromatin, indirectly decreasing cellular nucleosome turnover and restricting the availability of repressive proteins. If cellular histones or chromatin-modifying proteins could not be mobilized away from cellular chromatin, then repressive proteins would be less available to silence the HSV-1 genomes. Then, it would be far less critical for HSV-1 to be able to counteract silencing.

Mechanisms of Regulation of Nucleosome Dynamics

The stability and turnover of cellular nucleosomes are physiologically affected by several mechanisms, including: *(i)* the histone variants in the nucleosomes; *(ii)* their PTM; and *(iii)* the repositioning or eviction of nucleosomes by ATP-dependent chromatin remodelling complexes. These mechanisms most likely also modulate viral nucleosome turnover and stability.

Histone variants. Of all core histone variants, only the H3 variants H3.1 and H3.3 have been analyzed in HSV-1 chromatin [64]. Both are mobilized early during infection [99]. However, H3.3 is preferentially assembled into the viral chromatin at this time, whereas H3.1 is only assembled into it after the initiation of viral DNA synthesis [64]. The similar mobilization and differential assembly of H3.3 and H3.1 into HSV-1 chromatin indicates the specificity of nucleosome turnover and assembly during infection.

The endogenous free pool of H3.3 is increased in infected cells by mobilization of H3.3 from cellular chromatin [99]. Physiologically, H3.3 is assembled into cellular chromatin by three histone chaperones: HIRA, hDaxx, and DEK [107–109]. HIRA and hDaxx also participate in the assembly of HSV-1 chromatin [100]. Knockdown of HIRA decreases H3.3 interaction with HSV-1 genomes [64]. If HSV-1 nucleosomes strictly opposed transcription, then reduced H3.3 occupancy should increase viral transcription. Surprisingly, knockdown of HIRA decreases HSV-1 transcription [64]. HIRA knockdown also affects cellular chromatin dynamics, however, and could thus hinder the mobilization of H3.3 away from cellular chromatin. It would consequently decrease the pool of H3.3 available for silencing viral genomes. This decrease may affect the decondensation of decapsidated HSV-1 genomes, or their proper assembly into transcription competent chromatin. HIRA may also be important for viral nucleosome turnover during transcription (less dynamic viral nucleosomes would hinder transcription). The hDaxx/ATRX complex, which mediates the assembly of H3.3 into transcriptionally repressed chromatin, is recruited to HSV-1 genomes early during infection [100,110,111]. HIRA depletion may indirectly promote the hDaxx/ATRX-mediated assembly of repressive H3.3 nucleosomes with HSV-1 DNA, before ICP0 eventually disrupts this assembly complex [100].

H3.1 is only assembled into viral nucleosomes concomitantly with HSV-1 DNA replication [64]. Assembly is accompanied by a decrease in the pool of free H3.1 [99], and an increase in the level of total H3 occupancy on HSV-1 DNA [64,70]. Assembly of viral chromatin during HSV-1 DNA replication is also critical. Depletion of a component of the Caf1 complex that assembles H3.1 containing nucleosomes during cellular DNA replication, Asf1b, inhibits HSV-1 DNA replication [112]. Asf1 regulates cellular DNA replication fork progression by mediating the nucleosome disassembly and re-assembly ahead of and behind it, respectively [113]. Similar activities may be required for efficient viral DNA replication.

ICP0 targets the H3 variant CENP-A (and other centromeric proteins) for proteasomal degradation [80,114–116]. This degradation limits the potential for assembly of CENP-A nucleosomes with HSV-1 genomes. CENP-A is typically deposited in centromeric nucleosomes through replication-independent chromatin assembly [117]. The CENP-A chaperone, Holliday-junction

recognition protein (HJURP), is recruited to centromeres to mediate CENP-A nucleosome assembly [118–120]. Artificial targeting of HJURP to non-centromeric loci is sufficient for assembly of CENP-A nucleosomes, which in turn is sufficient to recruit constitutive centromere-associated proteins, such as CENPs-N, -M, -T, and –C [119]. HJURP recognizes Holliday-junctions, which may well form during circularization or replication of HSV-1 genomes. Nucleosomes containing CENP-A have slower turnover rates than those containing H3 [117], decreasing nucleosome dynamics and facilitating silencing.

Histone PTM, Acetylation. Histones are reversibly acetylated, by KAT (AKA histone acetyltransferases, HAT) and deacetylases (histone deacetylases, HDAC). Their acetylation neutralizes charge-dependent interactions with DNA and adjacent nucleosomes, promoting nucleosome turnover and increasing DNA accessibility.

The histones associated with HSV-1 genomes in lytically infected cells are enriched in H3K9ac and H3K14ac [50,121], PTM associated with active transcription and nucleosome instability. Consistent with such acetylation, the viral transcription transactivators VP16 and ICP0 recruit KATs and disrupt HDAC activity. VP16 recruits the KATs, GCN5, PCAF, and CBP/p300 to promote localized histone acetylation [60,86,106,122]. ICP0 also promotes histone acetylation through the stimulation of and interaction with PCAF, CBP/p300, and CLOCK, while disrupting HDACs [77,79,123–125]. Surprisingly, single or combinatorial knockdown of some of these KATs (CBP/p300, GCN5, PCAF) does not drastically affect IE gene transcription [69,89,126]. If anything, their depletion tends to enhance it [69,89,126]. However, global depletion of KATs would be expected to decrease the turnover of cellular nucleosomes. KAT-depleted cells likely have impaired nucleosome turnover before they are infected, and their ability to mobilize histones in response to infection is likely compromised, too. Viral gene expression would then have fewer obstacles to overcome in the knocked-down cells. Such effects on cellular nucleosome dynamics could very well account for the apparently paradoxical increase in IE gene expression in KAT depleted cells.

Global histone acetylation increases late during infection [50,121], but histones are mobilized to a basal degree before these changes occur [94,98]. The functional analysis of histone mobilization may be more sensitive than the morphological analysis of their PTM. Moreover, global evaluations cannot detect potentially relevant PTM in only the mobilized histones. The reduction in viral protein expression when CLOCK is depleted [125], for example, suggests that this KAT may have a more localized role during HSV-1 infection than p300, PCAF, or GCN5. The CLOCK/BMAL1 complex normally regulates circadian rhythms; it is consequently tightly regulated. CLOCK/BMAL1 is activated by SUMO-modification of BMAL1, which results in its localization to ND10s [127], ubiquitination, and proteasomal degradation [127]. However, ICP0 interacts with and stabilizes BMAL1 [125], stabilization which may promote localized CLOCK-mediated acetylation [128] of histones in the nuclear domains containing HSV-1 genomes. CLOCK may thus specifically promote viral nucleosome dynamics with little global effect on cellular nucleosome dynamics.

The interactions of ICP0 and VP16 with KATs and the dysregulation of HDACs indicate the important roles of these chromatin-modifying enzymes in the regulation of HSV-1 nucleosome dynamics and gene expression.

Histone PTM, Methylation. Reversible histone methylation is catalyzed by lysine histone-methyltransferases (KMTs) and lysine histone-demethylases (HDM). Histone methylation is a complex mechanism with mono- to tri-methylation of specific lysine residues contributing to gene activation or repression. For example, histone H3 methylation on lysines 4 (H3K4me) or 36 (H3K36me) correlate with gene activation, whereas methylation on lysines 9 (H3K9me) or 27 (H3K27me) correlate with gene repression. Some methylations are mutually exclusive, such as H3K4me and H3K9me (fully so) or H3K4me and H3K27me (partially so). The effects of methylation on histone dynamics are less direct than those of acetylation, phosphorylation, or poly-ADP-ribosylation, as methylation does not alter histone-DNA charge interactions.

The histones associated with HSV-1 genomes early after infections were modified with the repressive H3K9me3. HCF-1 then recruits a regulatory complex containing Set1/MLL1 KMT and LSD1/JMJD HDM to the viral IE promoters [61,102–104,129,130]. JMJD and LSD1 HDMs act in sequence to remove H3K9me3, such that the SET1/MLL1 KMT can then methylate H3K4 [61,68,102–104]. Removal of H3K9Me, and the subsequent methylation of H3K4, is critical for HSV-1 transcription and DNA replication [61,68,102–104]. Inhibition of JMJD or LDS1 increases histone occupancy and inhibitory PTM on the HSV-1 genomes, inhibiting HSV-1 transcription and DNA replication [68,103,104]. The accumulation of histones bearing repressive methylation in the absence of LSD1 or JMJD indicates that the replacement of H3K9me3 with H3K4me3 is critical in the regulation of HSV-1 nucleosome dynamics. Accumulation of H3K9me3 on HSV-1 chromatin also induces the subsequent association of HP1 and the formation of heterochromatin, further restricting viral nucleosome dynamics and promoting HSV-1 genome silencing.

SET7/9 methylation appears to hinder HSV-1 gene expression. SET7/9 generally methylates H3K4 to promote gene expression [131,132]. However, depletion of SET7/9 stimulates HSV-1 gene expression and replication [61]. This effect parallels the stimulation of HSV-1 gene expression in HIRA or KAT depleted cells. SET7/9 may therefore also function in the global regulation of nucleosome dynamics, as opposed to having only localized effects on HSV-1 nucleosomes. SET7/9 depletion may hinder the mobilization of histones from the cellular chromatin, which may in turn limit the availability of histones to silence viral gene expression.

Histone PTM, Phosphorylation. Phosphorylation directly contributes to histone exchange and nucleosome dynamics by introducing negative charges, which weaken interactions with DNA. However, the phosphorylation of histones in HSV-1 or cellular chromatin during infection remains largely unknown. The sole histone phosphorylation directly examined is that of H2A.X (referred to as γ-H2A.X). H2A.X phosphorylation typically occurs in response to cellular DNA damage. H2A.X is also phosphorylated during HSV-1 infection [133], perhaps due to the activation of some components of the cellular DNA damage response. γ-H2A.X localizes to the cellular chromatin at the periphery of viral replication compartments [133].

Histone phosphorylation also regulates nucleosome turnover indirectly. Phosphorylation of H3T118 promotes nucleosome disassembly by SWI/SNF chromatin remodelling complexes [134]. H3T118P or H3T32P also have high affinity for the histone chaperone NAP [135], an interaction that precludes H3 binding to DNA [135]. Increased NAP association therefore likely stimulates nucleosome disassembly and turnover. Phosphorylation of H3T11 stimulates JMJD2C activity towards H3K9me2/me3, whereas phosphorylation of H3T6 prevents LSD1 demethylation of H3K4 and stimulates preferential demethylation of H3K9 [136]. By such mechanisms, H3 phosphorylation could also modulate the demethylations critical for initiation of viral IE gene transcription [68,103,104].

Histone phosphorylation also stimulates transcription through the reduction of silencing stimuli. For example, phosphorylation of H3S28 displaces the PcG (polycomb group) KMT that mediates gene silencing though the establishment of H3K27me3 [137], and H3S10P impairs HP1 binding to H3K9me3. Via such mechanisms, histone phosphorylation inhibits nucleosome stability and heterochromatin formation [138,139], disrupting gene silencing and promoting nucleosome turnover. Phosphorylation also promotes gene expression more directly. For example, H3S10P promotes the activating GCN5 acetylation of H3K14 at the promoters of transcribed genes [140].

Linker histones have several phosphorylation sites in their N- and C-terminal tails [141]. H1 directly stabilizes chromatin and promotes the formation of higher-order chromatin structures [141]. It also hinders core histone acetylation and nucleosome remodelling by ATP-dependent chromatin remodelers [142,143]. These effects reduce nucleosome turnover. H1 phosphorylation promotes its disassociation from chromatin [144], and H1 chromatin exchange is enhanced in infected cells [94]. H1 mobilization may be required to permit the mobilization of core histones, or core histone mobilization may promote that of H1. However, whether HSV-1 chromatin contains H1 has not been evaluated; nor has the contribution of H1 phosphorylation to its mobilization in infected cells.

Histone PTM, Ubiquitination. All core histones are ubiquitinated, but ubiquitination of H2A and H2B is the most prevalent. Mono-ubiquitination of H2A is generally associated with transcription repression, whereas that of H2B contextually regulates transcription and chromatin structure. H2A and H2B are also ubiquitinated in response to DNA damage. HSV-1 encodes its own ubiquitin ligase, ICP0 [10,145,146], and thus modulates ubiquitination pathways. Although ICP0 mediated ubiquitination is well known to limit cellular antiviral responses, its effects on chromatin dynamics are not fully elucidated.

Monoubiquitination of H2A, on K119, is largely mediated by the E3 ligase component of polycomb repressive complex 1 (PRC1), RNF2 (Ring1 and Ring2). H2AK119ub facilitates the chromatin association of H1 [147], which in turn promotes the formation of higher-order chromatin structures to limit DNA access and repress gene expression [148]. H2AK119ub is sufficient to instigate chromatin compaction and gene repression, in that the PRC1- like 4 (PRC1L4) complex is recruited to chromatin independently of additional repressive PTM (such as H3K9me or H4K20me) [148]. H2AK119ub would limit HSV-1 nucleosome dynamics and promote silencing. Ubiquitination of H2A in HSV-1 nucleosomes has yet to be evaluated, as does the regulation and recruitment of the relevant E3 ligases.

The total nuclear levels of H2Aub decrease during lytic infection [149]. Such a global decrease would globally decrease nucleosome stability, promoting nucleosome turnover and increasing the availability of histones to silence viral genomes. Like H2B and H4, H2A is also mobilized during infection [150]. Viral mechanisms may also locally decrease H2A ubiquitination to specifically promote the turnover of HSV-1 nucleosomes.

Two E3 ubiquitin ligases, RNF8 and RNF168, typically catalyze the ubiquitination of H2A and H2A.X in response to cellular DNA damage. RNF8 localizes first to DNA lesions, but does not ubiquitinate nucleosomal H2A efficiently [151]. The formation of H2A polyubiquitin chains by RNF8 requires previous mono-ubiquitination on H2AK13-15 by RNF168 [151]. H2AK13-15ub is proposed to promote nucleosome exchange to facilitate DNA repair [151]. The H2A polyubiquitin chains recruit other proteins to the sites of DNA damage, including the E3 ligase BRCA1/BARD1 complex that further ubiquitinates H2A and H2B [152]. The decrease in ubiquitinated H2A (and H2A.X) during HSV-1 infection results from ICP0 mediated proteasomal degradation of RNF8 and RNF168 [81]. ICP0 also disrupts the redistribution of BRCA1 to the nuclear domains associated with HSV-1 genomes [153]. These activities of ICP0 suggest that H2A ubiquitination limits HSV-1 nucleosome dynamics to restrict viral gene expression. Under this model, the H2A in HSV-1 chromatin during latency or quiescence would be expected to be ubiquitinated. In contrast to its effects on RNF8 and RNF168, ICP0 does not alter the levels of RNF2 or 2A-HUB [81]. These ubiquitin ligases may thus still ubiquitinate a sub-population of H2A in the context of a global decrease in H2Aub.

Ubiquitination of H2BK120 (H2BK123 in yeast) stabilizes the nucleosome and promotes its assembly during transcription or DNA replication [154–156]. Nucleosomes with ubiquitinated H2B have slower turnover rates, resulting in apparently increased nucleosome occupancy [154]. Decreased H2Bub conversely enhances nucleosome dynamics, resulting in an apparent genome wide decrease in nucleosome occupancy [154,155] Ubiquitination of H2B in promoter nucleosomes inhibits transcription [155]. The slower turnover rates of H2Bub nucleosomes are proposed to prevent RNAPII binding or transcription complex assembly [154,155]. In contrast, ubiquitination of H2B in coding regions facilitates nucleosome reformation following passage of RNAPII, supporting transcription elongation [154,155]. H2Bub also stimulates the activating H3K4 and H3K79 methylations [154,157]. In contrast, H2Bub inhibits H3K36 methylation [155], a PTM enriched in transcribed genes (of yeast) which inhibits histone exchange during transcription [158]. The levels of ubiquitinated H2B decrease during infection independently of ICP0 [81]. ICP0 ubiquitination of a sub-population of H2B may promote HSV-1 transcription by regulating RNAPII transcription elongation.

Ubiquitination of H2B also promotes nucleosome reassembly during DNA replication and contributes to the progression of the replication fork [156]. In yeast, non-ubiquitinable H2B mutants destabilize the replisome, impairing replication fork progression [156]. They also have decreased H3 occupancy at the origins of replication and delayed H3 deposition during DNA replication [156]. The phenotype is consistent with defects in nucleosome formation, or stabilization, during chromatin assembly. Under such replication stress, homologous recombination (HR) dependent mechanisms may be activated to complete DNA replication [156]. The global decrease in ubiquitinated H2B during

infection may contribute to the removal of nucleosomes from the HSV-1 genomes for encapsidation. Loss of ubiquitinated H2B in HSV-1 chromatin during DNA replication may impair DNA replication and activate HR pathways. Efficient HR repair of cellular double-stranded breaks (DSB) requires NBS1, ubiquitination of H2B by RNF20, and SNF2H [159]. These proteins induce chromatin remodelling and promote the eviction of nucleosomes adjacent to the DSB to facilitate DNA resection [159]. SNF2H and NBS1 localize to the HSV-1 replication compartments, where they may well enhance nucleosome dynamics during HSV-1 DNA replication or genome encapsidation by similar mechanisms. Unfortunately, the localization of RNF20 in infected cells is still unknown.

Histone PTM, SUMOylation. SUMO modification contextually promotes transcriptional activation or repression. SUMOylation mediates the recruitment of chromatin-modifying factors. In turn, such factors modulate nucleosome dynamics to regulate transcription. Some of the SUMO-regulated repressive chromatin modifying-complexes, such as hDaxx/ATRX, CoREST/LSD1, and HDACs, as well as HP1 are important regulators of HSV-1 nucleosome dynamics. ICP0, a SUMO-targeted ubiquitin ligase (STUbL) [160], may very well disrupt the recruitment of repressive complexes to HSV-1 genomes by targeting essential SUMOylated proteins.

All core histones are SUMOylated [161–163]. Histone SUMOylation is generally associated with transcriptional repression [161,162], and opposes activating PTM, such as acetylation [162]. SUMOylation would thus be expected to impair nucleosome dynamics. However, SUMO-labeled H2B was associated with transcriptionally active euchromatin and excluded from heterochromatin [154]. Furthermore, SUMOylated H2B globally altered chromatin structure, enhancing nucleosome dynamics [154]. There is an increase in the levels of SUMO conjugated proteins during infection with ICP0 null HSV-1, and SUMO and SUMO-conjugating enzymes localize to nuclear domains adjacent to infecting HSV-1 genomes [160]. Histone SUMOylation may regulate HSV-1 nucleosome dynamics to promote or repress HSV-1 transcription. The SUMO-mediated recruitment of repressive complexes to the viral genomes, however, implicates this PTM in viral repression.

Histone PTM, Poly-ADP-ribosylation. Proteins are reversibly modified with mono- to poly-ADP-ribose (PAR) moieties. Linker and all core histones have ADP-ribosylation sites [164]. Histone H1 is a major PAR substrate, with glutamate ADP-ribosylation sites in the amino (N)- and carboxyl (C)-terminal tails and a lysine ADP-ribosylation site in the C-terminal tail. Core histones have one (H2A, H2B, H4) or two (H3) lysine ADP-ribosylation sites in their N-terminal tails. H2B has an additional glutamate modification site in its N-terminal tail. Typically, only a small percentage of histones are ADP-ribosylated at any given time. ADP-ribosylation adds a negative charge, which is compounded in poly-ADP-ribose chains. Histone ADP-ribosylation relaxes chromatin structure, potentially via charge-repulsions similar to those of acetylation. Consistently, ADP-ribosylation is associated primarily with transcriptionally active loci and chromatin that is extensively poly-ADP-ribosylated is more dynamic. For example, PARylation by the ADP-ribosyltransferase (ART) ARTD1 (PARP1) induces rapid transcription-independent loss of nucleosomes (H3) across the *Hsp70* gene after heat shock [165].

HSV-1 activates ARTD1, possibly as a result of activation of the DNA damage responses, globally increasing poly-ADP-ribosylation levels [166]. ARTD1, and the related ARTD5 (Tankyrase1), localize to the replication compartments [166–168]. However, their substrates, or the PARylation of histones, during infection are unknown. ADP-ribosylation of histones in HSV-1 nucleosomes would enhance their dynamics. Consistently with a role for PARylation in activating HSV-1 transcription, ICP0 triggers proteasomal degradation of the poly-(ADP-ribose) glycohydrolase (PARG) to promote the maintenance of PAR chains [166]. However, inhibitors of ADP-transferase activity only marginally decrease viral replication [166,168]. PARylation would likely also contribute to cellular nucleosome dynamics. As discussed, any inhibition of any mechanisms that mobilize histones to make them available to interact with viral genomes can lead to a variety of outcomes, depending on whether the modification plays a more prominent role in the viral or cellular nucleosomes.

ATP-dependent chromatin remodelling. Chromatin remodelling complexes are large multi-subunit complexes that catalyze the ATP-dependent restructuring and repositioning of nucleosomes. The ATP-dependent chromatin remodellers are classified into the SWI/SNF, ISWI, CHD, and INO80 families. The ISWI family mainly space nucleosomes via sliding. These complexes have roles in chromatin assembly, and transcription repression or activation. ISWI remodellers bind extrachromosomal DNA, with preference for longer DNA, to remodel and centre nucleosomes. Single-stranded DNA gaps, common in HSV-1 genomes, do not impede ISWI remodelling activity. SWI/SNF produce multiple outcomes including octamer ejection, dimer displacement and exchange, nucleosome sliding, and disome formation [169]. They also promote nucleosome exchange by facilitating octamer transfer in trans. Their BRG/BRM catalytic subunits have C-terminal bromodomains, which bind to acetylated histones and contribute to promoter targeting.

Chromatin remodellers from the ISWI (SNF2H) and SWI/SNF (BRG1, BRM, BAF155, BAF57, BAF170, and BAF60a) families interact with HSV-1 proteins and localize to the replication compartments [60,106,167]. SNF2H promotes HSV-1 gene expression and replication [105], and localizes to late replication compartments [167]. Knockdown of SNF2H decreases IE gene transcription, and viral replication, and increases H3 occupancy on HSV-1 promoters [105]. The apparent late reduction in H3 occupancy is SNF2H independent, however, suggesting that the largest effect of SNF2H is in the initial chromatin assembly and viral gene activation [105]. Consistent with this model, SNF2H is recruited by H3K4me-2 -3 [170], an important PTM in the activation of IE gene expression. VP16 recruits BRG and BRM to IE promoters [60,106]. BRG/BRM may also be recruited to acetylated HSV-1 promoters via bromodomains to regulate nucleosome dynamics during HSV-1 gene transcription. Depletion of BRG and BRM subunits unexpectedly tended to increase IE gene expression [69,89]. As other chromatin modifiers, however, BRG/BRM may well regulate the nucleosome dynamics in cellular chromatin as well. Their depletion would then impair the mobilization of histones from cellular chromatin, impairing the cellular antiviral responses that restrict viral replication. The available data suggest that chromatin remodelling by SNF2H is more relevant for HSV-1 nucleosome dynamics, whereas remodelling by BRG/BRM likely also regulates cellular nucleosome dynamics.

The mammalian family of INO80 chromatin remodelling ATPases exchange H2A for the variant H2A.Z. Nucleosomes containing H2A.Z, particularly in combination with H3.3, are highly unstable and promote chromatin relaxation and nucleosome exchange [33]. The NuA4 complex is recruited to cellular DSB, where the p400 ATPase component catalyzes the exchange of H2A with H2A.Z [171]. Subsequently, the Tip60 subunit mediates H4 acetylation [171]. The NuA4 complex thus initiates chromatin relaxation around the DSB to promote nucleosome dynamics and create a DNA template suitable for repair. The increased turnover of histones γH2A.X, H2A.X, and H3 within damaged chromatin requires the p400 ATPase activity and H2A.Z [171]. H2A.Z is mobilized during HSV-1 infection [150], although the presence of H2A.Z in HSV-1 chromatin or the activity of NuA4 during HSV-1 infection has yet to be evaluated. Tip60 is recruited to β- and γ-herpesvirus genomes, to stimulate lytic replication [172]. However, whether it is recruited as a component of the NuA4 complex or not was not evaluated. NuA4 is recruited to DSB independently of γH2A.X [171]. The NuA4 complex may thus also be recruited to HSV-1 DNA, which is devoid of γH2A.X, to mediate the exchange of H2A with H2A.Z in viral nucleosomes. The assembly of H2A.Z in HSV-1 nucleosomes would increase the instability of the viral chromatin and counteract cellular silencing attempts. However, H2A.Z and NuA4 would also be expected to regulate both cellular and viral chromatin dynamics. The potential effects of their depletions on HSV-1 gene expression, therefore, would depend on the balance of their activities on the cellular and viral chromatin.

3. Conclusions

The results of the original MCN protection and chromatin immunoprecipitation (ChIP) assays appeared to be incompatible. The former seemed to indicate that there were not enough nucleosomes on the HSV-1 genomes to regulate transcription (Figure 1). In contrast, the latter appeared to indicate that chromatin did regulate HSV-1 gene expression. It has become clear in the recent years that HSV-1, and cellular, chromatins are highly dynamic. With this new understanding, the results from ChIP, MCN accessibility, FRAP, and biophysical fractionations are entirely consistent with a model in which infected cells attempt to silence the infecting HSV-1 genomes by establishing silenced chromatin, but viral proteins disrupt this silencing by mobilizing histones away from the viral genomes (Figure 2). However, the mechanisms whereby histones are mobilized away from the cellular genome, then assembled in chromatin with viral genomes, and then mobilized away from them remain to be elucidated. The analyses of these mechanisms are complicated because the same general mechanisms are likely involved in the regulation of cellular and viral chromatin dynamics. The available evidence suggests that the same cellular proteins, complexes, and enzymes are likely involved in mobilizing the histones away from the cellular genome, and in mobilizing them away from the HSV-1 genomes. The first mobilization would make the proteins available to silence the HSV-1 genomes, whereas the second would de-silence the HSV-1 genomes and activate HSV-1 transcription. Consequently, perturbations of the steady state chromatin dynamics have opposing effects. They increase (or decrease) the availability of silencing proteins at the same time that they increase (or decrease) the ability of HSV-1 to mobilize these proteins away from its genomes. Most likely, new techniques,

ingenuity, innovation, and a little luck will all be needed to analyze the mechanisms of silencing and antisilencing that govern the regulation of viral gene expression by chromatin.

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Conflicts of Interest

The authors declare no conflict of interest.

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Review

Role of Polycomb Proteins in Regulating HSV-1 Latency

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Abstract: Herpes simplex virus (HSV) establishes a latent infection within sensory neurons of humans. Latency is characterized by the transcriptional repression of lytic genes by the condensation of lytic gene regions into heterochromatin. Recent data suggest that facultative heterochromatin predominates, and that cellular Polycomb proteins are involved in the establishment and maintenance of transcriptional repression during latency. This review summarizes these data and discusses the implication of viral and cellular factors in regulating heterochromatin composition.

Keywords: HSV; herpes; PRC; Polycomb; heterochromatin; latency; reactivation

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1. Introduction

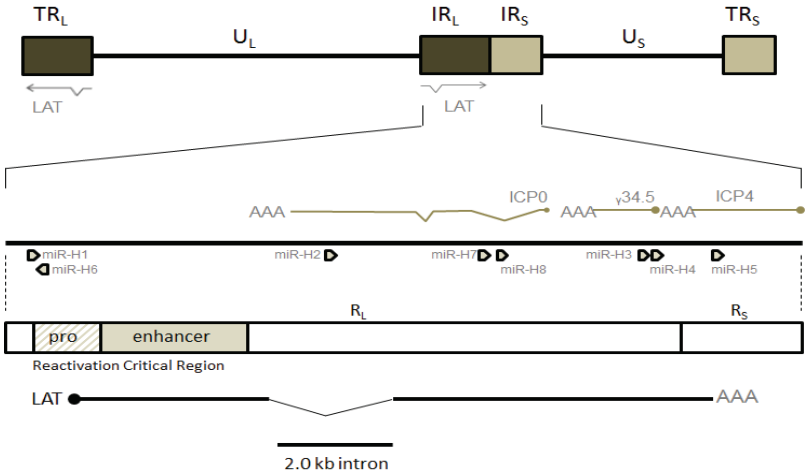
Persistent viral infections, especially those that establish latency, utilize epigenetic modifications to regulate transcription from their latent genomes as well as to regulate their entry and exit from latency. The goals of this review are to: (1) provide an overview of epigenetic regulation of Herpes Simplex Virus type 1 (HSV-1) gene expression during latency; (2) review the evidence for polycomb proteins playing a role in regulating heterochromatin deposition on the latent genomes; and (3) discuss potential mechanisms for the remodeling of heterochromatin to facilitate selective reactivation of HSV-1 from latency.

1.1. Evidence for the Role of Histone Post-Translational Modifications (PTMs) in Regulating Lytic and Latent Phases of Infection

Upon transport into the cell body of neurons, there is an ever-growing list of candidate viral and cellular factors that determine HSV's propensity towards either productive replication or descent into latency. During the latter, the non-random distribution of histones and functionally distinct PTMs partition the roughly 152 kb HSV-1 episome into regions with different chromatin profiles. As discussed later, regions associated with the three temporal classes of lytic genes display very dense deposition of histone marks typical of facultative or constitutive heterochromatin, conveying reversible or irreversible transcriptional repression. During this time, in an estimated third of latently-infected neurons, only non-coding transcripts are transcribed from the HSV-1 genome [1,2]. These include microRNAs and long non-coding RNAs, especially the extensively studied but still equivocal latency-associated transcript (LAT) (Figure 1). The LAT is transcribed as an 8.3–8.5 kb primary transcript. A single splicing event produces a remarkably stable 2.0 kb intron which is found at high concentrations in LAT-producing neurons, and a second splicing produces a 1.5 kb intron [3–5]. Based on the potential interactions of the LAT with a set of counter-acting cellular and viral factors, including chromatin remodeling proteins, the LAT may mediate the pivotal balance between ultimate outcomes for the cell-virus system, including uncontrolled lytic replication (termination of the cell), irreversible episome silencing (termination of the virus), or induction of apoptosis (termination of the cell and virus). The LAT plays varying roles in the conceptual models of latency proposed by different researchers, a conflict complicated by results that don't directly compare between experimental models (discussed below).

The chromatin profile of HSV-1 at any time during infection is commonly probed using chromatin immunoprecipitation (ChIP) of desired nucleosome components followed by DNA analysis. Early applications utilized semi-quantitative methods to quantify enrichment, but qPCR using primers specific to members of different HSV-1 gene classes has become the norm. The use of high throughput sequencing (ChIP-seq) holds promise for generating HSV-1 chromatin profiles with greater resolution and coverage. Though methods of analysis for ChIP data have been converging over the last decade, there remains some controversy as to what are the most meaningful and statistically valid means of quantifying the enrichment of any one chromatin mark on the viral genome. This shortcoming complicates the assembly of data provided by independent groups into a holistic map of heterochromatin on the HSV-1 genome.

Figure 1. Genome and Features of HSV-1. HSV-1 is comprised of a unique long (U_L) and unique short (U_S) region flanked by long and short repeat regions. The primary latency-associated transcript (LAT) transcript is located in the long repeat region and is 8.5 kb long, as shown in the expanded section. Also shown are key regulatory regions for the LAT as well as the 2.0 kb LAT intron. Note that lytic gene transcripts ICP0, ICP34.5 and ICP4 and miRNAs miR-H1 through miR-H8 are present in this region.



1.2. Animal Models Used to Study HSV-1 Latency

Despite its apparent tight co-evolution with humans, HSV-1 can produce infection in a variety of non-human animals, often with characteristic neuronal invasion and establishment of latency. For the purposes of studying HSV-1 epigenetics, mouse models are frequently utilized with the majority of data regarding latent HSV-1 chromatin dynamics generated using the footpad or corneal routes of infection. Following infection, the virus migrates along the axons of the sensory neurons afferent to these sites, and, for mice surviving the initial infection, latency is reliably and stably established in the dorsal root ganglia (DRG) or trigeminal ganglia (TG), respectively. Though the model's consistency engenders its utility, the difficulty in detecting any clinical shedding of virus during reactivation at the primary site of infection is one of the more important dissimilarities to human pathology. Some experimentalists have developed protocols to induce reactivation via thermal stress [6], immunosuppression [7], or sodium butyrate injection [8]. Far more commonly, reactivation is induced *ex vivo*; latently infected ganglia are explanted into culture medium, where isolation from their axonal processes and natural physiological environment serves as a stressor that efficiently and synchronously induces reactivation [9,10]. Alternatively, the rabbit ocular model, though limited by its expense, is considered the most analogous to human infection, allowing for reliable clinical reactivation in live animals using ocular iontophoresis of adrenergic agents [11]. For a more thorough review of the rabbit and mouse models of latent infection, see Webre *et al*. [11].

1.3. In Vitro *Models of HSV-1 Latency*

Cell culture systems are also frequently employed. Cell lines used to study the HSV-1 chromatin dynamics during lytic infection include HeLa cells (human cervical carcinoma) [12–16], Sy5y cells (human neuroblastoma) [17,18], Vero cells (monkey kidney epithelia) [14,18] and U2OS cells (human osteosarcoma) [14]. Additionally, quiescent infection can be established *in vitro* as a means of emulating latency. For such applications, several human fibroblast cell lines have been employed, and HSV-1 can be compelled to quiescence through the use of mutant strains which lack the ability to drive expression of initial lytic genes [19–21] or through the provision of exogenous inhibitors of lytic replication. Addition of complementing proteins *in trans*, or removal of inhibition can, in some ways, emulate reactivation. These *in vitro* systems allow for more precise genetic assays and more defined control over the cellular and viral life cycle progression, but have acknowledged limitations on physiological relevance. More recently, the development of dissociated cultures of primary neurons either from the adult mouse TG [22] or embryonic rat superior cervical ganglia [23] show great promise in mirroring many aspects of HSV-1 latency seen *in vivo*, including the stable transcriptional repression of lytic genes and the ability to reactivate following stimulation, in a manipulable *in vitro* setting (for a review see [10]).

1.4. Differences in Biological Properties of Different Strains Used to Study HSV-1 Latency

Special effort is required to integrate data obtained in distinct experimental systems; cell cultures lack extrinsic factors that may influence chromatin regulation such as exogenous immune effectors and signaling molecules. The animal systems, though more holistic as models, may have developed significant differences in epigenetic regulation strategies through evolutionary divergence, and the relevance of these differences is compounded in the context of a non-equilibrium host-pathogen interaction (*i.e.*, a pathogen evolved to carefully exploit the nuances of a phylogenetically distinct host). As a notable example, an HSV-1 KOS-derived LAT mutant exhibits a 5–10 fold increase in expression of lytic genes in latently infected mouse TGs, whereas a similar mutation in a 17*syn*+-based mutant results in a 3–154 fold decrease in expression of the same genes for latently infected rabbits [24,25]. Even more subtle experimental parameters may produce distinct outcomes. For example, phenotypic differences between wild-type and LAT mutants may be observable in Swiss Webster mice but not BALB/c mice [26] or mice inoculated via the cornea but not the footpad [27]. With these issues in mind, it is essential to employ carefully considered experimental controls. In addition, sequence analysis of many of the commonly used HSV-1 strains reveal differences, some of the most dramatic of which are in the LAT region. Furthermore, as the functionality of at least some HSV-1 proteins varies among different models of infection [10] the conclusions drawn therein may have limited predictive strength with regards to human infection.

2. Neuronal Basis of HSV Latency

A novel feature of HSV-1, compared to other viruses that spread hematogenously, is that once the virus gains access to a nerve terminus, it enters that neuron and travels to the cell body of the neuron in the sensory ganglion. HSV-1 does not tend to spread laterally to other neurons within the ganglia and instead is limited to transynaptic spread to neurons outside the ganglion, or transport back to the epithelium. Therefore the latent reservoir of the virus is limited to those neurons in the sensory and autonomic ganglia with projections that extend to the initial site of infection at the epithelial surface; this explains why lesions tend to recur at only the initial site(s) of infection. Therefore, in the familiar orolabial route of primary infection, the latent reservoir is comprised of neurons located in the mandibular/maxillary tract of the trigeminal ganglion with axons afferent to the mouth [28]. In contrast, infections of the eye establish latency within the ophthalmic tract of the TG [29]. The molecular basis for why HSV-1 establishes latency only in neurons is not known, though it is likely due to the absence of factors in at least some neurons that promote robust activation of the lytic transcriptional program.

It is important to note that sensory neurons in the peripheral nervous system represent a very diverse and highly specialized population of cells that serve to detect a wide range of different types of sensation including hot and cold, pain, vibration, moisture, and touch. Therefore it is not surprising that there are almost 2 dozen types of sensory neurons characterized to date whose representation differs in the various sensory ganglia and individual specialized ganglia, like the TG and DRG (for a review see [30]. Also not surprisingly, HSV-1 has been shown to establish latent infection preferentially in specific populations of sensory neurons within the TG [31,32]. Studies have used antibodies specific for functional receptors such as the high-affinity nerve growth factor receptor (trkA), molecules involved in pain sensation (substance P receptor), or cell surface markers that are expressed on different sub-populations of sensory neurons to identify those neurons that are infected with HSV [32,33]. These analyses revealed that HSV-1 tends to establish latency predominately within a subclass of trkA+ neurons expressing the cell surface molecule recognized by the monoclonal antibody A5. In contrast, HSV-1 tends to initiate a predominately productive infection within neurons expressing a cell surface molecule recognized by the monoclonal antibody KH10. The molecular basis that defines these neurons as supporting productive *vs*. latent infection are not known, but point out the roles that specific cell populations play in the biology of HSV-1 latency. In addition, it is important to note that while A5+ cells represent a defined population of HSV-1 latent neurons, they make up only about 25% of the latent reservoir, and further studies are needed to define the phenotypes of the other cells which support latency, and their contribution to the pool of reactivating virus.

3. Chromatinization of HSV-1 Genomes during Latency

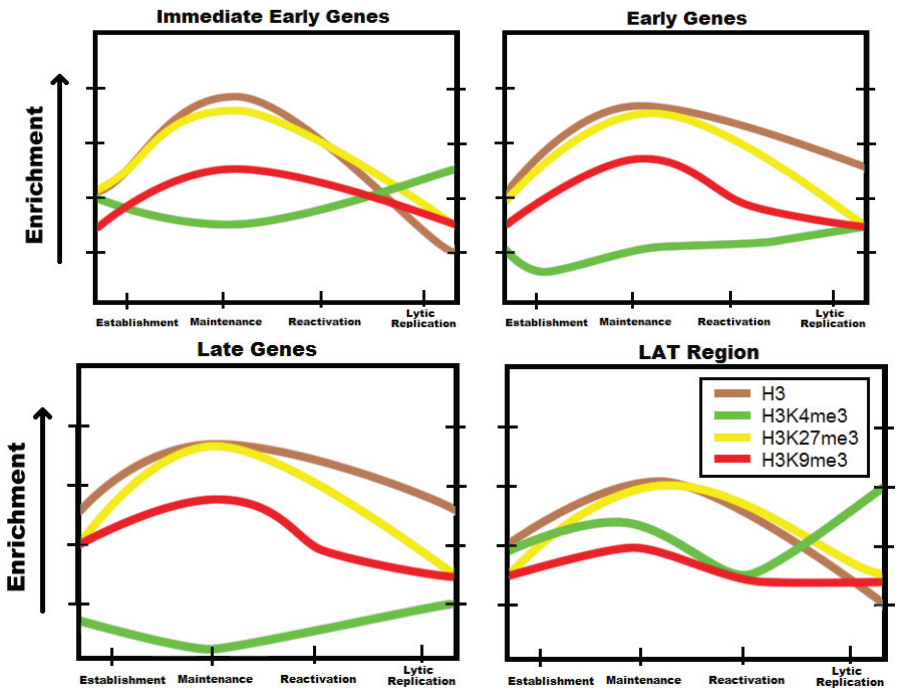
Histones are proteins that serve to package and condense DNA. The DNA strands are wrapped around an octamer core classically composed of histones H2A, H2B, H3 and H4. The large nucleosomal structure may serve to constrain or reveal binding sites depending on which histones are

present and where they are placed. HSV-1 has been shown to associate with histones *in vivo*, but does not encapsulate them in the virion and thus must recruit them from the host nucleus [18]. During primary lytic infection, HSV-1 is able to associate with histones as quickly as 1 hour post infection (hpi) [18]; these histones are only loosely associated with the virus and are not spaced at regular intervals [14,34,35]. As the virus enters latency in neuronal cells, histones begin to accumulate on the viral genome in appreciable amounts at 5 days post-infection (dpi) and increase over time [36]. When treated with micrococcal nuclease, the latent viral genome produces a classic ladder pattern on a gel or Southern blot [34]. This nucleosomal pattern is similar to those of eukaryotic DNA, suggesting that histones are recruited to the virus to regulate transcription in an active and deliberate manner.

The importance that chromatin structure plays in HSV-1 latency is further evidenced by the fact that histones associated with the latent viral genome are often modified to more tightly repress genes. The histone variant macroH2A, a repressive histone subunit that replaces H2A, is enriched in latent HSV-1, especially on lytic genes [37]. Furthermore, certain amino acid residues on the N-terminus of histones can be modified, changing their interactions with proteins and nucleic acids. A common posttranslational modification (PTM) to histones that is indicative of a transcriptionally permissive state is tri-methylation of histone 3 at lysine 4 (H3K4me3). These are most enriched in the promoter of active genes [38]. Two PTMs commonly associated with gene repression are methylation of H3K9 and H3K27. H3K9me2 and H3K9me3 are abundant on constitutive heterochromatin. In eukaryotes, these are areas of the genome that are tightly condensed and largely transcriptionally silent such as the pericentromeric regions [39,40]. H3K27 methylation, on the other hand, is a hallmark of transcriptionally repressed facultative heterochromatin. These marks are more labile and are present on genes that may be activated or repressed at specific time points such as developmental genes [39]. While both of these PTMs correlate with gene repression, they are regulated by different methylases and demethylases, suggesting that the virus is using multiple pathways to silence genes.

Figure 2. Trends in the chromatin profile of the viral genome through the HSV-1 life cycle for chosen epigenetic marks. Several groups working independently have demonstrated that the HSV-1 genome associates with a variety of investigated post-translational modifications, and that the density of any posttranslational modification (PTM) is differential with respect to each HSV-1 genetic loci and to each phase of the viral life cycle. Shown are epigenetic marks with special importance to repression during latency: histone H3, H3K4me3 (characteristic of transcriptional permissiveness), H3K27me3 (characteristic of reversible repression), and H3K9me3 (characteristic of irreversible repression). The general trends for temporal changes in enrichment of each mark are shown for the establishment of latent infection in neurons, the maintenance of transcriptional repression, the reactivation of the virus from latency, and the transition into productive replication. These trends, based on a survey of the literature for a variety of HSV-1 chromatin studies in distinct experimental models [8,12–20,36,37,41–46], represent a current conceptual model for regulation of latency in HSV-1. The trends for each class of

transcript represent overall average enrichment for the subset of representative genes of each class examined in the cited studies. For lytic genes, deposition of histones and heterochromatic PTMs become greater during the transition to latency, but are removed as the virus reactivates. In direct contrast, investigated regions of the LAT largely exhibit increased activation and decreased repression during latency, and more modest changes in the concentration of repressive PTMs occur throughout the cycle. Note the dynamics of H3K27me3, which reaches the highest levels of enrichment and undergoes the most dramatic changes. In our conceptual model, regulation of facultative heterochromatin through deposition and removal of this mark is a central determinant of the latency/lytic replication dichotomy. It should also be noted that in many experiments, PTMs representative of all transcriptional levels have been observed converging at loci, and that this may be representative of combined input from functionally distinct populations rather than general simultaneous occurrence of the PTM on HSV-1 genomes.



ChIP has been invaluable in elucidating the abundance of PTMs on the HSV genome (Figure 2). ChIP of both H3K9 and H3K27 have both been performed by several labs. H3K9me2 has been shown to associate at around 5 dpi on the promoters of lytic genes ICP4 and TK, and substantially increases at 10 to 14 dpi through 30 dpi [36]. H3K9me3 has also been shown to be present in the LAT promoter and enhancer as well as immediate-early and early genes [37,41]. While H3K9 methylation does play a part in latency, H3K27me3 seems to play a much more prominent role. It is also present in the same

genes as H3K9 methylation but in relatively higher amounts [37,41]. This PTM appears at 7 dpi; this is after initial viral association with H3 histones, suggesting that the histones are not initially methylated and are modified shortly after being recruited to the viral DNA [42]. Finally, H3K4 methylation, a mark associated with transcriptionally permissive genes, is very sparse on the genome. Interestingly, the only region found to have a relatively high abundance of H3K4me2 is in the LAT region [25]. This is logically consistent, as the LAT is abundantly transcribed during latency.

It is curious that methylation marks associated with opposing functions, and which would thus seem mutually exclusive, should occur in overlapping regions. One possible explanation is that these marks are present in a heterogeneous population of viral genomes. It is unclear whether viruses in the same cells have different chromatin marks or whether different cells harbor viruses with the same marks. This observation may also explain why the LAT region can be found with heterochromatin marks even though it is abundantly transcribed in several cells. These different PTMs may very well cause different levels of transcriptional repression. The aforementioned subpopulations of LAT-transcribing neurons might be correlated with a high enrichment of H3K4 methylation on the LAT region. Since H3K27me3 is commonly associated with facultative heterochromatin, enrichment of this mark on lytic genes may represent a loosely repressed state that allows the virus to more easily reactivate. Virus enriched in H3K9me3 may represent a smaller portion of the population that is more tightly repressed and difficult to reactivate.

4. Polycomb Proteins Role in Regulating Lytic Gene Activity during Latency

HSV-1 requires careful coordination of gene activity for successful lytic replication. This is also true for latency, as lytic genes must be turned off (and kept off) during latency and then turned back on in response to appropriate stressors, resulting in reactivation. The strength of lytic gene repression must be carefully modulated. If the repression is too strict, lytic genes may be silenced permanently, possibly resulting in inefficient or nonexistent reactivation and a subsequent failure to spread to further hosts. If the repression is too weak, then lytic gene activity may invite a vigorous immune response to sensory neurons harboring viral genomes (reviewed in [47]). HSV-1 has therefore adopted a strategy involving strong, yet dynamic and reversible gene silencing. During latency, the virus can utilize this strategy to escape immune surveillance. Then, during reactivation, the lytic genes can overcome the silencing for a brief burst of lytic replication to yield progeny virus.

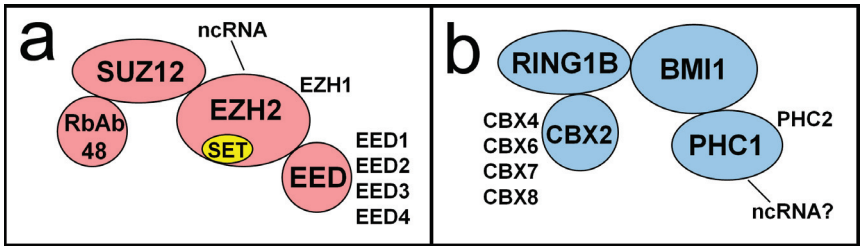
Early studies of epigenetic regulation of HSV-1 gene expression focused primarily on DNA methylation. A broad study using methylation-sensitive restriction endonucleases found no extensive methylation of HSV-1 genomes within the central nervous systems of latently-infected mice [48]. A later study focused tightly on specific CpG dinucleotides of HSV-1 genomes within DRG of latently-infected mice [43]. No significant CpG methylation was observed. In the same study, the authors found differential histone modifications across active and inactive portions of the latent HSV-1 genome, suggesting that “histone composition may be a major regulatory determinant of HSV latency.” These studies led to the hypothesis that cellular Polycomb Group (PcG) proteins could be

acting upon HSV-1 chromatin to repress lytic gene expression during latency in a manner similar to the silencing of cellular genes.

PcG proteins, originally identified in the fruit fly *Drosophila melanogaster*, are a set of proteins that interact with and modify chromatin to effect epigenetic changes and gene silencing (extensively reviewed in [49]). PcG proteins are essential for the silencing of a multitude of cellular gene loci, including genes that determine stem cell fate, *Hox* genes and other developmental regulators, and the mammalian inactive X chromosome. They are structurally and functionally conserved throughout higher eukaryotes and primarily function through two multiprotein complexes, the Polycomb repressive complex 1 (PRC1) and Polycomb repressive complex 2 (PRC2) (Figure 3). The most widely studied complexes are those of *Drosophila* and mammals. In *Drosophila*, the complexes are somewhat simpler and consist of fewer optional or alternative subunits. They are able to recognize and bind specifically to genomic regions termed Polycomb Response Elements (PREs) [50]. In *Drosophila* these consist of 5–7 bp binding motifs for different components of the PRC2 complex including Pho/Pho1, as well as transcription factors Sp1 and KLF (for a review see [51]). Mammalian complexes are more diverse in composition and there is little evidence for binding DNA in a sequence-specific manner. Despite these differences, their activities are highly similar. PRC2 catalyzes trimethylation of lysine 27 of histone H3 (H3K27me3) [52,53]. PRC1 catalyzes ubiquitylation of lysine 119 of histone H2A (H2AK119ub) [54] and also non-enzymatically participates in compaction of polynucleosomes [55]. These histone PTMs are hallmarks of Polycomb-mediated heterochromatin and gene silencing. ChIP studies have shown H3K27me3 [37,41,42], as well as components of PRC1 [37] and PRC2 [42], to be present on latent HSV-1 genomes, strongly suggesting a role for Polycomb-based silencing of lytic genes.

The mechanisms by which histone modifications promote gene silencing are varied and still under investigation. PRC2 negatively impacts the recruitment and binding of positive chromatin regulators and the deposition of activating modifications, such as histone acetylation. A ChIP-seq analysis of PRC2-negative *Drosophila* embryos demonstrated that PRC2 blocks recruitment of RNA Pol II to promoters [56]. The lack of PRC2 and subsequent reduction in H3K27me3 resulted in the association of Pol II with promoters of over 2,000 genes that showed no association in wild-type embryos. H3K27me3 is also known to recruit PRC1 to target loci [57]. Subsequent polynucleosome compaction by PRC1 reduces accessibility of promoters and prevents transcription factor binding. However, it must be noted that this is a dynamic process and that compacted chromatin regions are not set in stone. It is a method of limiting transcriptional initiation, but it is not an absolute obstruction. This offers some explanation of “leaky” HSV-1 lytic gene transcription during latency [25], or even observations of spontaneous reactivation events [58,59].

Figure 3. The core components of human polycomb repressive complexes are shown. Some alternate components are shown beside their more common canonical counterparts. (**a**) The SET domain of PRC2 component EZH2 catalyzes mono-, di-, and trimethylation of H3K27. EZH2 is also noted for several interactions with ncRNA; (**b**) The *C. elegans* homolog of PRC1 component PHC1 is a known RNA-binding protein. Whether or not this activity is conserved among mammalian PHC1 is under investigation.



In order to silence the correct genes, PcG proteins must first be recruited to the appropriate loci. In earlier models, PRC2 was designated the "establishment" complex where it was recruited to select loci and deposited the H3K27me3 mark. This mark then served as a binding site for PRC1, the "maintenance" complex which compacted nucleosomes and preserved silencing. While not inaccurate, later evidence shows that this model is incomplete and that PRC2 and PRC1 may be targeted by independent mechanisms [60]. In *Drosophila*, this is achieved primarily by proteins that bind specific PREs and then recruit PcG proteins. However, in mammals, only a few PRE-like elements have been identified [61,62]. It is therefore likely that most mammalian PcG association with chromatin represent more complex interactions, possibly mediated by association with specific transcription factors or other chromatin proteins. Mammalian regions associated with PcG complexes are often rich in CpG nucleotides [63] and also lack CpG methylation. As noted previously, no significant CpG methylation has been observed on latent HSV-1 genomes [43,48], perhaps opening the door for PcG recruitment. It is not yet known if HSV-1 plays an active role in preventing DNA methylation and recruiting PcG proteins to viral sequences. However, as discussed in the next section, reversible Polycomb-based gene silencing would seem to be ideal for the HSV-1 strategy of latent persistence and periodic reactivation.

PcG proteins may be targeted to select regions by multiple mechanisms, including interactions with transcription factors and non-coding RNAs (ncRNA). Several ncRNAs have been demonstrated to interact with EZH2, a member of PRC2. One example of these is RepA, a repeat region of the X-inactive specific transcript (XIST) [64]. A deletion of RepA in mice reduced H3K27me3 on the inactive X chromosome, implicating RepA in PRC2 recruitment [65]. While no direct ncRNA-PcG targeting partnership has yet been established, this is an intriguing mechanism to the field of HSV-1 epigenetics due to the high abundance and stability of the LAT RNA within sensory neurons, the site of HSV-1 latency. There is evidence that the LAT RNA, provided *in trans* in transgenic mice, reduces H3K27me3 enrichment on latent viral genomes. There is also evidence for an interaction between the

stable 2.0 kb LAT intron and PRC1 component PHC1 (unpublished data). The nature of this interaction and whether it directly impacts lytic gene silencing is still under investigation.

Several mechanisms have been proposed for the observed effects of LAT transcription on PcG-based silencing of lytic genes. One study, using HSV-1 strain KOS in a mouse ocular/TG model of infection, found that a deletion of the LAT promoter resulted in decreased H3K27me3 at lytic genes [41]. It was proposed that the LAT promoted the formation of heterochromatin at lytic gene promoters, paralleling the activity of RepA in recruiting PRC2 to the inactive X chromosome. Another study, using HSV-1 strain 17*syn*+ in a mouse footpad/DRG model of infection, published seemingly contradictory results [37]. Deletion of the LAT promoter resulted in significantly higher enrichment of H3K27me3 at lytic genes, suggesting that the LAT was acting to reduce silencing of lytic genes and maintain them in a state "poised" for reactivation. In this case, the LAT may act as a decoy to misdirect Polycomb-mediated silencing. Another possibility is that the LAT RNA may associate with transcription factors or histone demethylases during latency and target them to lytic genes to increase the efficiency of reactivation. This hypothesis is consistent with induced reactivation experiments in the rabbit eye model in which 17*syn*+ reactivates efficiently but 17ΔPst, the LAT deletion mutant, does not [66]. All of these hypotheses are intriguing and are the subject of further study.

As previously noted, the above studies used strains of virus that are already known to differ greatly in virulence and reactivation potential. The fact that they behave differently in regard to PcG recruitment and heterochromatin deposition is not surprising. The differing routes of infection and sites of latency must also be taken into account. The subunit composition of Polycomb complexes is likely to differ between TG and DRG, and perhaps even between subtypes of neurons within these tissues. These cell specific differences may explain why the PRC1 component Bmi1 was found to associate with latent genomes in one study [37], but not in another [41]. On a broader scale, differential use of Polycomb subunits may explain why LAT expression is observed in only one-third of infected neurons [1,67]. It may even be a factor in the observed anatomical preference of HSV-1 for the orofacial region and HSV-2 for the genital region.

5. The Predicted Role of Histone Demethylases in HSV-1 Reactivation

There are currently several histone demethylases with a large assortment of names but relatively focused functions. Homologs have been identified in several organisms including *H. sapiens*, *M. musculus*, *D. melanogaster*, *C. elegans*, *S. pombe*, *S. cervisiae*, as well as some prokaryotes. Their presence in the latter suggests an ancestral role divergent from histone modification. The following section will review the relevant demethylases with potential roles in regulation of alpha-herpesvirus infections; LSD1 (lysine demethylase 1, KDM1), JMJD3 (jumonji domain containing 3, KDM6B), and UTX (ubiquitously transcribed tetratricopeptide repeat, X chromosome; KDM6A) histone demethylases. The known histone demethylases that would be predicted to play a role in remodeling HSV chromatin to facilitate reactivation are described in Table 1.

Table 1. The predicted role of histone demethylases in HSV-1 reactivation.

Demethylase	Family	Specificity	Associated Complex	Biological Role	Inhibitor
LSD1 (KDM1A)	FAD-amine oxidase	H3K4me2/me1 H3K9me2/me1	HDAC1/ CoREST/ REST	Possible coordinated role with HDACs in transcription repression	Paraglyine [a], TCP, OG-L002
JHD3A/JMJD2A (KDM4A) JHD3C/JMJD2C/ GASC1 (KDM4C)	Jumonji [b]	H3K9me3/me2 H3K36me3/me 2 H1.4K26me [c]	NCoR complex [d]	KDM4C is a possible oncogene [e]	PCA, NOG, DMOG, ML324
JARID1A/RBP2 (KDM5A) JARID1C/SMCX (KDM5C)	Jumonji	H3K4me3/me2	Sin3/HDAC complex (KDM5A) NCoR/REST (KDM5C)	Notch signaling (JARID1A) NCoR-SMCX-REST complex functions in glial development	N/A [f]
UTX (KDM6A)	Jumonji	H3K27me3	MLL3/4, RbBP5, WDR5, and ASH2	Pluripotent stem cell differentiation (Hox gene regulation)	GSK-J4
JMJD3(KDM6B)	Jumonji	H3K27me3	RbBP5	Induced upon activation of macrophages by inflammatory stimuli Role in neuronal commitment	GSK-J4

[a] Monoamine-oxidase inhibitor; [b] Dioxygenases containing a Jumonji C(JmjC) domain with an active site containing Fe(II) and the co-factor α-ketoglutaric acid; [c] Somatic H1 isotype in humans; [d] NCoR has role in neural differentiation and hematopoiesis [68]; [e] Gene up regulated in cell lines derived from esophageal squamous carcinomas [69]; [f] No publication to date.

5.1. FAD-Amine Oxidase: Lysine Specific Demethylase 1 (LSD1/KDM1)

Lysine-specific demethylase 1 (LSD1) specifically demethylates H3K4me2/me1 as well as H3K9me2/me1 and is unable to demethylate tri-methylated lysine due to biochemical and biophysical constraints. In addition, LSD1 is dependent on protein partners within the Set1/MLL methyltransferase complex such as the transcriptional coactivator host cell factor-1 for its activity *in vivo* and requires the neuronal silencer co-repressor of RE1-silencing transcription (CoREST) factor to demethylate nucleosome-associated histones [70]. The use of small interference RNA to reduce levels of Set1 decreases levels of H3K4me3 and ultimately reduces replication of HSV-1 [13]. LSD1 also associates with the HDAC1-2/CoREST/REST complex and suggests a role with deacetylation. Inhibition of HDACs results in concomitant decreases in LSD1-mediated demethylation [71]. Displacement of HDAC1 from the complex by the immediate-early protein, ICP0, allows for association of components of the repressor complex—either HDAC1-2/LSD1/CoREST/REST or LSD1/CoREST—with ICP8 and may play a role in the emergence of DNA-replication compartments in HSV-1 infected cells [72].

RNAi depletion of LSD1 or inhibition of its enzymatic activity by monoamine-oxidases inhibitors or the highly selective OG-L002 increases enrichment levels of repressive chromatin and blocks viral gene expression for Varicella zoster virus, HSV-1, and Cytomegalovirus [72,73].

5.2. Jumonji-Domain Histone Demethylases: JMJD3 (KDM6B) and UTX (KDM6A)

The vast majority of lysine demethylases contain a conserved Jumonji-C domain motif (JmjC). The existence of a large family of Jumonji proteins that can demethylate mono-, di-, and tri-methylated lysine in a reaction mediated by Fe(II) and α-ketoglutarate catalysis provides an additional demethylation mechanism fundamentally different from LSD1.

UTX and JMJD3 belong to a subfamily of proteins that require a catalytically active JmjC domain to maintain demethylase activity. As well as possessing 84% sequence similarity, their Jumonji-domains share high structural conservation. Temporal expression of Hox genes—which are silenced in pluripotent cells—is mediated through the demethylation of H3K27me3 by UTX and is critical in mammalian embryogenesis. In addition to having a role in activated macrophages, JMJD3 has been identified as a protein specifically upregulated at the outset of neural commitment [74,75]. Previous studies have established that recombinant human UTX and JMJD3 that were overexpressed and purified from mammalian cells specifically remove methyl marks on H3K27 *in vitro* [76]. Decreases in di- and tri-methylation suggest that both UTX and JMJD3 may function as H3K27 demethylases *in vivo*. The association of UTX and JMJD3 with the H3K4 methyltransferase MLL family of proteins and components of the MLL complex (WDR5, RbBP5, and ASH2) suggests a physical role in balancing activation and repression [75] and may be a general phenomenon for most histone demethylases. It is unclear whether the switching of histone methyltransferase with demethylase activity on bivalent marks is cell signal specific or a result of high levels of PRC1/PRC2 within a cell—the maintenance of which is subverted by the HSV-1 LAT.

6. Summary and Discussion

The analysis of HSV-1 epigenomes during latency reveals a general consensus that: (1) the HSV-1 lytic genes are associated with heterochromatic histone modifications; (2) while both constitutive (H3K9me3) and facultative (H3K27me3) marks are present, the H3K27me3 marks predominate; and (3) the LAT-encoding regions of the genome display bivalent modifications of both repressive (H3K27me3) and active (H3K4me3 and H3K9,K14Ac) marks.

A number of studies suggest that a transient and rather global re-modeling of both the lytic and latent gene regions occur rapidly following stressors that induce reactivation, though productive reactivation occurs in only a fraction (<5%) of latently infected neurons. These observations suggest that while there are initial stress-induced changes in chromatin to a large proportion of the genomes, there are downstream effectors of productive phase transcription that operate only in a sub-set of cells. Whether this reflects different neuronal population that regulate transcription differently, differences in established epigenome profiles that vary from cell to cell, or the magnitude of stress-induced signaling

that reaches a given cell remains to be determined, and ultimately may require single-cell analyses to sort out. None-the-less, the identification of the spectrum of epigenetic marks that are present on the latent genomes sets the stage for identifying the remodeling proteins that ultimately play an essential role in the initial stages of HSV reactivation.

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Conflicts of Interest

The authors declare no conflict of interest.

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Review

Current Understanding of the Role of the Brd4 Protein in the Papillomavirus Lifecycle

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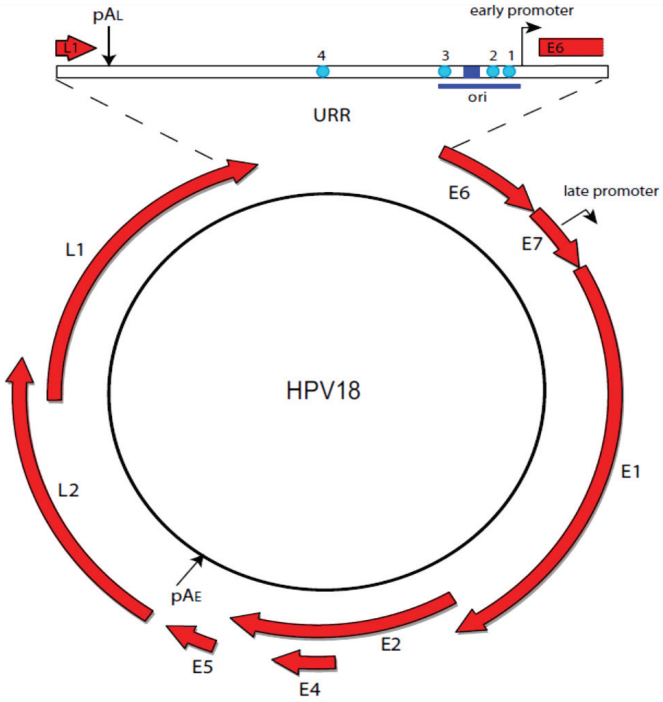
Abstract: The Brd4 protein is an epigenetic reader that is central to regulation of cellular transcription and mitotic bookmarking. The transcription and replication proteins of many viruses interact with Brd4. We describe the multiple roles of Brd4 in the papillomavirus lifecycle.

Keywords: Brd4; BET protein; HPV; papillomavirus; transcription; chromatin; replication; tethering; partitioning; bromodomain

1. Introduction

Papillomaviruses (PVs) are an ancient group of viruses that have coevolved along with their hosts for millions of years. Each viral type infects only a particular host species and is trophic for a specific anatomical niche in the stratified epithelium of the skin or mucosa of the host. Papillomavirus infection is persistent and results in clinical outcomes, such as asymptomatic infection, verrucae, plantar and filiform warts and condylomata acuminata. A subset of oncogenic HPVs is associated with carcinomas of the oropharyngeal and anogenital tracts [1–3]. Despite the diversity of pathogenesis associated with papillomavirus infection, all papillomaviruses have similar small dsDNA genomes of approximately 8 kbp (see Figure 1), and each encodes only six to eight genes. Papillomaviruses rely on hijacking and manipulating host factors to maintain their lifestyle.

Figure 1. HPV18 genome. The circular dsDNA genome of HPV18 (7,857 bp) is shown. Viral open reading frames are depicted as red arrows. The URR (upstream regulatory region) is expanded to show transcription and replication regulatory elements. Binding sites for the E2 protein (cyan circles) and the E1 binding site (blue rectangle) are shown. The early promoter and replication origin (ori) are indicated.



One key, cellular regulatory protein hijacked by all papillomaviruses (and other viral families) is a cellular chromatin binding protein, Brd4. Brd4 is an essential cellular protein that binds and marks chromatin to regulate several transcriptional processes. In this review, we will summarize the current knowledge pertaining to the role of Brd4 in papillomavirus infections.

2. Papillomaviruses

2.1. The Papillomavirus Lifecycle

The stratified epithelium of the skin or mucosa consists of several layers of keratinocytes in various stages of differentiation, overlying the basal layer of proliferative cells. Papillomaviruses gain access to cells in the basal layer through a microabrasion. They infect these keratinocytes, induce their proliferation by expression of the viral E7 protein and establish a persistent infection therein. The viral genome is maintained as a low copy, extrachromosomal element in the nucleus of these cells, and only

low levels of viral gene products are produced. When the infected cells divide, some of the daughter cells begin the process of stratification and differentiation and progress towards the surface of the epithelium. The papillomavirus lifecycle is finely tuned to this process of differentiation; viral transcription and replication switches from early to late modes as the cell differentiates. Vegetative viral DNA replication is initiated in the mid-layers of differentiated cells, and capsid synthesis is confined to the most differentiated layers of cells.

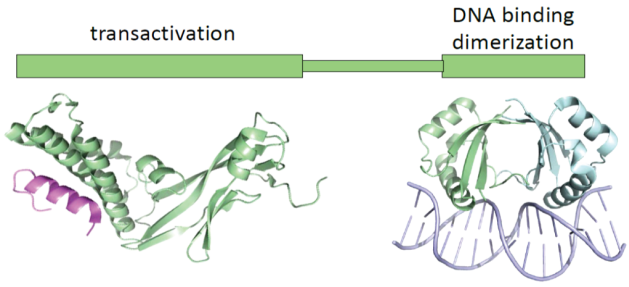
2.2. Viral Transcription

Papillomavirus genomes can be divided into three regions (see Figure 1). The upstream regulatory region (URR) contains transcriptional enhancers and promoters and the origin of replication. The early region encodes the E (early) proteins that are expressed at early and intermediate stages of infection. Last is the late region that contains the L (late) genes. In the most undifferentiated cells, viral RNAs are transcribed from the early promoter, terminate at the early polyadenylation site (pAE) and encode the E6, E7, E1, E2, E4 and E5 proteins (reviewed in [4]). As the cells progress through differentiation, the late promoter is activated, resulting in an intermediate class of transcripts that use the late promoter and the early polyadenylation signal. These transcripts encode high levels of the E4, E1 and E2, the latter two being required for high level vegetative viral DNA replication in the mid-layers of the epithelium. Exclusively late transcripts are expressed at an even later stage of differentiation; they use both the late promoter and late polyadenylation signal (pAL) and encode the L1 and L2 capsid proteins. Complex patterns of viral mRNA species are generated by the use of alternative splicing and viral gene expression is highly regulated by differential splicing and polyadenylation (reviewed in [4]).

The E2 protein is the primary regulator of early viral transcription [5]. E2 consists of a conserved amino terminal “transactivation” domain linked to a C-terminal DNA binding/dimerization domain by a poorly conserved linker (see Figure 2) [6]. E2 specifically binds to a consensus sequence of ACCGNNNNCGGT, several copies of which are located in the viral URR [7] (see Figure 1). E2 can activate transcription from certain viral promoters [5,8–10], but the early viral promoter in the well-studied, oncogenic alpha-PVs is primarily repressed by E2 [11–13]. Early studies showed that E2 could repress the early promoter by sterically hindering the association of TBP and Sp1 with promoter elements adjacent to E2 binding sites 1 and 2 [14–16]. Subsequent studies have shown that much of this E2-mediated repression is mediated through interaction with factors that modulate cellular chromatin processes [17–20]. Disruption of E2-mediated repression (often due to HPV integration) results in increased expression of the E6 and E7 oncogenes [11,12,21]. In fact, reintroduction of the E2 protein into cervical cancer-derived lines (such as HeLa) leads to rapid senescence, as viability of these cells is completely dependent on E6 and E7 expression [22–24].

Shorter forms of the E2 protein that are lacking the transactivation domain also repress HPV transcription, in part by competing for binding to E2 binding motifs [25,26]. One of the best characterized is the E8^E2 protein (encoded from a spliced transcript), which also represses through the recruitment of chromatin-associated repressive factors [27]. As described in detail below, E2 interacts with the Brd4 protein, and this association is pivotal to transcriptional regulation of HPVs.

Figure 2. Structure of the papillomavirus E2 protein. The two domains of the E2 protein are shown. The transactivation domain structure is of HPV16 E2 bound to the Brd4 C-terminal motif (CTM). The Brd4 C-terminal peptide (residues 1343–1362) is shown in magenta. This structure is from the pdb file, 2NNU. The DNA binding domain is of the HPV18 DNA binding domain bound to DNA (shown in light purple) from the pdb file 1JJ4.



2.3. Disruption of E2 Function by HPV Integration

In the majority of HPV associated cancers, the HPV genome is integrated into the host genome (reviewed in [28]). The viral genome is most often integrated in such a way as to disrupt the E2 gene and, thus, alleviate transcriptional repression of the early viral promoter [22–24,29]. In turn, this causes dysregulation of the E6 and E7 oncogenes and promotes malignant progression [30].

2.4. Viral Replication

Papillomavirus replication requires the E1 protein, the E2 protein and the origin of replication [31–33]. E1 is the primary replication protein, an ATP-dependent helicase that binds specifically to the origin and unwinds it in a bidirectional manner to permit access by the cellular replication machinery (reviewed in [34]). E2 functions to load the E1 helicase onto the origin [35]. As depicted in Figure 1, the minimal origin consists of the E1 binding site flanked by E2 binding sites and overlaps elements regulating the early promoter.

There are three modes of viral replication in the viral lifecycle. The first is a limited amplification that occurs when the virus particle infects a basal keratinocyte. The virus must undergo a few rounds of unlicensed DNA replication to establish the genome as a low copy, nuclear, extrachromosomal element. This phase requires E1, E2 and the minimal replication origin. The second phase of replication maintains the viral genome at a low copy number during division of the proliferating basal cells. This also requires E1, E2 and the minimal origin of replication, but there is an additional requirement for additional E2 binding sites in *cis* to the origin, thus implicating the E2 protein in maintenance replication [36]. Subsequent studies showed that the role of E2 was to tether the viral genomes to the host chromatin [37–39]. The interaction of E2 with Brd4 is crucial for the tethering function of many papillomaviruses and will be discussed in detail below. Further investigation showed that the E1 protein was not always necessary for maintenance replication and, presumably, in this

situation viral replication, is initiated by cellular factors, while the genome is retained by the E2 protein [40,41].

The third phase of replication is vegetative amplification, when progeny virions are produced in large numbers in differentiated cells. This requires the E1 and E2 proteins, and their expression is upregulated in differentiated cells [42,43]. There is evidence that the mode of replication changes in differentiated cells [44], and incorporation of Rad51 into replication foci indicates that the virus may replicate using a recombination directed replication mechanism [45,46]. Furthermore, the cellular ataxia telangiectasia mutated (ATM) DNA damage response pathway is required for vegetative replication in differentiated cells [47]. Nuclear foci formed by either expression of the E1 and E2 proteins [48–51] or the replicating viral genome [46,47] recruit multiple cellular proteins required for the cellular DNA damage response and repair pathways.

2.5. Differences in Transcription and Replication among Papillomaviruses

To date, there are over 240 named papillomavirus genomes that have been classified into 37 different genera [52]. The best studied are human viruses from the alpha and beta genera that infect primarily the mucosa and skin, respectively. The human mu virus, HPV1, and the ungulate delta virus, BPV1, are also well characterized. While each of these viruses has a similar organization of genes to that of HPV18 (an alpha-PV) shown in Figure 1, the number and position of the E2 binding sites can vary considerably. BPV1 has 11 E2 binding sites in the URR and six elsewhere in the genome [53], while most human alpha viruses have only the four E2 sites shown in Figure 1. In BPV1, E2 is primarily an activator of transcription [5], while E2 predominantly represses the major early promoter of alpha-PVs.

All viruses require the minimal replication origin and the E1 and E2 proteins to initiate replication, but the requirements for maintenance replication are more complex. E2 binding sites are essential for initiation of replication and for transcriptional regulation making it very difficult to separate and elucidate the role of individual sites in maintenance replication of the viral genome. Maintenance replication is best understood for BPV1, where it has been shown that at least eight E2 binding sites are required for persistent replication [36]. In the alpha-PV HPV31, only three of the four E2 binding sites are required for maintenance replication of the viral genome [54]. In agreement with this finding, using a novel complementation assay, we find that a region encompassing the 3’ half of the URR of HPV18 (containing E2 binding sites 1–3) is sufficient for long-term maintenance in the presence of the E1 and E2 proteins [55].

3. The Brd4 Protein

3.1. Brd4 Structure and Function

Brd4 was first described as an unusual chromatin binding factor that remained bound to chromosomes throughout mitosis [56]. It is a member the BET (bromodomain and extra-terminal

domain) family of chromatin binding proteins and, therefore, its name was changed from MCAP (mitotic chromosome-associated protein) to bromodomain containing protein 4 (Brd4) [57]. Brd4 is an essential protein [58] that is ubiquitous in proliferating cells. The tandem bromodomains of Brd4 interact with acetylated tails of H3 and H4 histones [59], and Brd4 has been shown to be a mitotic bookmark that marks genes, which are expressed shortly after mitotic exit [60,61]. Brd4 decompacts chromatin and recruits transcriptional initiation and elongation factors to rapidly activate early G1 genes post-mitosis, as well as later in interphase [62]. Brd4 recruits the transcriptional elongation factor, p-TEFb, to promoters to enhance phosphorylation of the C-terminal tail (CTD) of RNA polymerase II promoters to stimulate transcription [63,64]. Brd4 further promotes transcription by directly phosphorylating the RNA polymerase II CTD [65]. This fundamental role of Brd4 in transcriptional regulation places it at the center of many diverse biological activities.

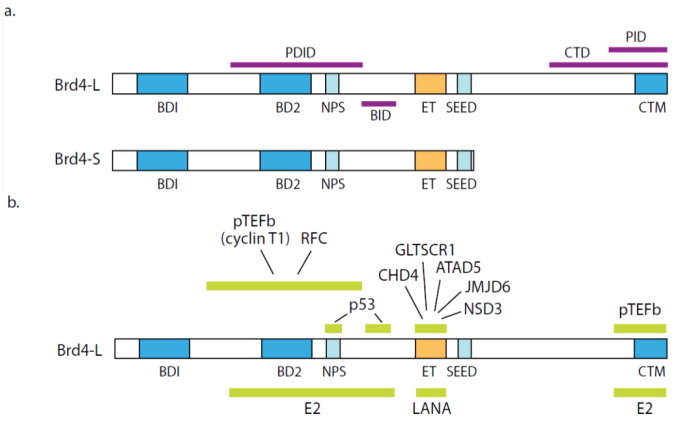
The Brd4 gene encodes two proteins; the short form of Brd4 contains the two bromodomains and the extra-terminal (ET) region (important for many protein-protein interactions), while the longer form of Brd4 has an additional, long unique C-terminal region (see Figure 3). The structures of both bromodomains and the ET domains have been solved [66,67]. The bromodomains bind to specific acetylated lysines on H3 and H4, but BD2 (bromodomain 2) can also interact with acetylated residues in other proteins, such as cyclin T1 (the p-TEFb subunit) and the relA subunit of NFκB [67–69].

The extra-terminal domain consists of three alpha-helices, and it seems to be a site of protein-protein interaction [70]. It binds proteins involved in transcriptional regulation, such as NSD3 (also known as WHSC1L1), a histone methyl transferase, JMJD6, a histone demethylase, and CHD4, a component of the NuRD (nuclear remodeling and deacetylase) repressor complex. Thus, Brd4 has the potential to assemble multifaceted positive and negative regulatory complexes on promoters [71].

The function of the C-terminal region is not well-characterized, except for the last 100 amino acids, which is important for interacting with the papillomavirus E2 protein [72] and with p-TEFb [73] (see Figure 3). Thus, p-TEFb interacts with two independent regions of the Brd4 protein [68].

p53 has also recently been shown to be a binding partner of Brd4, and analysis of this interaction revealed a conformational switch in Brd4 structure and protein-protein interactions modulated by CK2 phosphorylation [74]. A region between the second bromodomain and the ET domain contains two interaction regions, BID (basic residue enriched interaction domain) and NPS (N-terminal cluster of phosphorylation sites), that modulate this switch. When unphosphorylated, the NPS region interacts with the PDID (phosphorylation dependent interaction domain) that encompasses bromodomain 2, thus preventing Brd4 from binding to acetylated histones (see Figure 3). p53 binds to the BID region when Brd4 is unphosphorylated. Upon phosphorylation of NPS, p53 is released from binding to BID, and both p53 and BID now associate with phosphorylated NPS, thus exposing the bromodomain 2 in PDID and activating the complex [74].

Figure 3. Structure and function of the Brd4 protein. (**a**) Domains of the Brd4 protein. The domains shown are BD1 (bromodomain I: residues 58–169); BD2 (bromodomain 2: residues 349–461); NPS (N-terminal cluster of phosphorylation sites: residues 484–503); BID (basic residue enriched interaction domain: residues 524–579); ET (extra-terminal domain: residues 600–678); PDID (phosphorylation dependent interaction domain: residues 287–530); SEED (Ser/Glu/Asp-rich region: residues 695–720); CTM (C-terminal motif: residues 1325–1362); CTD (dominant negative domain of Brd4: residues 1047–1362) and PID (pTEF binding region: residues 1209–1362) [73]. (**b**) Viral and cellular interacting partners of Brd4. See text for references.



3.2. Association of Brd4 with Disease

The first association of Brd4 with human disease was the discovery that the aggressive NUT midline carcinoma (NMC) was due to a translocation (t(15;19)(q13;p13)), resulting in the fusion of Brd4 with NUT (nuclear protein in testes) [75]. The NUT protein recruits the histone acetyl transferase, p300, resulting in a feed-forward loop of histone acetylation and Brd4-NUT recruitment, which gives rise to hyperacetylated, but inactive, chromosomal foci [76]. Sequestration of Brd4 and p-TEFb to these foci promotes proliferation of Brd4-NUT cells. This can be reversed and differentiation restored, by siRNA to Brd4, by histone deacetylase inhibitors that promote global acetylation and by BET protein-specific histone mimics [77–79].

Brd4 is also a modifier of breast cancer metastasis [80] and a promising target for several cancers, because of its fundamental role in transcriptional processes. Targeted inhibition of BET bromodomain binding is potentially therapeutic for glioblastoma, lung adenocarcinoma, ALL (acute lymphoblastic leukemia) and MLL (mixed-lineage leukemia) [81–84,85].

4. Brd4 and Papillomaviruses

Brd4 was discovered to be a major interactor of the papillomavirus E2 protein by proteomic analyses [20,72], yeast two hybrid screening [86]. It was also investigated as an E2 target, because the analogous tethering protein in KSHV (LANA) interacts with the BET family member, Brd2 [87–89]. The E2 protein had previously been shown to bind and tether viral genomes to host mitotic chromosomes [37–39,90], and the Brd4 protein colocalized completely with these chromatin bound speckles of E2 [72,87,89,91]. The E2 protein is a multifunctional protein involved in papillomavirus transcription, maintenance and partitioning of extrachromosomal viral genomes and initiation of viral DNA replication. Many subsequent studies have dissected the role of Brd4 in each of these processes.

4.1. Interaction between Papillomavirus E2 and Brd4 Proteins

The Brd4 protein binds primarily to the transactivation domain of the E2 proteins (see Figure 2) [72]. The transactivation domain contacts a peptide at the extreme C-terminus of the Brd4 protein [91], and a C-terminal domain of Brd4 (CTD; residues 1047–1362) has proven useful as a dominant-negative inhibitor of the E2-Brd4 interaction [72]. The region of the E2 transactivation domain that makes contact with the Brd4 CTD is highly conserved; yet, there is a wide range of binding affinities between Brd4 and E2 proteins from different papillomaviruses [92]. The C-terminal DNA binding domain of E2 does not seem necessary for Brd4 binding, but the dimerization function of this domain greatly increases E2-Brd4 binding both *in vivo* and *in vitro*. There are hints that there may be additional contacts between E2 and Brd4: HPV11 E2 binds to the Brd4 CTD, but also to a region encompassing bromodomain 2 (BD2). The DNA binding domain of the KSHV LANA protein (a tethering protein analogous to E2 and with a DNA binding domain of similar structure) interacts with the ET domain of Brd4 [93,94]. It is likely that the interaction between Brd4 and the E2 protein will be multifactorial.

4.2. Brd4 Modulates the Stability of the E2 Proteins

Several groups have noted that the E2 protein is stabilized by interaction with Brd4 [95,96] or with the Brd4 CTD [97]. This interaction prevents proteasomal degradation of E2 by the E3 ligase cullin-3 [97] and may enhance many E2 functions, such as transcriptional regulation and stable tethering of genomes on host chromatin.

4.3. The Role of Brd4 in Viral Transcription

The E2 protein can both activate or repress viral transcription, depending on whether it binds to sites that are distal or proximal to promoter elements [5,8–10]. Binding of E2 to promoter proximal sites represses the early viral promoter in the oncogenic alpha-PVs [11–13]. A number of mutational analyses identified residues in the transactivation domain of E2 that were important for E2-mediated transcriptional activation or replication [98–103]. Prominent were two highly conserved residues (R37 and I73) that when mutated, abrogated E2-mediated transactivation, but not replication. Subsequently,

it was shown that these residues were located on two adjacent alpha helices on the same face of the E2 transactivation domain [104], and later, these residues were shown to make direct contact with a C-terminal peptide (residues 1343–1362) of Brd4 [91]. In addition to binding to the Brd4 C-terminus, HPV11 E2 also interacts with Brd4 residues 280–580, which encompasses the BD2 bromodomain.

It became clear that Brd4 was essential for the transcriptional activation function of E2. A dominant negative C-terminal peptide encompassing the Brd4 CTD interfered with the E2-Brd4 interaction and inhibited transactivation by many papillomavirus E2 proteins [92,105,106]. Initially, the role of Brd4 in transcriptional repression was controversial, but it has now been proven that Brd4 is also involved in E2-mediated transcriptional repression [18–20]. However, in some cases, the dominant negative Brd4 CTD interferes with E2-mediated transactivation, but not repression, implying that there are additional or alternative modes of interaction between the Brd4 and E2 proteins [18]. Another factor that contributes to repression of the HPV early promoter is the histone acetyl transferase complex, NuA4/TIP60 [18,107]. TIP60 preferentially acetylates K14 of histone H3 and K5, K8, K12 and K16 of histone H4 [108], which are all targets of the Brd4 bromodomains [66]. Notably, the HPV E6 protein destabilizes TIP60, thereby alleviating repression of its own promoter [107].

One of the key functions of Brd4 is to recruit p-TEFb to promoters to stimulate elongation of RNA polymerase II transcription. The C-terminal region of Brd4 interacts with both E2 and p-TEFb, suggesting that these complexes are mutually exclusive. Brd4 recruitment of P-TEFb to the early promoter is required for viral transcription, and E2 disrupts this interaction [109]. In this study (unlike [18]), the dominant negative BRD4 CTD was able to inhibit E2-mediated repression.

Most HPV repression studies have analyzed viral transcription in cervical carcinoma-derived cell lines that harbor integrated HPV genomes. However, E2 may preferentially repress integrated genomes compared to episomally replicating genomes, and so, further studies are needed to define the role of Brd4 in HPV transcription [110].

4.4. The Role of Brd4 in Viral Genome Replication

Most studies have indicated that the E2-Brd4 interaction is important for transcriptional regulation and tethering of viral genomes to host chromatin. Mutated E2 proteins that are unable to bind Brd4 are able to efficiently support transient replication of an origin containing plasmid [87,98,99,105,106,111–113]. Ilves *et al.* demonstrated that the dominant negative Brd4 CTD could inhibit the replication of BPV1 genomes or origins in rodent cells, but not in human C-33A cells [113]. Furthermore, this inhibition was not dependent on the interaction of Brd4 with E2 (at least through the R37 and I73 residues) and was not specific for papillomavirus replication [113].

Wang *et al.* find that Brd4 is recruited to foci formed by HPV16 E1 and E2 in a replication origin-dependent fashion in C-33A cells [114]. An E2 protein mutated in both Brd4 interacting residues (R37 and I73) is defective in replication, thus leading the authors to propose that Brd4 is required for replication. However, as previously found by others, E2 proteins with a single substitution in I73 are not defective in replication, despite an inability to bind to Brd4 [87,99,105,106,111–113]. Because downregulation of Brd4 has detrimental effects on cell growth and proliferation (making it

difficult to interpret HPV replication experiments), Wang *et al.* demonstrated that Brd4 could stimulate HPV replication *in vitro* [114].

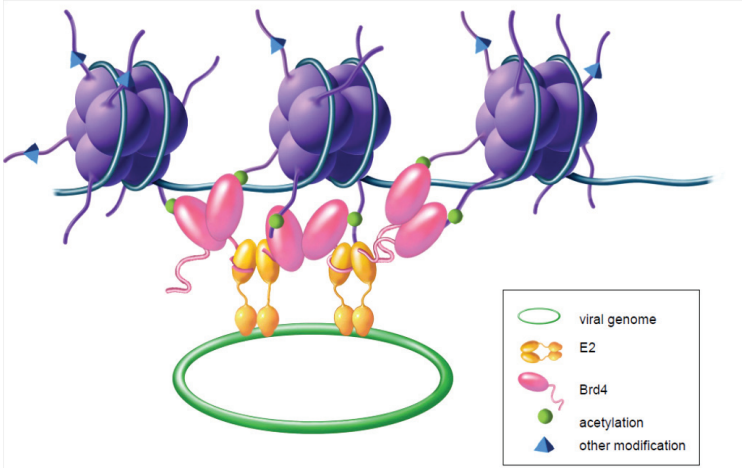
Somewhat similarly, we find that Brd4 is recruited to replication foci formed by the E1-E2 proteins in keratinocytes [49], in a process that is completely dependent on E1, E2 and Brd4. However, we find that Brd4 is displaced to the periphery of these foci in the presence of an actively replicating origin or genome, and Brd4 is no longer required for their formation [115]. Brd4 is also found in a satellite pattern around late replication foci that contain amplified genomes in differentiated keratinocytes [115], but it does not seem to be essential for viral DNA amplification [116]. The HPV replication foci induce a cellular DNA damage response and recruit repair proteins [46–50], and it is tempting to speculate that Brd4 is involved in these processes. The replication factor, RF/C, is found in Brd4 [117] and E2-Brd4 protein complexes [20,118]. Furthermore, the alternative RFC1 subunit, ATAD5, which is involved in the DNA damage response, interacts with the ET domain of Brd4 [71], suggesting that it might play a role in HPV replication. Clearly, more studies are required to elucidate the exact role of Brd4 in the papillomavirus replication process, but there are several hints that Brd4 might be involved in viral and cellular DNA replication and repair processes.

4.5. The Role of Brd4 in Viral Genome Maintenance and Partitioning

The E1 and E2 proteins support transient replication, but long-term persistence of viral-derived DNA requires additional E2 binding sites in *cis* to the replication origin [36]. The first clue to the role of E2 in viral DNA persistence was the observation that both viral DNA and the BPV1 E2 protein are localized in punctate foci on the host mitotic chromosomes [37]. This led to the model (as shown in Figure 4) that E2 associates with host chromosomes through the transactivation domain [39], while the DNA binding domain binds to E2 sites in the viral genomes and tethers them to the host chromosomes to promote retention and partitioning [119]. This tethering mechanism ensures that the low copy viral genome is retained in the nucleus and is partitioned to daughter cells.

At least for BPV1, the cellular target that mediates mitotic tethering is the Brd4 protein [72,87,89]. Brd4 is usually observed as a diffuse cloud (if at all) around the condensed mitotic chromosomes [59], but in the presence of E2, both proteins colocalize in punctate foci [89]. Brd4 has a high on-off rate and can be easily extracted from chromatin [59], but E2 dramatically stabilizes the interaction of Brd4 with chromatin forming a stable anchor in interphase and mitosis [89]. As described above, two highly conserved residues in the transactivation domain of E2 (R37 and I73) mediate the interaction with Brd4, and mutations in these residues abrogate the interaction of E2 with Brd4 and mitotic chromatin [87]. The DNA binding domain of E2 is not required for the interaction with Brd4, but it is required to link the viral genomes to chromatin [119]. The dimerization function of the E2 C-terminal domain greatly increases the affinity of Brd4 for chromatin both *in vivo* and *in vitro*, most likely by promoting the assembly of higher order E2-Brd4 complexes [120]. Expression of the dominant negative Brd4 CTD resulted in inhibition of E2 mitotic chromosome binding and loss of BPV1 genomes from BPV1 transformed cells [89,113,121]. Moreover, BPV1 E2-mediated plasmid maintenance could be reconstituted in *Saccharomyces cerevisiae* by exogenously expressed Brd4 [122].

Figure 4. Model of E2-mediated tethering of the viral genome to host chromatin. The Brd4 protein interacts with acetylated lysine residues on histone tails protruding from the host nucleosomes (shown in purple). The papillomavirus E2 protein interacts with the C-terminal region of Brd4 through the E2 transactivation domain. The DNA binding-dimerization domain of E2 links the viral genome to the chromatin complex.



Although the E2 proteins from all papillomaviruses interact with the C-terminal region of Brd4 (through the R37 and I73 residues in E2), there are differences in the strength of binding [92,118]. Furthermore, not all E2 proteins are as readily observed bound to mitotic chromosomes as BPV1 E2 [92,123]. A careful analysis of the E2 proteins from numerous different papillomaviruses showed that there were three different phenotypes of mitotic chromosome binding that segregated perfectly according to the phylogeny of papillomaviruses [123]. These groups were comprised of the alpha-papillomaviruses, a large genus that encompasses mainly human viruses that infect the oral and genital mucosa; the beta and gamma papillomaviruses, another large group containing mainly human viruses that infect the cutaneous epithelium in an asymptomatic manner and a diverse group of viruses from the delta (BPV1), mu (HPV1), kappa (OcPV1 and SfPV1) and other genera. The E2 proteins from this latter group bind tightly to Brd4, stabilize its association with interphase chromatin and colocalize with Brd4 on the arms of mitotic chromosomes in punctate dots [92,123].

In contrast, the beta-PV E2 proteins bind strongly to pericentromeric regions of mitotic chromosomes that overlap the loci for the ribosomal RNA genes [123,124]. The determinants of the E2 protein required for this perichromosomal binding are quite different from those required for the E2 Brd4 chromosomal foci. The primary requirements are the E2 DNA binding domain and a short peptide from the hinge region that facilitates interaction with chromatin [125]. Phosphorylation of this peptide by PKA (protein kinase A) stabilizes the E2 protein and promotes chromosomal binding [126] in a manner analogous to that of the KSHV LANA tethering protein [127]. However, the beta E2 proteins do have high affinity for Brd4, and if the chromosomal binding peptide in the hinge is

mutated, E2-directed foci of Brd4 can be observed on mitotic chromosomes [128]. The significance of these two binding modes has yet to be determined, as beta-PV genomes do not readily replicate in cell culture.

For alpha-PVs, the mechanism of E2-mediated viral genome tethering and E2-Brd4 chromosomal binding is still elusive. Alpha-PV E2 proteins bind to Brd4 relatively weakly, they do not stabilize the association of Brd4 with host chromatin and cannot be easily detected on mitotic chromosomes, except in late telophase [123,129]. When cells are pre-extracted before fixation, the alpha-PV E2 proteins bind to the peri-centromeric regions of host chromosomes in a Brd4-independent manner similar to that of the beta-PVs [123]. Difficulties in detecting alpha-PV E2-Brd4 mitotic foci have led to the proposal of other targets, such as the mitotic spindle [130], a mitotic kinesin-like protein, MKlp2 [131], ChlR1 (an ATP-dependent DNA helicase important for sister chromatid cohesion) [132] and TopBP1 [129]. Furthermore, HPV31 genomes that encode a Brd4 binding defective E2 protein (an I73L mutation) can still maintain extrachromosomal viral genomes and undergo amplification in differentiated keratinocytes [54,105]. Recent findings indicate that Brd4 colocalizes with nuclear foci formed by the alpha-PV E1 and E2 proteins [114,115], and we find that the alpha-PV E1-E2 protein complex binds to the same regions of host chromatin as the stable HPV1 E2-Brd4 complex in C-33A cells [133].

Therefore, many questions remain, and it seems that the interaction of E2 and Brd4 with host chromatin is complex. The tethering mechanism is likely to be coupled with transcriptional and replication processes. Silla *et al.* have shown that simple attachment of genomes to chromatin is not sufficient [134]; chromatin attachment and transactivation functions must cooperate to ensure proper plasmid segregation. A genome-wide ChIP-on-chip analysis showed that BPV1 E2 and Brd4 were bound to transcriptionally active regions of chromatin, perhaps to ensure that the viral genome localized to transcriptionally active regions of the nucleus [135]. Brd4 is recruited to HPV replication centers containing alpha-PV proteins that do not tightly associate with Brd4 [114,115]. Thus, transcription, replication and genome partitioning are most likely intertwined processes.

4.6. Association of Brd4 with Other Viruses

Papillomaviruses are not the only viruses that have discovered the versatility and usefulness of BET proteins [136]. The EBNA and LANA tethering proteins of the gamma herpes viruses, EBV and KSHV, interact with Brd2 and Brd4 for transcriptional regulation [88,93,137,138]. Polyoma viruses also recruit Brd4 to viral replication centers [139]. Brd4 represses HIV expression by competing with the HIV TAT transactivator for recruitment of p-TEFb to the HIV promoter [73]. Inhibition of Brd4 with BET inhibitors reactivates latent HIV with great therapeutic potential [140–143].

5. Conclusions

Viruses have always alerted us to the key players in cellular processes. Papillomaviruses, in particular, have small genomes with limited coding capacity and rely almost completely on using and

manipulating cellular factors for viral processes. Brd4 is clearly a central player in HPV biology, and a complete understanding of its role in essential viral processes will provide deeper insight into its role in host biology.

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Conflicts of Interest

The authors declare no conflict of interest.

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Review

Biochemical and Functional Interactions of Human Papillomavirus Proteins with Polycomb Group Proteins

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Abstract: The role of enzymes involved in polycomb repression of gene transcription has been studied extensively in human cancer. Polycomb repressive complexes mediate oncogene-induced senescence, a principal innate cell-intrinsic tumor suppressor pathway that thwarts expansion of cells that have suffered oncogenic hits. Infections with human cancer viruses including human papillomaviruses (HPVs) and Epstein-Barr virus can trigger oncogene-induced senescence, and the viruses have evolved strategies to abrogate this response in order to establish an infection and reprogram their host cells to establish a long-term persistent infection. As a consequence of inhibiting polycomb repression and evading oncogene induced-senescence, HPV infected cells have an altered epigenetic program as evidenced by aberrant homeobox gene expression. Similar alterations are frequently observed in non-virus associated human cancers and may be harnessed for diagnosis and therapy.

Keywords: cervical cancer; biomarker; histone methylation; tumor suppressor

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1. Introduction

1.1. Human Papillomaviruses

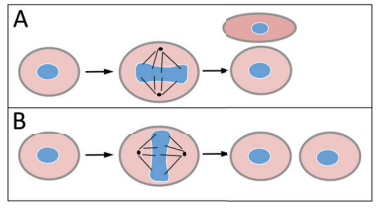
Human papillomaviruses (HPVs) are members of the *Papillomaviridae,* a large family of viruses that infect epithelial cells. Papillomaviruses consist of approximately 8 kb double stranded circular DNA genomes packaged into 55 nm icosahedral capsids. Only one of the two strands is transcribed and encodes five to six "early" (E) open reading frames (ORFs) that encode non-structural regulatory proteins and two "late" (L) ORFs, L1 and L2, from which the major and minor capsid proteins, respectively, are translated. Papillomaviruses exhibit exquisite species specificity, and as a consequence there are no heterologous animal systems to model the natural course of HPV infections.

Almost 200 HPV genotypes have been identified, and they are categorized by the degree of sequence similarity into specific papillomavirus genera [1]. The alpha HPVs have received considerable experimental attention, since they are classified as "high-risk" and "low-risk" based on the propensity for malignant progression of the lesions that they cause. Low-risk HPVs such as HPV6 and HPV11 cause generally benign warts of the genital or oral mucosa whereas infections with "high-risk" HPVs cause lesions of these same target tissues that can undergo malignant progression [2]. Almost 100% of human cervical cancers as well as a large fraction of other anogenital tract and oral carcinomas are caused by high-risk alpha HPV infections [3]. Even though highly efficacious prophylactic vaccines—that prevent infection with the most highly abundant low-risk HPVs (HPV6 and HPV11) and high-risk HPVs (HPV16 and HPV18)—are on the market, the acceptance of these vaccines in the US population has been low [4]. Moreover, because of their cost, these vaccines are not universally available to populations in low-income countries, where cervical carcinoma remains one of the leading causes of cancer death in women [5]. In addition, cervical carcinoma generally develop years or decades after the initial infection and since the prophylactic vaccines do not protect from pre-existing infections or prevent malignant progression, it will be decades before these vaccines will have a noticeable impact on the incidence of HPV-associated cancers [6]. Hence, infections with high-risk alpha HPVs will continue to be one of the most common sexually transmitted diseases and a major cause of morbidity and mortality for decades to come.

Like other viruses, HPVs are obligatory intracellular parasites that need to reprogram the infected host cell to establish an infection and complete their life cycles and produce viral progeny. In the case of HPVs that is a particularly challenging endeavor. First of all, despite extensive splicing due to their limited coding potential, HPVs can only produce a limited number of non-structural proteins. Only two of these, E1 and E2, are mechanistically involved in viral genome replication. E1 has ATPase and DNA helicase activity, and in complex with E2, it binds the viral origin of replication, where it assembles into a hexameric complex that forms a scaffold for binding cellular replication proteins [7]. HPVs do not encode any other proteins that are rate-limiting for replication such as nucleotide biosynthesis enzymes or a DNA polymerase, and these viruses are acutely dependent on the availability of the cellular DNA synthesis machinery to replicate their genomes. Moreover, the productive HPV life cycle is coupled to the differentiation status of the infected squamous epithelial

cell [8]. The squamous epithelium is the largest organ of the human body and is in a constant state of self-renewal. Within this tissue, basal epithelial cells are the only cells that undergo cell division and are the targets of HPV infection. To maintain the integrity of the multilayer epithelial structure, basal epithelial cells divide asymmetrically. One daughter cell retains undifferentiated, DNA synthesis-competent basal cell characteristics, whereas the other daughter cell is poised to undergo cell cycle withdrawal and terminal differentiation, while it is pushed towards the surface of the epithelium and is eventually sloughed off. When the epithelium is injured, however, some basal cells can also undergo symmetrical division to replenish the population of basal cells and close the wound (Figure 1). Since the productive HPV life cycle including production of viral progeny only occurs in terminally differentiated cells, the virus needs to encode proteins that uncouple cell cycle withdrawal from the differentiation program to allow differentiating cells to remain DNA synthesis competent so as to permit viral genome replication. The viral E6 and E7 proteins, which in high-risk HPVs have potent oncogenic activities, are the major drivers of this process. A second and equally important aspect of the viral life cycle is the ability of high-risk HPVs to establish a long-term persistent infection of undifferentiated, basal epithelial cells [8]. One attractive model is that during persistent infection, cells harboring HPV genomes either undergo division only infrequently or that they preferentially undergo symmetrical cell division, yielding two equal basal like cells where the genome is maintained but no infectious progeny is produced. This model has not been tested experimentally, mostly because there are no adequate experimental systems that allow long-term study of a squamous epithelium *in vitro*.

Figure 1. Cell division in a squamous epithelium. (**A**) Asymmetric cell division of a basal epithelial cell yields two unequal daughter cells. One remains an undifferentiated, DNA synthesis competent basal epithelial cell whereas the other daughter is a differentiating epithelial cell that will terminally withdraw from the cell division cycle. Human papillomaviruses (HPV) genomes segregated into such cells will retain these cells in a DNA replication competent state and complete their full replicative life cycle including viral progeny synthesis. (**B**) Symmetric cell division will yield two basal like cells. This mode of cell division is to replenish basal epithelial cell during wound healing. After the initial infection, HPVs establish a long-term persistent infection of these cells where the viral genome is maintained at a low copy number. The viral genome undergoes replication every time the cell replicates its genome. See text for details.



In high-risk HPV-associated cancers the viral genome frequently integrates during malignant progression and only two viral genes, E6 and E7 are consistently expressed in cervical carcinoma cells [9,10]. E6 and E7 each have potent transforming activities and can immortalize primary human epithelial cells and induce genomic instability that drives development of fully transformed, carcinogenic cells [11]. Expression of E6 and E7 in transgenic mice from a keratin K14 promoter that drives their expression in basal epithelial cells in combination with low dose estrogen treatment causes cervical carcinoma development [12]. Cervical carcinoma cells are "addicted" to E6/E7 expression and undergo cell cycle arrest, senescence or cell death when E6/E7 expression is extinguished [13–15]. Hence, HPV E6/E7-mediated cellular reprogramming for the purpose of establishing and maintaining a long-term persistent infection and to generate viral progeny is a risky proposition for the infected host cell as well as the virus, as it can lead to cancer formation and the ultimate demise of the host and the viral genome that is trapped within that host.

In addition to targeting specific transcriptional programs, HPV E6 and E7 proteins are known to affect epigenetic control mechanisms to more globally perturb the transcriptional competence of the infected host cells. Not surprisingly, high-risk HPV E6 and E7 proteins have been reported to biochemically interact with and/or functionally alter a variety of epigenetic enzymes including DNA methylases and histone modifying enzymes. This article focuses on interactions of the HPV E7 protein with components and regulators of Polycomb group (PcG) proteins.

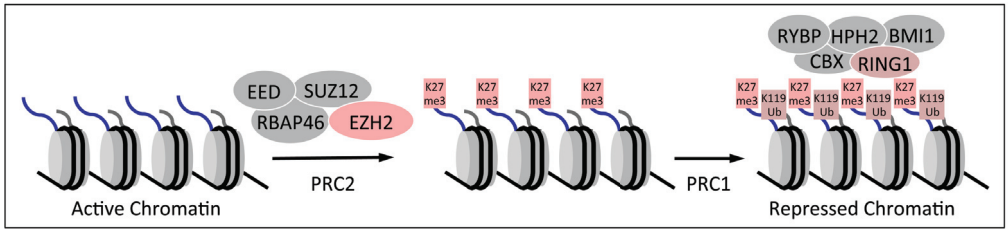
1.2. Polycomb Group Proteins and Homeobox Genes

PcG genes were discovered in *Drosophila melanogaster* and named to denote a mutant phenotype that, in addition to segmentation defects, displays formation of aberrant sex combs on the legs of male flies. PcG proteins form macromolecular repressor complexes, polycomb repressive complexes (PRCs), that globally regulate transcriptional competence of genes that play important roles in cell fate specification and maintenance of stem cell pools [16].

There are two major PRC species, designated PRC1 and PRC2, which play critical roles in epigenetic gene silencing. PRC2 contains the EZH2 enzyme, which places a trimethyl mark on lysine 27 of histone H3 (H3K27me3). This repressive mark causes chromatin compaction and gene silencing. PRC1 occupies H3K27me3 marked chromatin and further silences the chromatin by mono-ubiquitination of lysine 119 on histone H2A (H2AK119Ub) (Figure 2). Some PRC1 complexes can also silence gene expression in the absence of repressive H3K27me3 marks since H2AK119Ub marked chromatin is a binding site for the L3MBTL2 protein, which then establish repressive structures [17] that are particularly important in pluripotent stem cells [18].

There is compelling evidence that PRCs regulate epithelial cell differentiation as well as expansion of basal cell pools during wound healing [19–21]. As described in more detail above, HPVs may need to target both of these processes to allow for viral progeny synthesis and establishment of long-term persistent infection, respectively. Hence, PRC components and molecules that regulate their activity are attractive targets for HPV proteins.

Figure 2. Polycomb mediated silencing of gene expression. The polycomb repressive complex 2 (PRC2) silences chromatin by trimethylating lysine residue 27 (K27me3) of histone H3, causing chromatin compaction. H3K27me3 marked chromatin is bound by PRC1, which further silences gene expression by monoubiquitinating lysine residue 119 (K119Ub) of histone H2A.



The best-known transcriptional targets of PRCs are the homeobox (*HOX*) genes, which encode a family of related transcription factors that contain a homeodomain. *HOX* family members are frequently dysregulated during carcinogenesis [22,23] and germline *HOXB13* mutations have been linked to an increased risk for the development of prostate cancer [24]. HOX proteins are not only downstream transcriptional targets of PRCs, but they also mediate PRC repressor activities on some of their target genes [25].

2. Dysregulation of Homeobox Gene Expression in Cervical Cancers

Not surprisingly, dysregulated HOX gene expression has also been reported during cervical carcinogenesis. In an early study, *HOX* gene expression was analyzed in a series of cervical carcinoma lines and compared to expression in normal cervical epithelium. These studies documented aberrant expression of *HOXA1*, *B2*, *B4*, *C5*, *C10* and *D13* in some of the cancer lines but there was no evidence for coding mutations in *HOX* genes [26]. Differential *HOXC5* expression was also observed in a study where *HOX* gene expression in normal keratinocytes was compared to the HPV16 positive SiHa cervical carcinoma line. This study also provided evidence for increased expression of *HOXC8* in SiHa cells [27], and another study provided additional evidence for *HOXB4* overexpression in cervical carcinomas [28]. Collectively, however, these results are difficult to interpret and may simply reflect the differences in differentiation states of the normal keratinocytes and the cervical carcinoma lines that were analyzed.

The most compelling evidence for aberrant *HOX* gene expression in cervical carcinoma was provided by a study performed with clinical specimens. Gene expression analysis of normal cervix, high-grade premalignant lesions and frank cancers provided strong evidence for upregulation of *HOXC10* expression during cervical cancer progression. Using cell culture based experiments the authors were able to link *HOXC10* expression to the acquisition of the invasive phenotype during cervical carcinogenesis [29].

3. Association of HPV16 E7 with Polycomb Group Proteins

3.1. HPV16 E7

The HPV16 E7 ORF encodes a low-molecular size, 98 amino acid protein that lacks intrinsic enzymatic and specific DNA binding activities. E7 is consistently expressed in cervical cancer even after integration of the viral genome into a host chromosome. It exerts its biological activities through associating with and functionally reprogramming host protein complexes that play critical roles in signal transduction. The best-studied cellular target of HPV16 E7 is pRB, the protein encoded by the retinoblastoma susceptibility gene (RB1), and the related p107 (RBL1) and p130 (RBL2) proteins [11]. However, additional important cellular targets of HPV16 E7 exist, and with the development of proteomic methods, identification of putative transformation targets for the HPV E7 protein has been a mainstay of HPV research [30].

3.2. Association of HPV16 E7 with E2F6 Containing Polycomb Repressive Complexes

One of the first proteomic studies with twin-epitope tagged HPV16 E7 ectopically expressed in HeLa cells, led to the identification of multiple previously unappreciated cellular HPV16 E7 targets, including the E2F6 transcriptional repressor [31,32]. E2F6 is a non-canonical member of the E2F family of transcription factors as it lacks the C-terminal binding site for pRB family member, and hence its transcriptional activity is not modulated by pRB binding. Like other E2F family members, E2F6 associates with a dimerization partner (DP1 or DP2) to form a transcriptionally active DNA binding complex. The E2F6/DP1 or DP2 heterodimer functions as a transcriptional repressor that is expressed in late S-phase, presumably to down-regulate E2F transcriptional target genes and stimulate S-phase exit [33,34]. HPV16 E7 associates with the C-terminal repressor domain of E2F6 and abrogates its repressive activity on E2F6 target genes. The ability to target E2F6 is shared with low-risk HPV E7 proteins, and the simian vacuolating virus 40 large tumor antigen (SV40 TAg) and the adenovirus (Ad) E1A protein also share the ability to inhibit E2F6 repression [32].

E2F6 is also a component of PRCs and several, apparently distinct, E2F6 containing PRCs have been described [35–38]. Examination of E7 associated proteins revealed evidence for the presence multiple additional PRC components, including BMI1, PCGF2 (MEL-18), CBX4 (hPC2), RING1, MGA and L3MBTL2 [32]. Many of these proteins have been specifically described as components of E2F6-associated PRCs, and hence it is likely that HPV16 E7 can associate with and potentially modify activities of E2F6 containing PRCs. It has not been investigated whether low-risk HPV E7 proteins, SV40 TAg or Ad E1A can also associate with E2F6 containing PRCs.

Detection of E2F6 containing nuclear dots, presumably representing E2F6 containing PRCs were diminished in HPV16 E7 expressing cells, and an HPV16 E7 mutant that does not associate with E2F6 had no effect on the number of detectable E2F6 nuclear dots [32]. This finding is consistent with the model that E7 binding may alter the composition of E2F6 containing PRCs. This model, however, has not yet been tested experimentally.

3.3. Decreased Trimethylation of Lysine 27 of Histone H3 in HPV16 E7 Expressing Cells

Analysis of H3K27me3 levels in HPV16 E7 expressing primary human keratinocytes revealed a dramatic decrease in the H3K27me3 mark compared to donor and passage matched control vector expressing keratinocytes. Interestingly, there was no comparable decrease in the H3K27me2 and H3K27me1 marks [39]. Similar decreases in the H3K27me3 mark were also detected in clinical specimens of HPV16 positive high-grade premalignant squamous intraepithelial lesions (SILs) of the cervix [39,40]. Studies with a U2OS human osteosarcoma cell line with tetracycline-inducible E7 expression showed that the observed decrease of the repressive H3K27me3 mark is a direct and immediate effect of HPV16 E7 expression [39]. H3K27 staining diminishes within 72 hours of E7 induction and is restored when E7 expression is switched off. These results may provide an alternative explanation for the observed loss of detection of E2F6 containing PRCs in HPV16 E7 expressing cells.

4. Modulation of Polycomb Group Protein Expression by HPV16 Oncoproteins

4.1. Increased EZH2 Expression in HPV E7 Expressing Cells

The enzymatic PRC2 component, the H3K27 methyltransferase EZH2, is a bona fide human oncogene that is overexpressed and amplified in human tumors [41–43]. EZH2 transcription is regulated by E2F transcription factors [41]. HPV16 E7 targets pRB, p107 and p130 for proteasomal degradation and associates with E2F6 and therefore interferes with E2F transcriptional repressor activities, causing deregulated and increased expression of E2F transcriptional targets. Consistent with this model, HPV16 E7 transcriptionally activates EZH2 through its E2F sites and EZH2 is highly overexpressed in cervical lesions and tumors [44]. Interestingly, cervical carcinoma cells appear addicted to EZH2; depletion caused G1 cell cycle arrest and a low level of apoptosis.

High levels of EZH2 expression in HPV-positive cervical carcinomas and HPV E7 expressing cell lines and their apparent addiction to EZH2 is particularly remarkable since the H3K27me3 mark is decreased, not increased, in such cell lines. Different potential mechanistic explanations have been proposed to account for this apparently paradoxical finding. There is evidence that the enzymatic activity of EZH2 in PRC2 complexes is negatively regulated by AKT mediated phosphorylation at serine residue (S) 21 [45]. Since HPV16 E6 and E7 have both been reported to cause AKT activation [46,47] it is conceivable that PRC2 complex-associated EZH2 enzymatic activity may be low despite high-level overexpression in HPV16 positive lesions and cancers. A recent exciting study has shown that EZH2 phosphorylation on S21 acts as a functional switch that promotes EZH2 association with an androgen receptor containing transcription factor complex and in this context EZH2 acts as a polycomb independent activator of gene expression [48]. This study also provided compelling evidence that the oncogenic activity of EZH2 in castration-resistant prostate cancer cells is based on this polycomb independent activity of EZH2 [48]. EZH2 has also been detected in PRC complexes that catalyze H1K26 methylation [49]. It will be important to determine the

posttranslational modifications of EZH2 and the biochemical composition of EZH2 complexes in HPV16 E7 expressing cells.

4.2. Modulation of BMI1 Expression by HPV E6 and E7

Expression of other PcG proteins in HPV16 E6/E7 expressing cells has also been studied. Levels of the PRC1 components SUZ12 and EED were reported to be unchanged but the levels of BMI1 were dramatically reduced in HPV16 E6/E7 expressing cells [40]. These results were somewhat surprising since BMI1 is a well-known oncogene that is frequently overexpressed in human tumors including cervical carcinomas [50–53], and it has even been suggested that BMI1 autoantibodies may be useful as a biomarker of cervical carcinomas [54].

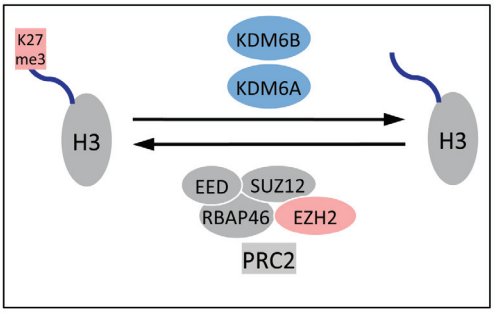
In another study, HPV16 E6 was shown to increase BMI1 expression in primary human keratinocytes, and levels remained high in HPV E6/E7 immortalized keratinocyte lines. Most intriguingly, BMI1 could substitute for E6 and cooperate with E7 to cause keratinocyte immortalization [55]. This result is consistent with an earlier study that showed that BMI1 depletion in the HPV18 positive HeLa cervical carcinoma line caused S-phase depletion and G1 growth arrest [56].

5. Modulation of PRC Regulators by HPV16 E7

5.1. Upregulation of the H3K27 Demethylases KDM6A and KDM6B

H3K27 trimethylation is a dynamic process that is reversed by two demethylases, KDM6A (UTX) and KDM6B (JMJD3) (Figure 3). A mechanistic explanation for the dramatically decreased H3K27me3 levels in HPV16 positive cervical lesions and cancers was provided by the finding that KDM6A and KDM6B are expressed at markedly higher levels in these cells [39].

Figure 3. PRC2 mediated gene silencing is reversible. The EZH2 histone methyl transferase in PRC2 silences gene expression by adding the trimethyl mark on lysine residue 27 of histone H3, and this mark can be removed by one of the two H3K27 specific demethylases, KDM6A or KDM6B. See text for details.



Even though KDM6A and KDM6B have identical catalytic activities and substrate specificities, they have different chromatin targets. KDM6A is encoded on the X-chromosome but escapes X-inactivation [57]. A related gene, UTY, is encoded on the Y chromosome but the UTY protein appears to lack histone demethylase enzymatic activity [58].

The fact that KDM6A and KDM6B have non-overlapping and non-redundant biological activities and that KDM6A may have biological activities that are independent of enzymatic activities have been impressively validated in genetically engineered mouse models (GEMMs). Studies with KDM6A$^{-/-}$ GEMMs revealed that KDM6A plays an important role in activating cardiac differentiation [59]. KDM6A$^{-/-}$ female mice died at E12.5 with defects in neural tube, yolk sac, and cardiac development, whereas approximately 20% of KDM6A$^{-/-}$ male mice survived. These animals are fertile, but are smaller and have a reduced life span [58,60,61]. Studies with embryonic stem (ES) cells derived from these GEMMs and GEMMs expressing enzymatically inactive KDM6A, revealed that KDM6A regulated embryonic development may be independent of its enzymatic activity [61].

KDM6B$^{-/-}$ GEMMs show perinatal lethality due to respiratory failure, which is caused by a developmental defect of the respiratory neuronal network [62]. KDM6B may also have biological activities that may not dependent on H3K27 demethylation. In macrophages KDM6B is induced by NF-kB in response to inflammatory stimuli and modulates expression of PRC silenced genes [63]. This KDM6B activity also does not appear to primarily depend on H3K27 demethylation [64].

The observed non-redundant activities of KDM6A and KDM6B may reflect incorporation of the two proteins into different multiprotein complexes. KDM6A is associated with H3K4 methyl transferase activity containing Mixed-Lineage-Leukemia (MLL)2/3 complexes [65,66]. KDM6A containing MLL2/3 complexes might link H3K27 demethylation to H3K4 methylation, and KDM6A/MLL2/3 complexes can convert transcriptionally silenced chromatin to a transcriptionally competent state. KDM6A mutations have been detected in some tumors [67], whereas KDM6B is upregulated in some cancers, particularly metastatic prostate carcinomas [68].

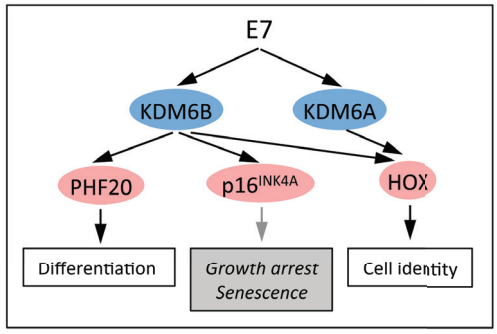
HPV16 E7 upregulates expression of KDM6A and KDM6B, at least in part at the level of transcription. The exact mechanisms of transcriptional induction of KDM6A and KDM6B expression remain to be determined. Upregulation of KDM6B, however, is clearly independent of HPV16 E7-mediated pRB degradation and activation of canonical E2F transcription factors [39].

5.2. KDM6B Controls Epithelial Differentiation

Genes that are specifically expressed during skin differentiation frequently carry H3K27me3 repressive marks in undifferentiated basal epithelial cells and are subject to PRC transcriptional regulation. Differentiation causes removal of these marks and relief from PCG repression. Even though KDM6B levels remain unchanged during differentiation, the removal of the repressive H3K27me3 mark is mostly mediated by KDM6B. This is based on the observation that KDM6B depletion in keratinocytes inhibited differentiation whereas ectopic expression accelerated differentiation. KDM6B enzymatic activity was required for acceleration of epithelial differentiation [69]. These results predict that HPV16 E7 expression not only changes *HOX* gene expression but also induces expression of

genes that are normally only expressed in differentiating epithelial cells (Figure 4). This might play an important role in the viral life cycle as the switch from early to late viral gene expression is mediated by differentiation cues.

Figure 4. HPV16 E7 causes increased KDM6A and KDM6B expression. E7 mediated KDM6A and KDM6B induction affects multiple transcriptional programs, including keratinocyte differentiation and cell identity. KDM6B induction represents a cellular defense response to E7 oncogene expression that is to trigger cell cycle arrest and senescence. HPV16 E7 short-circuits this response by targeting the retinoblastoma tumor suppressor for degradation. Several other viruses including EBV have developed strategies to subvert such cellular defense responses. See text for details.

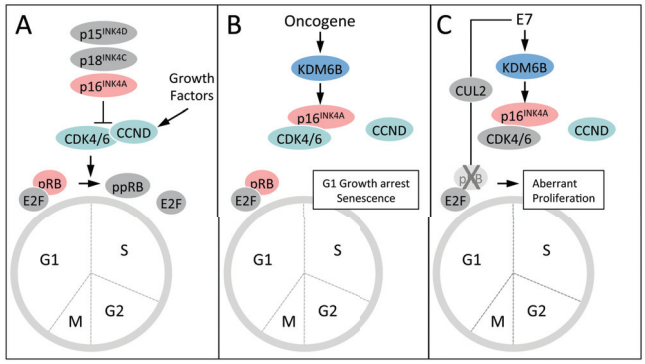


5.3. KDM6B Mediates the Oncogene-Induced Senescence (OIS) Response to RAS/RAF

Oncogene induced senescence (OIS) is one of the cell intrinsic tumor suppressor responses that has evolved to eliminate aberrantly proliferating and therefore potentially tumorigenic cells from the proliferative pool. OIS was initially discovered with the *RAS* oncogene and is signaled through transcriptional upregulation of the $p16^{INK4A}$ tumor suppressor [70] (Figure 5). In some cell types, particularly in mouse cells, the partially overlapping gene encoding $p14^{ARF}$ (or $p19^{ARF}$ in mouse cells) is also induced and causes activation of the p53 tumor suppressor by inhibiting ubiquitination by MDM2 [71]. The $p16^{INK4A}$ protein is a component of the retinoblastoma tumor suppressor pathway and inhibits CDK4/6-cyclin D mediated pRB phosphorylation and S-phase entry (Figure 5). It is frequently mutated, deleted or transcriptionally silenced in human tumors and tumor cell lines, supporting the notion that overcoming the OIS response is a major bottleneck in human cancer development.

Expression of $p16^{INK4A}$ in normal human cells is generally low because the gene is silenced by H3K27 trimethylation and PRCs. In response to RAS/RAF stimulation, KDM6B expression is transcriptionally activated, potentially through AP1, causing removal of the H3K27me3 mark and enhanced $p16^{INK4A}$ expression. This then signals G1 arrest and senescence through activation of the pRB tumor suppressor. Notably, however, RAS/RAF stimulation does not induce KDM6A expression nor does it consistently cause de-silencing of the $p14^{ARF}$ promoter [71,72].

Figure 5. Induction and abrogation of the Oncogene Induced Senescence (OIS) tumor suppressor response by HPV16 E7. (**A**) The retinoblastoma tumor suppressor (pRB) pathway regulates S-phase cell cycle entry. Activity of pRB is regulated by phosphorylation by cyclin dependent kinase 4 or 6 (CDK4/6). CDK4/6 activity is activated by association with a D-type cyclin (CCND) and negatively regulated by association with one of several small molecule inhibitors ($p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$), which are induced in response to different growth suppressive stimuli. CDK4/6 phosphorylation of pRB at the G1/S boundary inactivates the transcriptional repressor activity of the pRB/E2F complex and signals S-phase entry. (**B**) Oncogenic stimuli such as RAS/RAF signaling or HPV16 E7 expression causes KDM6B expression and de-repression of $p16^{INK4A}$ expression, which inhibits CDK4/6 activity and pRB phosphorylation, causing G1 cell cycle arrest and senescence (OIS). (**C**) HPV16 E7 has evolved to evade OIS by targeting pRB for ubiquitin mediated proteasomal degradation.



5.4. HPV16 E7 Induces the Cervical Cancer Biomarker $p16^{INK4A}$ through KDM6B

The $p16^{INK4A}$ tumor suppressor is an important biomarker for high-risk HPV-associated lesions and cancers and is induced by E7 [73,74]. It was initially thought that E7 induced $p16^{INK4A}$ through E2F activation [75], but there are no E2F response elements in the $p16^{INK4A}$ promoter and a pRB binding/degradation deficient HPV16 E7 mutant that does not activate E2F transcription, induces $p16^{INK4A}$ expression as efficiently as wild-type HPV16 E7 [39]. More recent studies have clearly shown that high level $p16^{INK4A}$ expression in cervical lesions and cancer represents a readout of an E7 induced OIS response [39]. How E7 triggers the response and how it is signaled to the KDM6B promoter is unclear, but E7 proteins also blunts OIS signaling by targeting pRB for ubiquitin dependent proteasomal degradation. Indeed, earlier studies have shown that pRB degradation, not merely binding, is necessary to fully inhibit pRB induced senescence [76]. Notably, $p14^{ARF}$ expression is also increased in E7 expressing cells but in contrast to $p16^{INK4A}$, depletion of KDM6B does not decrease $p14^{ARF}$ levels or the H3K27me3 mark at the $p14^{ARF}$ promoter [39].

Abrogation of the $p16^{INK4A}$ OIS block in primary lymphocytes also represents a major roadblock to transformation by Epstein-Barr virus (EBV) [77]. The EBV proteins EBNA3A and EBNA3C cooperate to epigenetically silence the $p16^{INK4A}$ promoter through a mechanism that involves the transcriptional repressor C-terminal binding protein (CtBP) [78,79]. Transcriptional silencing of other PRC controlled genes, such as the apoptosis regulator BIM, likely also contribute to EBV transformation [80]. Similarly, the hepatitis B virus (HBV) X protein downregulates the PRC2 protein SUZ12, which is essential for placing the H3K27me3 mark [81].

It is interesting to note that high-level $p16^{INK4A}$ expression has also been detected in a number of other cancer types, including lung, prostate, breast and ovarian cancers [82–85]. In most cases, high level $p16^{INK4A}$ expression is linked to pRB mutations [86], and it will be important to determine whether $p16^{INK4A}$ expression is also mediated by KDM6B in such tumors. As mentioned previously, KDM6B upregulation has been reported in metastatic prostate carcinomas [68].

Cervical carcinoma cells are “addicted” to HPV E6/E7 oncogene expression and undergo growth arrest, senescence and cell death when expression of the viral oncoproteins is silenced [13–15]. Loss of HPV E6 or E7 expression can each individually induce senescence through re-activation of the p53 and pRB pathways, respectively [87–89]. It will be interesting to determine whether epigenetic mechanisms in general and PcG group proteins in particular are involved in signaling induction of senescence responses in these cases.

5.5. Dysregulated Homeobox Gene Expression in HPV16 E7 Expressing Cells

In contrast to RAF/RAS stimulation, HPV16 E7 also causes increased expression of the H3K27-specific demethylase KDM6A [39,40]. It is not clear whether KDM6A expression also represents a cellular tumor suppressive defense response, how E7 triggers it and what the transcriptional consequences of KDM6A expression may be. The fact that KDM6A has been classified as a putative tumor suppressor [67] may support the model that KDM6A may also be induced as a consequence of E7 triggering a cell intrinsic tumor suppressive defense response. Therefore it will be important to determine the mechanisms of E7 mediated KDM6A induction, the transcriptional consequences of KDM6A expression and the mechanisms by which E7 subverts KDM6A tumor suppressor activity.

It is known that KDM6A controls expression of *HOX* genes and indeed, *HOX* gene expression is dysregulated in HPV16 E7 expressing cells [39]. As pointed out in a previous section, *HOX* gene expression is frequently dysregulated in human cancers, including cervical carcinomas, and some *HOX* genes have been shown to contribute to cancer formation. Aberrant *HOX* gene expression in E7 expressing cells suggests that *HOX* gene dysregulation observed in cervical carcinoma cells is caused by HPV E7 expression and suggests that HPV infection causes reprogramming of the epigenetic make up of epithelial cells.

5.6. KDM6A and KDM6B are Essential in Cervical Carcinoma Cells

It is not intuitively obvious that KDM6A and/or KDM6B upregulation by HPV16 E7 would contribute to E7 transformation, since abrogation of downstream transcriptional consequences, such as $p16^{INK4A}$ mediated OIS signaling, is critical for HPV, EBV and possibly HBV cell transformation. Hence, it was surprising that KDM6A as well as KDM6B depletion dramatically inhibited viability of the HPV16 positive CaSki cervical carcinoma line [39]. This finding suggests that survival of HPV E7 expressing cells is critically dependent on KDM6A and KDM6B targets. Even though it cannot be ruled out that these enzymes may have substrates other than methylated H3K27, it is likely that these represent transcriptional targets. The full delineation of KDM6A and KDM6B targets represents a major effort, but it is feasible by determining the full spectrum of human genes that have altered H3K27me3 marks in response to KDM6A or KDM6B using chromatin immunoprecipitation followed by DNA sequencing (ChIPSeq) in combination with expression profiling.

6. Concluding Remarks

Dysregulation of the histone code and activation of transcriptional programs in the "wrong" cell type is a hallmark of carcinogenesis. The REST transcriptional repressor, for example, is responsible for silencing neuronal-specific gene expression in non-neuronal cells. REST is a major tumor suppressor in colon, lung and breast cancers [90–92] and may be key to the neuro-endocrine character of some of these tumors. Similarly, PcG group proteins have been implicated in human carcinogenesis and some, including *BMI1* and *EZH2* are bona fide oncogenes that are overexpressed in many tumor types. The H3K27 demethylases KDM6A and KDM6B have also been implicated in carcinogenesis and the finding that KDM6B is the major mediator of OIS further supports the involvement of these enzymes in cancer formation.

One of the most exciting possibilities is that, unlike genetic mutations, epigenetic alterations may be reversed by inhibiting the enzymes that cause them. Even though some functions of KDM6A and KDM6B appear to be independent of their enzymatic activities, a recently developed KDM6 selective inhibitor was show to affect proinflammatory signaling in macrophages [93], even though previous studies suggested that KDM6B modulation of expression of PRC silenced genes in response to inflammatory stimuli [63] may not primarily depend on H3K27 demethylation [64]. Given the result that KDM6A as well as KDM6B expression is essential for viability to cervical carcinoma cells, KDM6 inhibition should be evaluated as a therapeutic modality for some human cancers.

Acknowledgments

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Conflicts of Interest

The authors declare no conflict of interest.

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Review

Regulation of Human Cytomegalovirus Transcription in Latency: Beyond the Major Immediate-Early Promoter

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Abstract: Lytic infection of differentiated cell types with human cytomegalovirus (HCMV) results in the temporal expression of between 170–200 open reading frames (ORFs). A number of studies have demonstrated the temporal regulation of these ORFs and that this is orchestrated by both viral and cellular mechanisms associated with the co-ordinated recruitment of transcription complexes and, more recently, higher order chromatin structure. Importantly, HCMV, like all herpes viruses, establishes a lifelong latent infection of the host—one major site of latency being the undifferentiated haematopoietic progenitor cells resident in the bone marrow. Crucially, the establishment of latency is concomitant with the recruitment of cellular enzymes that promote extensive methylation of histones bound to the major immediate early promoter. As such, the repressive chromatin structure formed at the major immediate early promoter (MIEP) elicits inhibition of IE gene expression and is a major factor involved in maintenance of HCMV latency. However, it is becoming increasingly clear that a distinct subset of viral genes is also expressed during latency. In this review, we will discuss the mechanisms that control the expression of these latency-associated transcripts and illustrate that regulation of these latency-associated promoters is also subject to chromatin mediated regulation and that the instructive observations previously reported regarding the negative regulation of the MIEP during latency are paralleled in the regulation of latent gene expression.

Keywords: cytomegalovirus; latency; gene expression; chromatin

1. Introduction

Human cytomegalovirus (HCMV) is an opportunistic pathogen that, like all herpes viruses, can establish a latent infection that persists for the lifetime of the host. In healthy individuals both primary infection and the reactivation of latent virus rarely causes any significant clinical symptoms due to a robust immune response in the host [1,2]. In contrast, infection or reactivation in immuno-suppressed transplant patients or immune-compromised late stage AIDS sufferers represents a major source of morbidity and mortality [3–5]. Furthermore, HCMV infection in utero remains the leading viral cause of infectious congenital disease [6]. As it is now clear that a major contribution to HCMV-mediated disease is due to the reactivation of latent virus, [2,4,7–9] a number of laboratories have studied intensively the mechanisms that activate and repress viral immediate early gene expression during lytic and latent infection, respectively.

2. Histones, Chromatin and Gene Expression

The 'histone code hypothesis' predicts that the signature of the post-translational modifications of histone proteins directly impacts on the transcriptional activity of the cell [10–12]. In the broadest terms, a promoter can be transcriptionally active or transcriptionally silent and this is influenced principally by the acetylation and methylation states of its associated histones [13–15]. As such, pan acetylation of histones H3 and H4 is directly linked with a promoter that is capable of transcription [14]. In contrast, trimethlyation at lysine residues 9 and 27 on histone H3 and the subsequent recruitment of heterochromatin protein 1 (HP1) or polycomb proteins, respectively, is indicative of a transcriptionally repressed promoter [16–19]. As with all biological systems caveats do exist. For instance, histone methylation is not exclusively indicative of repression—dimethylation at lysine residue 4 on histone H3 is a marker of a promoter that has been recently active [20]. Additionally, histone phosphorylation, which occurs at serine (10 & 28) and threonine (11 & 29) residues in the N terminal of histone H3, has been linked with both transcriptional activation and repression of promoter activity depending on the phase of the cell cycle [21].

The regulation of the major immediate early promoter (MIEP) during latency and reactivation has been the subject of a number of recent reviews and, thus, it will not be covered extensively, here. Suffice to say, the regulation of the MIEP during latency and reactivation exhibits many hallmarks associated with chromatin-mediated regulation of eukaryotic gene expression. The MIEP in latently infected CD34+ haematopoietic cells or circulating monocytes isolated from healthy seropositive donors is predominantly associated with HP1 [22]—a marker of a transcriptionally silenced promoter that is recruited to promoters via an interaction with histone H3 trimethylated on lysine 9 (H3K9) [19]. However, differentiation to a mature dendritic cell (DC) phenotype is concomitant with high levels of acetylation of the histones bound to the MIEP and a lack of HP1 binding [22]. Importantly, the differences in the pattern of the modifications of the histones bound to the MIEP in undifferentiated or differentiated myeloid cells correlates directly with the ability to detect of IE gene expression in these different cell types in healthy individuals [22–26].

It is now well accepted that differentiation-dependent changes in the post-translational modifications of histones around the MIEP regulate latency-associated repression and reactivation of MIEP activity. However, much less is known about viral gene expression associated with latent infection and whether this is also regulated by histone modifications. With the advent of more sensitive high throughput screening techniques, the identification of a small number of latency associated transcripts (which, in most cases, are also expressed during lytic infection) has been possible. These transcripts expressed during latent infection are from viral genes dispersed throughout the viral genome, suggesting that no one region is particularly 'latently active' but rather that latency-associated transcripts are regulated independently by specific promoters.

In this short review, we will present the current status of our knowledge regarding the regulation of expression of the latency associated transcripts of HCMV and its relationship to post-translational modifications of histones.

3. Human Cytomegalovirus Latent Gene Products

A key biological property of all herpes viruses is their ability to undergo latent infection during which time only a subset of viral genes are expressed. Latent herpes simplex virus (HSV) gene expression is predominantly restricted to one locus—the latency associated transcript (LAT) region [27]. Alternate splicing generates multiple non-coding RNAs with functions ranging from anti-apoptosis [28], anti-sense mediated inhibition of lytic gene expression [27,29] and the generation of miRNAs that influence gene expression during latency [30]. In contrast, a number of patterns of Epstein-Barr virus (EBV) latent gene expression have been reported based on the phenotype of the infected lymphocyte [31]. The initial infection and transformation of a resting B cell (latency III) is concomitant with the expression of a number of gene products including EBNA-1, -2, 3A-C, EBNA-LP, LMP-1, 2A and B, as well as the untranslated EBER transcripts. It is hypothesised, based on studies of cell lines and the analysis of tissue ex vivo that the level of EBV latent gene expression is down-regulated (latency II - EBNA-1, LMP-1, LMP-2A and the EBERs) with the LMP proteins thought to drive the differentiation into latently infected memory B cells [32–36]. Consistent with this, circulating immunoblastic lymphomas (latency III) are rarely detected *in vivo* likely due to the robust immune response directed against EBV in the host promoting their elimination. Indeed, the most common phenotype (latency 0) is genome carriage in the absence of latent gene expression [37]—although LMP-2A transcripts have been detected in these cells [38]. However, dividing memory B cells that display a latency I phenotype (EBNA-1) have also been detected *in vivo* [35].

In contrast to HSV and EBV, the analysis of latent HCMV gene expression is in its relative infancy. Latent transcripts arising from the major immediate early region had been identified in specific populations of granulocyte-macrophage progenitors by the Mocarski laboratory in the 1990s [39,40] and US28 transcripts were also reported in an experimentally infected THP1 leukaemia cell line [41], but the application of high throughput molecular approaches led to the identification of a number of novel latent transcripts. Microarray technology employed by the Shenk and Slobedman laboratories detected transcription from a number of different loci in experimentally latently infected myeloid

progenitor cells [42,43]. Subsequent work confirmed that some of these products were also expressed during natural latency and included UL138 [44], UL111A [43] as well as the UL81-82 anti-sense transcript (UL81-82ast) [45,46]. Although it is worth noting that the latent transcriptomes reported by these studies are not entirely overlapping, likely due to different cell types and viral strains used in the analyses. Indeed, the possible effects of using different viral strains is no better illustrated than by recent work showing that the expression of UL144 during latency appears to be dependent on the strain of virus used [47] and is discussed in more detail later.

The selective expression of viral genes during latent infection is likely to depend on similar mechanisms known to modulate the MIEP during latent and lytic infection. Importantly, aberrant expression of viral gene products not required during latency would likely risk triggering a robust host immune response, from the high memory T cell population known to recognise lytic antigens in normal HCMV carriers, with no benefit to the virus. Consequently, tight control of viral gene expression during latency is likely to be of some import. Since, most of the viral genes expressed during lytic infection are regulated by the prodigious activity of the IE72 and IE86 proteins [48–52], a failure to express a number of genes during latency can likely be attributed to the lack of IE expression resulting from extensive silencing of the MIEP which is clearly observed in latently infected myeloid progenitor cells (reviewed in [53]). Clearly then, gene expression during latency must be subject to mechanisms of regulation which are independent of the functions of viral IE gene products. In this review, we will use the regulation of two gene products—UL81-82ast and UL144—to illustrate potential mechanisms for the control of latent gene expression by HCMV in myeloid progenitor cells.

4. The UL81-82 Antisense Transcript—LUNA

Of all the putative latent transcripts, the factors controlling expression of the UL81-82 antisense transcript (UL81-82ast) during latent and lytic infection are arguably the best understood [46,47,54]. UL81-82ast was identified by Bego *et al.* [45] whilst searching for the UL81 transcription detected in the previous study by Goodrum *et al.* [42]. Bego *et al.* identified RNAs spanning the UL81 region in naturally latent monocytes and showed that transcription of this RNA actually occurs from the opposite strand encoding UL81 (hence the designation UL81-82ast), giving rise to a putative 133aa serine rich protein called LUNA (Latency Unique Nuclear Antigen). Although there is no known function attributed to LUNA during natural latency, there is some recent evidence that expression of LUNA may impact on HCMV carriage and reactivation *in vitro* [55].

Superficially, at least, the regulation of LUNA promoter activity during lytic [54] and latent [46,47] infection appears to be mediated by very different mechanisms. However, closer scrutiny reveals that a number of the key proteins involved in latent or lytic LUNA transcriptional regulation actually exhibit overlapping functions—this provides an interesting model for the complex regulation of herpes virus gene promoters during different phases of infection.

4.1. Regulation of LUNA during Latent Infection

After confirmation that the expression of LUNA could be detected in experimentally and naturally latent CD34+ cells, a very simple question was asked—is the LUNA promoter associated with histone proteins during latency and, if so, what post-translational modifications do these histones have? Chromatin Immunoprecipitation analyses of the LUNA promoter alongside the MIEP showed that the promoter is indeed associated with histones [46]. Furthermore, the LUNA promoter, in contrast to the MIEP, was associated with acetylated histones [22,46]. Thus, in the latent phase of infection, the LUNA promoter is associated with histone post-translational modifications that support gene expression.

4.2. Regulation of LUNA during Lytic Infection

Although classified as a latent transcript, LUNA, like other latency associated transcripts, is also expressed during lytic infection [45]. Broadly speaking, the regulation of viral gene expression during lytic infection is subject to regulation by histone proteins [56–61] and, consistent with this including LUNA, the transfected LUNA promoter is responsive to the histone deacetylase inhibitor, Trichostatin A (TSA). Furthermore, increased expression of LUNA was detectable in virally infected cells incubated with TSA [54]. Further investigation identified that the expression of IE72 was critical for LUNA expression during lytic infection. Interestingly, IE72 has been hypothesised to drive early and late viral gene expression via the sequestration of inhibitory histone deacetylase activity [59]. However, unlike reported for other IE72-responsive genes LUNA gene expression in an IE72 null background was not rescued by TSA alone [54] suggesting that, although histone proteins may play a role, additional regulatory mechanisms besides IE72-mediated sequestration of histone deacetylases were needed for LUNA promoter activation [54]. Further work showed that activation of the LUNA promoter during lytic infection involved IE72 overcoming the repressive activity of the cellular repressor hDaxx and its binding partner, ATRX [54] (Figure 1A,B). Both hDaxx and ATRX have also been shown by a number of groups to exert a profound phenotype on the activity of the MIEP and these cellular proteins are targeted by incoming tegument protein, pp71 [60,62–65].

These observations are consistent with the view that chromatin architecture, whilst providing the framework for controlling gene expression, is not the only factor involved in LUNA promoter regulation. Indeed, it is interesting to note that parallels occur in other herpes viruses. EBV encoded BNRF1 disrupts the hDaxx:ATRX interaction to relieve early gene expression from the transcriptional silencing imposed by this complex also [66].

4.3. LUNA Is Regulated by the GATA Transcription Factors during Latency

The obvious question that arises from these observations is what drives differential LUNA promoter activity during latent infection? During latency LUNA expression must be independent of IE72 (by definition) whereas LUNA gene expression during lytic infection is IE72-dependent. Clues to the regulation of the LUNA promoter during latency came from a bioinformatics approach identifying

a number of putative transcription sites in the LUNA promoter, including the GATA family of transcription factors [47]. GATA transcription factors are expressed in the haematopoietic cell lineage and have differential expression profiles dependent on the cell fate [67]. GATA-2 is a transcription factor expressed in early myeloid progenitors and declines in levels as myeloid cells differentiate and is sometimes referred to as the 'master regulator' of haematopoietic progenitor cells [68,69]. Certainly, GATA-2 is essential for haematopoiesis providing credence for such a grandiose moniker [69]. Furthermore, a lack of GATA-2 results in a severe defect in the generation of the granulocyte/ macrophage progenitor population [68]—a cell type which represents an important site of HCMV latency in the myeloid lineage [24,39,70]. Pertinently, GATA-2, along with other family members, has been shown to interact with chromatin modifying enzymes [71,72]. Amongst these interaction partners are histone acetyltransferases (pCAF and CBP/p300) which are important for histone and non-histone protein acetylation, including the GATA proteins themselves [71–73]. Similarly, GATA proteins also interact with histone deacetylases (HDACs) with GATA-2 interacting with HDACs-3 and -5, but not HDAC-1 [74] and thus, like a number of proteins, GATA-2 can promote the deacetylation as well as acetylation of target proteins including itself.

Interestingly, the LUNA promoter is GATA-2 responsive in transfection assays [46,47] and we have observed that GATA-2 over expression can rescue LUNA gene expression in ΔIE72 virus infected cells (M.R. and J.S., unpublished observations). Furthermore, a study that assessed the impact of HCMV latency on the expression of cellular miRNAs identified that mir92a was down-regulated in latently infected cells [75]. Interestingly, a target of mir92a is GATA-2 and, consistent with this, GATA-2 expression levels were elevated in latently infected cells [75]. Thus, at least in latency, it can be argued that HCMV promotes a micro-environment that supports the expression of LUNA by modulating the availability of the GATA-2 transcription factor. Taken together, these observations argue that during latency high levels of GATA-2 bind to the LUNA promoter and propagate a chromatin environment that is pro-transcriptional. However, during lytic infection, depletion of hDaxx:ATRX complex is required for LUNA gene expression to occur [54]. A pertinent question is why, then, does this complex not repress LUNA promoter activity in myeloid progenitors? One possible explanation is that there are differences in the relative balance of transcriptional activators and repressors which determine the activity of the LUNA promoter in different cell types. The viral MIEP provides a paradigm for this type of differential regulation in undifferentiated and differentiated myeloid cells (Figure 1). High levels of transcriptional repressors that bind the MIEP are present in early myeloid progenitors (Figure 1C) [22,76–80]. Furthermore, HCMV binding and entry into undifferentiated myeloid cells has been suggested to up-regulate transcription factors that would promote an environment repressive for the MIEP [81]—an event that does not occur upon infection of permissive fibroblasts [82]. In contrast, myeloid cell differentiation is concomitant with changes in both the absolute levels of repressors as well as the activation of transcriptional activators of the MIEP [22,83–85]. In the context of the LUNA promoter, the hDaxx:ATRX complex is likely to be stable in many cell types.

Figure 1. A model for the regulation of the major immediate early promoter (MIEP) and Latency Unique Nuclear Antigen (LUNA) promoter during lytic and latent infection. (**A**,**B**) Lower levels of GATA-2 expression in permissive differentiated cell types favour the recruitment of ATRX/Daxx to GATA-2 binding sites in the LUNA promoter to establish a repressive phenotype. As the lytic infection proceeds (**B**), IE72 is expressed which sequesters ATRX/Daxx complexes thus removing them as binding competitors at the LUNA promoter. This potentially increased GATA-2 binding which would promote LUNA gene expression in differentiated cells; (**C**) Latency is established following the infection of non-permissive haematopoietic CD34+ cells. High levels of transcriptional repressors bind to the MIEP and promote the formation of a repressive chromatin structure via the recruitment of histone modifying enzymes (histone methyltransferases (HMT) and histone deacetylases (HDAC). The relatively lower availability of transcriptional activators is possibly exacerbated by the exclusion of the major MIEP transactivator, pp71, from the nucleus in CD34+ cells. In contrast, high levels of GATA-2 are present in CD34+ cells and thus promote LUNA gene expression. In lytic infection, the scales are tipped towards transcriptional activation of the MIEP by relatively higher levels of activators versus repressors and the co-operative activity of pp71.

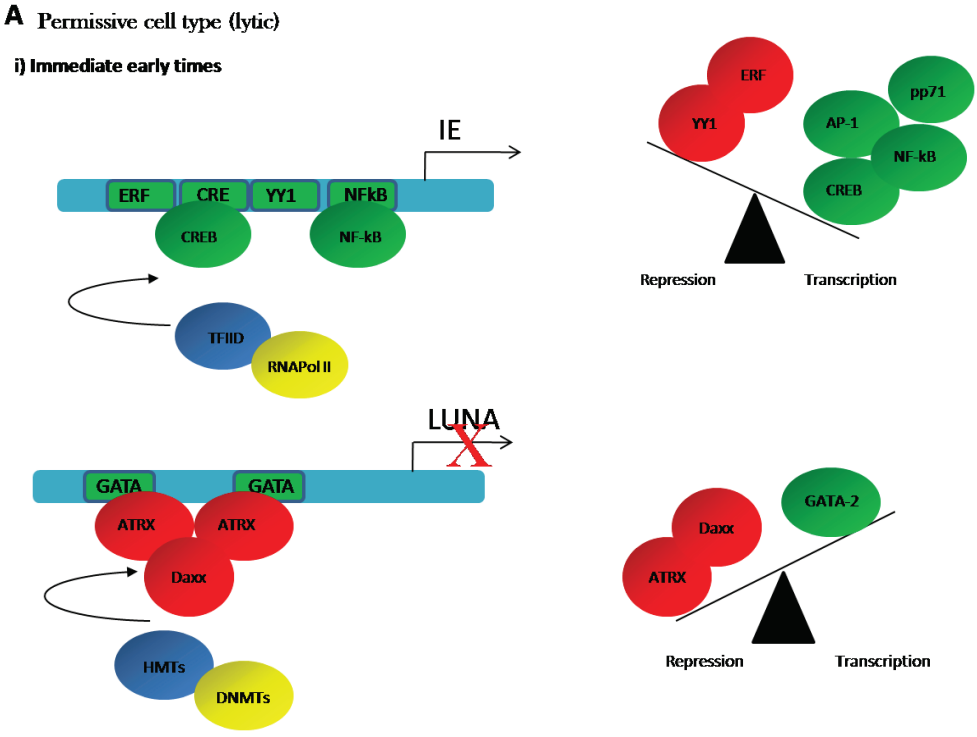
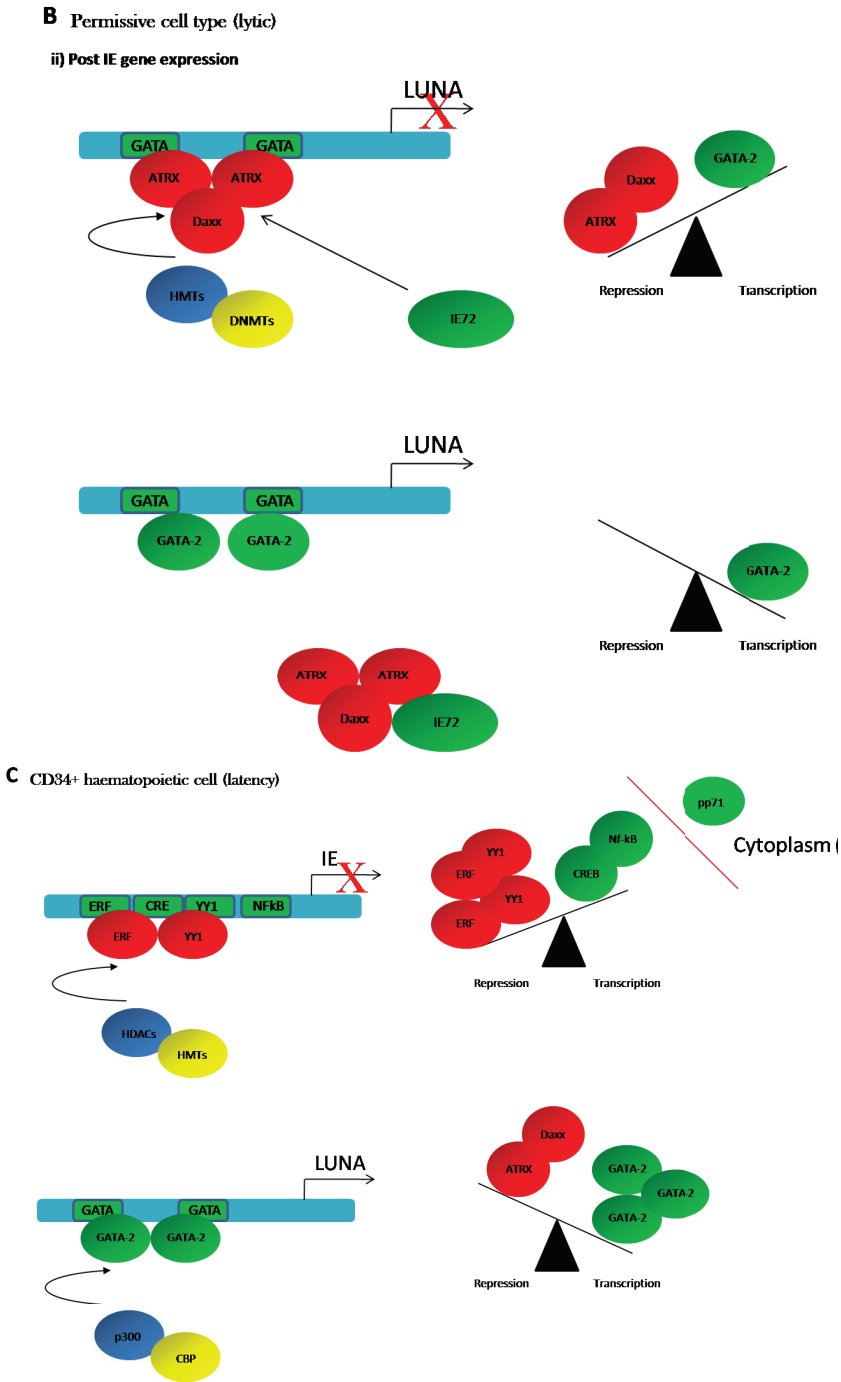


Figure 1. *Cont.*



However, we hypothesise that in early myeloid cells high levels of GATA-2 (which would alone act as positive regulator of the LUNA promoter) out-compete the repressive hDaxx:ATRX complex (Figure 1C). In contrast, in more differentiated cells with functional levels of repressive hDaxx:ATRX, but lower levels of GATA-2, this GATA-2 mediated competition of hDaxx:ATRX repression would not occur (Figure 1B) and hDaxx:ATRX-mediated repression would need to be overcome by e.g., IE72 during lytic infection (Figure 1B). Consistent with this view, as stated above, over-expression of GATA-2 into ΔIE72 infected fibroblasts can drive LUNA gene expression. In essence, the low levels of GATA-2 in more differentiated cell types that HCMV lytically infects may result in the additional requirement of IE72 activity against hDaxx:ATRX to promote LUNA gene expression during lytic infection. Provocative evidence in support of the competition hypothesis is derived from a number of biological properties of ATRX. Firstly the N terminal region of ATRX encodes domains that bind to histones—particularly methylated histone H3 on lysine 9 [86] and, thus, associates with heterochromatic DNA where it establishes a functional interaction with the hDaxx protein [87] to promote transcriptional repression. Furthermore, in addition to encoding helicase and ATPase functions [88], the ATRX protein contains coiled-coiled regions that allow direct binding to DNA—with affinity for DNA regions encoding GATA protein binding sites [89] and thus could provide direct competition with GATA transcription factors for DNA promoter occupancy.

5. TNF Receptor Superfamily Member—UL144

The UL144 gene product was originally identified as an orthologue of the tumour necrosis factor receptor superfamily member, herpes virus entry mediator (HVEM) [90,91]. As such, UL144 like HVEM can interact with BTLA to inhibit the proliferation of activated T cells [92]. As well as this intercellular function, UL144 has been shown to modulate intracellular signalling to regulate further immune responses to infection by hijacking NF-kB signaling [93,94]. UL144 recruits cellular TRAF-6 to promote CCL22 expression [94]—a cytokine that has been shown to promote the migration of Th2 and T regulatory cells which could impact on immune-surveillance and clearance of infected cells by the Th1 T cell repertoire [95]. The activity of UL144 shares characteristics with the LMP2A gene product of EBV which is expressed in persistently infected B lymphocytes [32,35] and also results in increased CCL22 production [96]. Furthermore, although UL144 expression during latency was not identified in a previous study [42], the UL144-148 region of HCMV was shown to be important for more efficient establishment of latency in an *in vitro* system [44] and a subsequent re-investigation of UL144 expression during latent infection showed that UL144 was, indeed, expressed during HCMV latency [47].

5.1. UL144 Is Expressed during Latency in a Strain-Specific Manner

In lytic infection, the UL144 protein is expressed with early kinetics reaching steady state levels by 48 hours post infection [90]. Although suggestive that expression is dependent on IE gene expression during lytic infection, the first insights into the regulation of the UL144 promoter came from an

analysis of different HCMV isolates during latent infection of myeloid progenitor cells and, interestingly, suggested some parallels to those observed with LUNA [47]. Analyses showed that UL144 expression did occur during latency but that this appeared to be isolate dependent [47]. Importantly, previous studies [42,43] that had failed to identify UL144 expression during latency were performed using strains of HCMV that were either UL144 deficient (AD169) or UL144 null for expression during latency expression (TB40/e and VR1814) - based on the these recent observations of Poole *et al.* [47]. In contrast, the HCMV sequence reference strain, Merlin [97], was UL144 positive for expression during latency. Thus the differences between these and previous data were easily reconciled and, furthermore, provided a level of cross validation for the respective studies.

5.2. UL144 Expression during Latency Is Dependent on GATA-2 Binding Sites

The observation by Poole *et al.* [47] that all the HCMV isolates analysed routinely expressed UL144 during lytic infection, even though this was not the case for latent infection, suggested that differential expression of UL144 during latency was a function of UL144 promoter activity specifically in myeloid progenitor cells. Sequence analysis of UL144 promoters showed that, although a high level of conservation was evident, a UL144 positive phenotype during latency correlated with the presence of putative GATA-2 binding sites in the UL144 promoter. Consistent with this, UL144 reporter constructs based on the Merlin Ul144 promoter were GATA-2 responsive whereas TB40/e-based UL144 reporter constructs were not. Additionally, the deletion of the putative GATA-2 sequences was sufficient to render the Merlin UL144 promoter inactive [47].

Direct evidence for a physical interaction between GATA-2 and UL144 promoter correlating with gene expression was also obtained by performing chromatin immunoprecipitation assays on infected myeloid cells. Crucially, GATA-2 immuno-precipitation with the UL144 promoter was only observed in cells infected with strains of HCMV that supported UL144 expression [47]. Furthermore, GATA-2 binding correlated with the detection of histone H3 di-methylated at lysine 4 bound to the UL144 promoter. In themselves, these data argue that the UL144 promoter is regulated by post-translational histone modifications around the UL144 promoter during latent infection likely modulated by GATA-2. Pertinently, the parallels with the LUNA promoter are overt [46]. Although LUNA expression during latency is independent of viral strain used, the LUNA promoter binds GATA-2 and is also responsive to GATA-2 in transfection assays [47].

6. Concluding Remarks

It is becoming increasingly clear that the regulation of cytomegalovirus gene expression during all phases of lytic infection involves post-translational modification of histone proteins (reviewed in [98]). Work from a number of laboratories has now demonstrated quite clearly that extensive chromatinisation of HCMV genomes occur [56–58,60,61,99,100] and that, during lytic infection, at least, the virus has to modify chromatin to control viral gene expression [59,101]. What is now also becoming clearer is that a complex interplay between cellular transcription factors and higher order chromatin structure

with viral promoters is equally as important for the latency-associated regulation of viral gene expression [22,46,102].

Although the exact order of events controlling viral gene expression is still not fully understood, a model for the control of latent viral gene expression, based on a number of instructive observations, can be made. Firstly, the viral genome in the virion is naked—no histone proteins can be detected [56,58] consistent with a failure to detect histone proteins in purified virions by mass spectrometry [103]. This argues that the incoming viral genomes are subject to cellular responses that promote chromatinisation of the viral genome. Whether this is true for all foreign DNA is unclear but the association of plasmids and non-viral vector delivered DNA with histone proteins is documented *in vitro* [104] and *in vivo* [105]. The association of herpesvirus genomes with ND10 bodies—sites of extensive accumulation of histone modifying enzymes in the cell—is intrinsically repressive (reviewed in [106]). Indeed, the concerted attempt made by HCMV (and other herpes viruses) to disrupt these structures suggests they are anti-viral (reviewed in [107]). Consistent with a global repression of viral gene expression immediately post infection, studies of histone proteins at low MOIs show that a substantial number of incoming viral genomes are associated with methylated histone proteins [56] and that this response could be mediated by the action of a number of cellular proteins (*i.e.*, hDaxx, PML and Sp100) that accumulated at ND10 bodies. Consequently, the virus, through the action of pp71 [62,63,65,108] and subsequently IE72 [109–112] targets these ND10 structures to quickly overcome repression and initiate a lytic infection, especially at high MOIs.

During latent infection, however, it is likely that the same initial events occur—the genome is chromatinised immediately upon infection and that the same intrinsic immune defences are activated. However, the failure of pp71 to translocate to the nucleus and inactivate these defences upon infection of myeloid progenitors (Figure 1A) has been argued to be one possible mechanism to aid the establishment of latency [113]—although whether this is mediated solely by an interaction with hDaxx is still not clear [114]. Regardless of the exact mechanism, there is no doubt that the failure of a major viral transactivator of the MIEP (pp71) to enter the nucleus would have a significant impact upon activation of IE gene expression. Nevertheless, there are also longstanding observations that the MIEP—even after transfection—is transcriptionally less active in myeloid progenitors [77,115]. The prevailing hypothesis is that this latent phenotype is driven by the presence of high levels of transcriptional repressors in myeloid progenitors, such as YY1 and ERF, promoting long term silencing during latency via interactions with histone deacetylase and histone methyltransferase enzymes (Figure 1C). However, maybe a more refined model would suggest that levels of promoter occupancy and the recruitment of co-factors is critical to the establishment of latency rather than the absolute levels of ubiquitous transcription factors. Nevertheless, it is likely that the known interactions of YY1 and ERF with histone deacetylases and methyltransferases contribute significantly to the repressive ‘chromatin phenotype’ observed in naturally latent CD34+ haematopoietic cells around the viral MIEP [22].

It is also likely that these same mechanisms which promote silencing of the MIEP in latency are also important for driving viral latent gene expression. GATA-2 is, like YY1 and ERF, highly

expressed in myeloid progenitor cells [68] and, while YY1 and ERF are binding to the MIEP mediating repression [76,79], GATA-2 binding to the LUNA and UL144 promoters drives their activity [47,54]. Interestingly, an in silico analysis of the promoters of other latently expressed genes identified putative GATA binding sites which may be important for the expression of the vIL10 and UL138 gene products. It is highly likely that histones are also recruited to latent promoters immediately post infection and, akin to the binding of ERF and YY1 to the MIEP resulting in the recruitment of histone deacetylases and histone methyltransferases, it is highly plausible that the binding of GATA-2 to the UL144 and LUNA promoters in latently infected monocytes [47] results in the recruitment of, for example histone acetyltransferases, generating the histone signature we observe in latently infected cells [46]. This 'open' chromatin conformation would support transcription and is consistent with viral latent gene transcription observed during HCMV latency [45–47]. Clearly, it remains to be seen whether GATA transcription factors are solely responsible for all the latent HCMV transcription that has been reported. Indeed, the regulation of UL138 gene expression during latency has been shown to be dependent on LUNA gene expression [55] in a long term monocyte culture model of latency [116] by, as of yet, an unidentified mechanism. However, the expression of viral genes containing binding sites for transcription factors highly expressed in the myeloid progenitor cells is highly plausible and provides further evidence for the key role cellular mechanisms of gene regulation play in the control of gene expression during persistent virus infection.

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Conflicts of Interest

The authors declare no conflict of interest.

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Review

Histone Deacetylases in Herpesvirus Replication and Virus-Stimulated Host Defense

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Abstract: Emerging evidence highlights a critical role for protein acetylation during herpesvirus infection. As prominent modulators of protein acetylation, histone deacetylases (HDACs) are essential transcriptional and epigenetic regulators. Not surprisingly, viruses have evolved a wide array of mechanisms to subvert HDAC functions. Here, we review the mechanisms underlying HDAC regulation during herpesvirus infection. We next discuss the roles of acetylation in host defense against herpesvirus infection. Finally, we provide a perspective on the contribution of current mass spectrometry-based "omic" technologies to infectious disease research, offering a systems biology view of infection.

Keywords: herpesvirus; HDAC; IFI16; acetylation; NF-κB; p53; proteomics; metabolomics; chemoproteomics

1. Introduction

Protein acetylation has recently emerged as a critical regulatory factor during herpesvirus infection. Recent studies have linked chromatin remodeling and acetylation/deacetylation events to the regulation of promoter activity and maintenance of viral latency during herpesvirus infection. These

studies examined herpes simplex virus 1 (HSV-1), human cytomegalovirus (HCMV), Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) [1–4], thereby pointing to similarities among alpha-, beta- and gamma-herpesvirus infections. During latent infection, the HSV-1 genome is assembled into an ordered nucleosome-associated structure; however, during active lytic infection, the structure of viral chromatin exists in a more disordered state [5,6]. In lytic stages of infection, histones associate with herpesvirus promoter regions, and chromatin adopts a conformation consistent with more actively transcribed host genomic regions [6]. Moreover, it is well established that the post-translational modification of herpesvirus-associated histone tails is important for the regulation of viral gene transcription [7–11].

Given the significance of acetylation during viral infection and its requirement for essential host functions, knowledge of the regulation of the enzymes controlling protein acetylation is critical for understanding viral pathogenicity and host defense. This position is further emphasized by the recent finding that acetylation is a more prominent post-translational modification than previously thought, with several thousand host protein acetylations identified to date [12,13]. Among the enzymes involved in regulating protein acetylation are the human histone deacetylases (HDACs), which remove acetylations from their substrates [14]. Along with the numerous histone acetyltransferases (HATs) responsible for lysine acetylation, HDACs control the activity of substrates by acting as components of diverse multi-protein complexes with co-repressor functions (reviewed in [15,16]). HDACs themselves are a conserved family of proteins that evolutionarily predate histones, indicating that their interactions with non-histone proteins are integral to their cellular functions [17]. Given the array of human deacetylases, which encompasses eleven Zn^{2+}-dependent HDACs and seven NAD^{+}-dependent sirtuins (SIRTs), as well as their ability to participate in multiple protein complexes, viral interactions with members of this enzymatic family are likely to be protein-specific and individually regulated. HDACs have been linked to viral replication and pathogenesis during infection with a variety of human pathogens, including herpesviruses, hepatitis B and C, HIV-1 and HPV (reviewed in [18]). It is therefore not surprising that, during co-evolution with their hosts, viruses have gathered finely tuned mechanisms for targeting HDACs to either appropriate or inhibit their enzymatic activities. These observations highlight the importance of understanding the mechanisms of HDAC regulation during viral infection.

In this review, we summarize the mechanisms employed by herpesviruses to perturb the functions of this important family of host transcriptional regulators, the histone deacetylases. Next, we discuss the roles of acetylation in regulating host defense mechanisms against herpesvirus infection. Finally, we provide a perspective on the promise of emerging "omic" technologies for gaining a systems biology view of infection and an in-depth understanding of virus-induced changes within cellular pathways.

2. Viral Control of HDAC Complexes

Histone deacetylases are critical chromatin-associated transcriptional regulators responsible for the removal of lysine acetylations within targeted genomic regions, thereby promoting compact chromatin organization and repression of transcription. HDACs perform their repressive functions as components

of numerous multi-protein co-repressor complexes, including the nucleosome remodeling and deacetylase (NuRD), co-repressor of RE1 silencing transcription factor (CoREST), mSin3A co-repressor, nuclear co-repressor (NCoR) and mitotic deacetylase (MIDAC) complexes [19–26]. The diversity of HDAC-containing complexes was clearly demonstrated by a recent proteomics-based study that profiled the interactions of all eleven human HDACs and assessed the relative stabilities of interactions within protein complexes [27]. This study demonstrated that HDACs can be part of numerous pre-assembled functional complexes that can associate with transcriptional factors for effective regulation of downstream gene expression. Moreover, these studies provided evidence that HDACs play roles not only in chromatin remodeling and transcriptional regulation, but also in diverse cellular processes, including cell cycle progression and RNA processing. Therefore, these results offer a valuable platform for examining changes in HDAC interactions during herpesvirus infection. The modulation of host protein interactions in response to infection provides both a mechanism for virus-mediated control of host protein functions for the benefit of viral replication, while also serving as a signal for the activation of host immune responses to counteract infection (summarized in Table 1 and Figure 1).

Table 1. Human histone deacetylase (HDAC) interactions with viral proteins during herpesvirus infection. HSV-1, herpes simplex virus 1; HCMV, human cytomegalovirus; EBV, Epstein-Barr virus; KSHV, Kaposi's sarcoma-associated herpesvirus.

Enzyme	Virus	Interaction	Functional consequence	Ref.
HDAC1	HSV-1	ICP8	Redistribution of HDAC1/CoREST and LSD1 to cytoplasm	[28–31]
	HSV-1	ICP0	Disrupts CoREST association; localizes HDAC1/ICP0 to ND10 bodies	[28,32–35]
	HSV-1	US3	Upstream effector of HDAC1 phosphorylation	[32,36,37]
	HCMV	pUL29/28	Associates with HDAC1/HDAC2 and NuRD to promote expression of viral genes	[38,39]
	HCMV	pUL38	Associates with HDAC1/HDAC2 and NuRD complex members via pUL29/28 bridge	[38,39]
	HCMV	IE86	Co-expression promotes MIEP repression	[40]
	EBV	ENBA3C	Represses Cp promoter via association with co-repressor complexes (e.g., mSin3A and NCoR)	[41,42]
	EBV	TRF2	Deacetylation of OriP; promotes stability of latent genome	[43]
	KSHV	ORF50 promoter	Proposed to modulate promoter acetylation status and LANA acetylation	[2,44]
HDAC2	HSV-1	US3	Upstream effector of HDAC2 phosphorylation	[32,36,37]
	HCMV	pUL29/28	Associates with HDAC1/HDAC2 and NuRD to promote expression of viral genes	[38,39]
	HCMV	IE2	De-represses pUL54 promoter; promotes localization of HDAC2 to replications sites	[45]
	HCMV	pUL38	Associates with HDAC1/HDAC2 and NuRD complex members via pUL29/28 bridge	[38,39]
	EBV	ENBA3C	Represses Cp promoter via association with co-repressor complexes (e.g., mSin3A and NCoR)	[41,42]
	EBV	TRF2	Deacetylation of OriP; promotes stability of latent genome	[43]

Table 1. *Cont.*

Enzyme	Virus	Interaction	Functional consequence	Ref.
HDAC3	HCMV	IE1	Increased acetylation at viral promoter	[46,47]
	HCMV	IE2	Increased acetylation at viral promoter	[46,47]
HDAC4	HSV-1	ICP0	Relieves MEF2-binding domain-mediated repression	[48]
HDAC5	HSV-1	ICP0	Relieves MEF2-binding domain-mediated repression	[48]
	KSHV	ORF50 promoter	Proposed to modulate promoter acetylation status and LANA acetylation	[2,44]
HDAC7	HSV-1	ICP0	Relieves MEF2-binding domain-mediated repression	[48]
	KSHV	ORF50 promoter	Proposed to modulate promoter acetylation status and LANA acetylation	[2,44]

Figure 1. Summary of HDAC functions during herpesvirus infection. (**A**) Response to alpha-herpesvirus infection (HSV-1); (**B**) response to beta-herpesvirus infection (HCMV); (**C**) response to gamma-herpesvirus infection (EBV and KSHV).

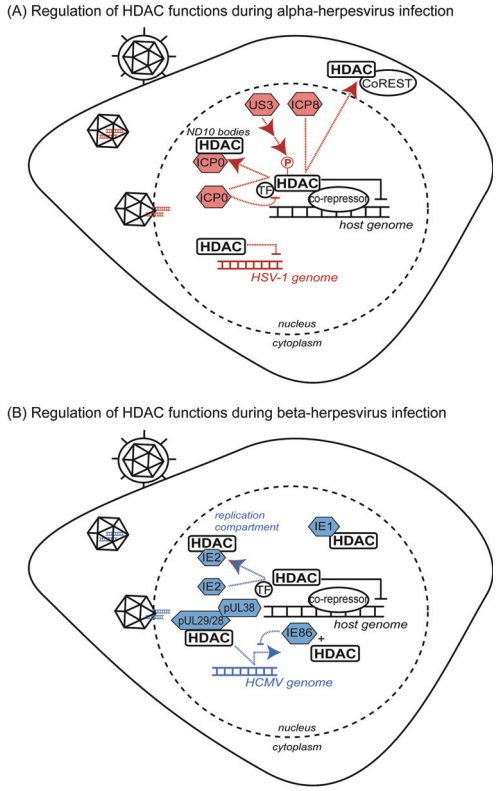
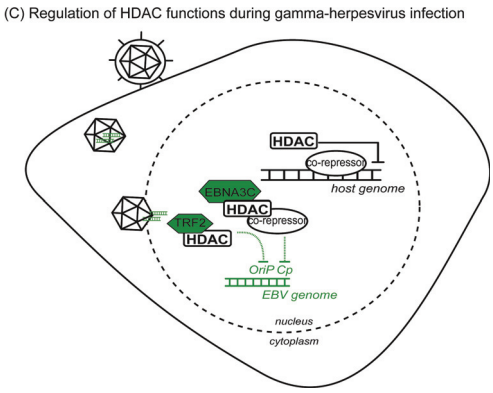


Figure 1. *Cont.*



2.1. Infection with the Alpha-Herpesvirus HSV-1 Promotes Misregulation of HDAC1/HDAC2-Containing Complexes

Several studies have started to uncover the means through which HDACs are recruited to viral protein-associated complexes and to viral genomes. As components of the CoREST complex, HDAC1 and HDAC2 act as repressors of host gene transcription within the nucleus. In the absence of infection, HDAC1 is observed within distinct nuclear structures in complex with CoREST and LSD1; however, upon HSV-1 infection, the HDAC1/CoREST/LSD1 complex is redistributed to compact structures that also contain the viral protein ICP8 (Figure 1A and Table 1) [28]. Resembling viral replication compartments, these structures suggest a function for this complex in active transcription of the viral genome [28]. As LSD1 is an established histone demethylase, this association can couple demethylase and deacetylase activities for coordinated regulation of histone post-translational modifications associated with the viral genome [28–30]. It is possible that the association of separate viral factors could trigger preferential association of specific accessory demethylases with the core CoREST complex. Upon infection, components of the HDAC1/CoREST/LSD1 complex are observed to translocate to the cytoplasm, indicating that this complex or its members may adopt new functions in response to viral infection (Figure 1A) [31]. Intriguingly, the redistribution of these complex members appears to be temporally regulated, with HDAC1/CoREST accumulating in the cytoplasm at four hours post-infection (hpi) and LSD1 changing localization at 8 hpi [28]. The HDAC1-CoREST interaction also contributes to a host defense mechanism against herpesviruses, which is discussed in Section 3.2 of this review.

Interestingly, the viral protein kinase U_S3 can enhance expression of viral reporter genes, similar to the phenotype observed during HDAC inhibition, suggesting that a U_S3-mediated kinase cascade may be an upstream regulator of HDAC activity [32]. Both HDAC1 and HDAC2 hyperphosphorylation during herpesvirus infection depends on the activity of U_S3, yet HDAC phosphorylation appears to be an indirect effect of U_S3 activity, suggesting the involvement of additional viral or host kinases

(Figure 1A) [36,37]. In the absence of infection, phosphorylation is an established regulator of HDAC1 and HDAC2 activity and protein associations. Specifically, HDAC1 phosphorylation promotes binding to members of co-repressor complexes, including the Sin3a (RbAp48 and mSin3A), NuRD (RbAp48 and MTA2) and CoREST complexes [49]. Similarly, mSin3 and Mi2 (member of the NuRD complex) preferentially associate with phosphorylated HDAC2 [50]. The activity of viral kinases and perturbations in kinase signaling pathways could alter the phosphorylation landscape of these proteins, ultimately contributing to aberrant regulation of HDACs and rearrangement of HDAC complexes.

Phosphorylations can directly mediate HDAC protein interactions; however, a second level of regulation results from phosphorylation-dependent changes in HDAC localization. Class IIa HDACs (HDAC4, -5, -7 and -9) shuttle between the nucleus and the cytoplasm through a mechanism that relies on site-specific phosphorylation of 14-3-3 binding sites [51–54]. Interestingly, the subcellular distribution of class IIa HDACs has also been observed to be dynamic during infection. HDAC4, -5 and -7 co-localize with ICP0 within the nucleus during HSV-1 infection (Figure 1A) [48]. ICP0 was shown to physically interact with these enzymes via the conserved *N*-terminal extensions shared by class IIa HDACs and absent in their class I counterparts [48]. ICP0 serves to relieve repression of MEF2 induced by the binding of HDAC *N*-terminal domains to MEF2. However, transcription mediated by the HDAC *C*-terminal deacetylation domain was not relieved by ICP0, consistent with continued co-localization of HDACs with SMRT, another member of the nuclear co-repressor complex [48]. bICP0 has also been reported to interact with HDAC1 during bovine herpesvirus 1 infection to modulate Mad-dependent transcription, suggesting that this virus-host protein interaction may represent a common point of virus-mediated control of host protein complex formation [33]. Therefore, the phosphorylation status of HDACs significantly contributes to the regulation of both protein interactions and localizations in the context of infection. Recent results have demonstrated that Aurora B-dependent phosphorylation of class IIa HDACs modulates cell cycle-dependent functions of these enzymes, indicating that HDAC phosphorylations are both spatially and temporally regulated [55]. The importance of temporally regulated phosphorylations during viral infection remains to be established.

Altogether, multiple viral factors, including ICP0, ICP8 and U_S3, promote changes in the localization and functional roles of HDACs and co-repressor complexes during HSV-1 infection to promote viral reproduction. Further investigation of viral protein interactions with HDACs will contribute to development of a comprehensive understanding of the roles of individual HDAC family members during HSV-1 infection.

2.2. Beta-Herpesvirus HCMV Proteins Target Class I HDACs to Modulate Viral Gene Transcription

Modulation of HDAC complexes is also apparent during beta-herpesvirus infection. The HCMV proteins pUL29/28 and pUL38 interact with the HDAC1/HDAC2-containing NuRD complex in order to promote expression of immediate-early viral genes (Figure 1B and Table 1) [38,39] (Figure 1B). Immunoaffinity purification of pUL38 from cells infected with HCMV identified six components of the NuRD complex (Mi2β, MTA1, MTA2, HDAC1, HDAC2 and RbAp48/46 [38]), and the pUL38-NuRD

interaction was shown to depend on the presence of the viral pUL29/28 protein [39]. Co-purification studies demonstrated that pUL29/28 binds to HDAC1 and MTA1 [39]. While pUL29/28 was also shown to interact with the Sin3A complex, this association seemed less stable than its interaction with NuRD, indicating preferential association of viral proteins to specific co-repressor complexes [39]. The pUL38/pUL29/28/NuRD complex forms early during infection and persists through 72 hpi, suggesting a role throughout multiple stages in the HCMV lifecycle [39]. While pUL29/28 and pUL38 exist in a complex together, these proteins also possess independent functions within the host cell. pUL38 interacts with TSC2, a component of the tuberous sclerosis tumor suppressor protein complex (TSC1/2), to maintain an active mTOR pathway during infection [38], whereas pUL29/28 was not shown to associate with TSC2 [39]. Moreover, pUL29/28 association with NuRD is independent of pUL38 and sufficient for transcriptional activation of the HCMV major immediate early promoter (MIEP). Treatment of cells infected with a pUL29/28-deficient virus with the HDAC inhibitor trichostatin A (TSA) rescued the decreased expression of immediate early genes induced by pUL29/28 depletion, demonstrating that this virus protein interaction with HDAC1/NuRD is important for stimulation of immediate early RNA production [39].

Similarly, HDAC inhibition was shown to relieve repression of other MIEP-dependent viral genes, as treatment with TSA rescued IE86-mediated autorepression [40]. IE86 interacts with HDAC1 and the histone methyltransferases G9a and Suvar(3–9)H1, likely contributing to changes in chromatin organization at the promoter region (Figure 1B) [40]. Co-expression of IE86 and HDAC1 enhanced repression of MIEP expression through both Rb-independent and -dependent mechanisms, indicating the potential involvement of multiple HDAC-containing complexes that differ in their association with Rb [40].

The immediate early proteins, IE1 and IE2, are also known to interact with HDACs to facilitate HCMV replication. IE1 and IE2 both interact with HDAC3, while IE2 was shown to also interact with HDAC2 (Figure 1B) [45,46]. Similar to the observations on IE86, HDAC inhibition rescued viral growth defects associated with IE1-deficient HCMV strains. Loss of IE1 triggers reduced histone H4 acetylation levels at the MIEP and the UL44 early promoter, indicating that HDAC activity at these regions is IE1-dependent [46]. IE2 interaction with HDAC3 is also thought to promote loss of deacetylation at viral promoter regions through a similar mechanism [46]. Intriguingly, overexpression of HDAC3 has been demonstrated to reduce activity from the MIEP and to limit HCMV infection [8]. As IE1 and IE2 are splice variants of the same mRNA sequence, domains common to both proteins could be important for association with structurally similar HDACs. Moreover, indirect association of immediate early proteins with bridge proteins could allow for interaction with multiple HDACs and HDAC-containing complexes [46,56,57]. Indeed, IE2 has been shown to associate with HDAC2 to de-repress expression from the viral polymerase (pUL54) promoter [45]. HDAC2 also exhibits altered localization during HCMV infection, co-localizing with IE2 at viral transcription and replication sites (Figure 1B); however, co-expression of IE2 alone was insufficient to induce HDAC2 redistribution, suggesting that additional viral factors contribute to its regulation during infection [45]. Interestingly, while the acetylation levels at the pUL54 promoter were observed to increase during the course of infection (from 6 hpi to 72 hpi), the acetylation at MIEP exhibited a different pattern—increasing at

24 hpi and diminishing at 48 hpi and 72 hpi. Therefore, HDAC-mediated regulation of viral promoter regions appears to be both promoter-specific and temporally regulated [45].

Careful regulation of gene expression is critical for progression through the viral lifecycle. The interaction of HCMV proteins with HDACs provides a mechanism by which viral gene programs can capitalize on the existence of an established host transcriptional regulatory system. HCMV proteins appear to display preference for individual HDAC-containing complexes, allowing for finely-tuned appropriation of host protein functions. Association of HDACs with viral promoters further allows modulation of the acetylation status of the viral genome, while simultaneously limiting the population of HDACs that would otherwise be available for interaction within typical host complexes.

2.3. The Gamma-Herpesviruses EBV and KSHV Regulate HDAC-Containing Co-Repressor Complexes through Protein Interactions and Phosphorylation-Dependent Signal Cascades

The gamma-herpesvirus EBV also modulates the activity of HDACs and HDAC complexes through the interaction of the viral Nuclear Antigen 3C (EBNA3C) protein with HDAC1 and HDAC2 (Figure 1C and Table 1) [41,42]. Careful regulation of viral gene expression is necessary for efficient replication, but also for maintenance of latency. The EBNA3C-HDAC interaction promotes the association of HDAC1 with the DNA binding protein CBF1/RPB-Jκ for both autorepression of EBNA3C and repression of Cp-responsive genes (Figure 1C) [41]. CBF1/RBP-Jκ is also reported to interact with HDAC1 and SMRT as part of a repressor complex [41,58]. As SMRT also associates with HDAC3 and class IIa HDACs, it is possible that multiple co-repressor complexes are recruited by EBNA3C to modulate complex-specific functions during infection [23,59]. Indeed, EBNA3C was subsequently shown to interact with HDAC1, HDAC2 and the co-repressor complexes mSin3A and NCoR as part of an EBNA3C-ProTα complex during EBV infection [42]. ProTα may serve as a bridge between EBNA3C and HDACs, as these co-repressor complexes were also demonstrated to associate with ProTα alone.

Viral gene transcription is further regulated by exploiting class IIa HDAC association with MEF2, which recruits HDACs 4, 5 and 7 to inhibit the viral BLZF1 promoter (Zp) [60,61]. Specifically, the de-phosphorylation of MEF2D has been shown to be important for regulating HDAC-mediated repression of Zp [61]. A consequence of this interaction is limited production of the immediate early viral protein BLZF1, which is a transcriptional activator important for exit from latency [60,61]. The temporal regulation of EBV gene expression is not only connected to HDAC transcriptional repressive functions (as in the case of MEF2), but also to deacetylation activity. HDAC1 and HDAC2 stably associate with the telomere repeat factor 2 (TRF2), which binds the EBV origin of plasmid replication (OriP) (Figure 1C) [43]. Based on the loss of HDAC/TRF2 binding to OriP during G1/S, along with a loss of deacetylation at OriP upon HDAC inhibition, this HDAC/TRF2 complex has been proposed to delay replication initiation from OriP in order to promote the stability of the latent EBV genome [43]. Thus, the temporal regulation of genome acetylation status and gene expression by HDACs is important for the preservation of latent stores of EBV within the host cell.

In addition to protein interactions, signaling pathways activated by viral infection affect HDAC activities, possibly through signal-dependent post-translational modification. The KSHV protein vGPCR has been reported to induce signaling pathways that converge on HDACs, among other targets [62]. vGPCR induces expression of the ORF50 promoter, which produces the protein RTA (replication and transcription activator) that, in itself, is sufficient for lytic reactivation of KSHV [62,63]. vGPCR activates PKC and PKD signaling pathways associated with regulation of ORF50 expression [62]. These kinases are well-established regulators of nuclear export of class IIa HDACs [51,64], indicating that HDAC phosphorylation during infection may be an important strategy for virus-mediated control of HDAC activity and complex formation. vGPCR signaling was reported to reduce the activity of deacetylases, as shown for both HDACs and sirtuins [62]. HDACs 1, 5 and 7 were shown to associate with the ORF50 promoter during KSHV latency, and the acetylation status of the latency-associated nuclear antigen (LANA) was dependent on HDAC activity [2,44]. The KSHV LANA protein can also interact with the HDAC co-repressor complex mSin3 during EBV infection [65]. Given the importance of temporal coordination of viral gene programs, cell cycle-dependent kinase cascades could provide an additional level of regulation of gene expression during infection. Interestingly, ORF50 is also capable of repressing p53 transcriptional activity through an interaction with the CREB binding protein, CBP [66,67]. As p53 activity is regulated by acetylation, it is tempting to speculate that p53-associated responses to viral infection are, in part, due to misregulation of deacetylase complexes [68,69].

Altogether, these studies have demonstrated that gamma-herpesviruses EBV and KSHV co-opt HDAC complexes to repress viral gene expression. These mechanisms likely allow for maintenance of the viral genome in a latent state until conditions are advantageous for viral replication. Viral infection further alters HDAC activity through activation of phosphorylation signaling cascades, thereby modulating the phosphorylation states and activities of individual HDAC enzymes.

3. Host Employment of HDACs and Acetylation in Defense against Herpesviruses

Comprehensive acetylome studies have revealed that histone and non-histone protein acetylation is comparable in frequency to phosphorylation [12,13]. One study alone identified over 3,500 acetylations across three human cell types using immunoaffinity isolation of acetylated substrates coupled with mass spectrometry analysis [13]. Interestingly, acetylations within several important effectors of innate immunity—RIG-I, IRAK4, OAS2, TRIM25 [13] and, most recently, IFI16 [70]—have been identified, suggesting that acetylation provides dynamic control of important mammalian innate immune functions. Indeed, increasing evidence suggests that HDACs are regulators of inflammatory response, immune signaling and myeloid differentiation [71–74].

3.1. IFI16 Acetylation and Host Detection of Viral DNA

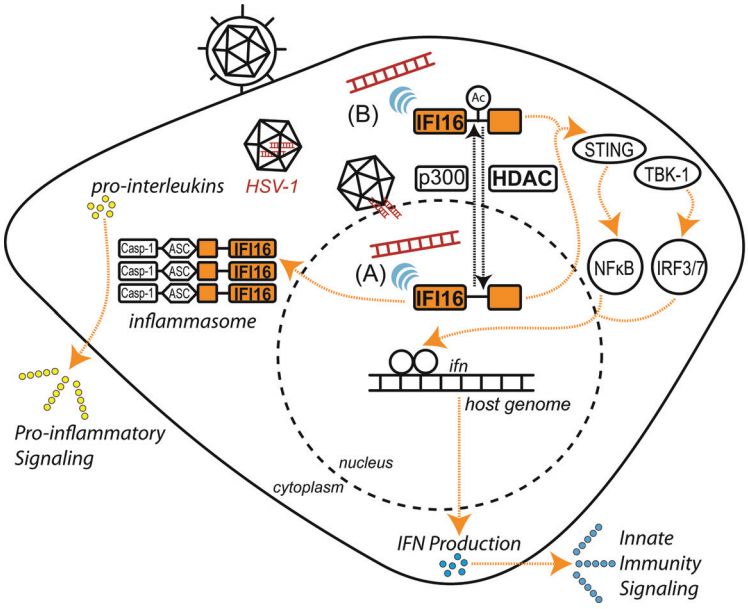
In mammals, induction of host innate immunity in response to viral infection begins with the detection of pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) by intracellular pattern recognition receptors (PRRs). Several classes of PRRs have been characterized,

including AIM2-like receptors (ALRs), retinoic acid-inducible gene I-like receptors (RLRs), nucleotide oligomerization domain-like receptors (NLRs), Toll-like receptors (TLRs) and an assorted array of cytoplasmic DNA receptors [75]. These PRRs are predominantly localized to the cytosol, plasma membrane and endosome to sense and respond to a wide range of invading pathogens. Surprisingly, recent studies have extended the range of host immuno-surveillance to the nucleus, where the DNA sensor IFI16 selectively detects the double-stranded DNA genome of herpesviruses during infection [70] (Figure 2).

As an ALR family member, IFI16 senses nuclear and cytoplasmic herpesvirus DNA, inducing pro-inflammatory and interferon responses via distinct pathways to limit viral replication and spread (Figure 2) [70,75–80]. In the cytoplasm, IFI16 associates with the signaling adapter protein STING (stimulator of interferon genes) upon binding of viral DNA, subsequently activating IRF3 and NF-κB transcription factors, which then translocate to the nucleus and induce robust interferon-β (IFN-β) expression [75,80]. However, DNA sensing is not restricted to the cytoplasm, as IFI16 has also been demonstrated to bind herpesvirus DNA in the nucleus during early stages of infection to induce IFN-β expression [70]. Intriguingly, following detection of viral DNA, IFI16 and the additional ALR AIM2 form oligomeric, multi-protein complexes known as inflammasomes [76,78,79,81,82]. In the cytoplasm, these inflammasomes recruit and activate caspase-1, which, in turn, processes the pro-inflammatory cytokines pro-IL-1β and pro-IL-18 to their secreted, biologically active forms. Until recently, the underlying mechanisms regulating these localization-dependent innate immune functions of IFI16 were not well-understood. Li *et al.* identified two acetylations within IFI16 that regulate its sub-cellular distribution [70]. Partly mediated by the HAT p300, acetylation of Lys99 and Lys128 within the nuclear localization signal (NLS) promotes cytoplasmic retention of IFI16, whereas HDAC activity promotes its nuclear import. Nuclear localization of IFI16 was shown to be essential for recognition of nuclear herpesviral DNA during infection [70,76,77], indicating that HDACs may play a critical role in IFI16-mediated DNA sensing. Thus, modification by acetylation provides a means for expanding the range of IFI16-mediated immuno-surveillance of double-stranded DNA viruses and may function as a toggle for additional localization-dependent functions (Figure 2). Observations of the multiple patterns of IFI16 behavior during viral infection suggest that this protein may have varied functions in immune response. While acetylation of IFI16 is critical for positioning this DNA sensor in the appropriate cellular compartment prior to infection, the roles of IFI16 acetylation and associated HDAC functions during infection require further investigation.

IFI16 is not the only PRR known to be acetylated. RIG-I, a sensor of viral double-stranded RNA that acts through the MAVS adapter protein to induce IFN signaling [83], is acetylated at Lys858 within its *C*-terminal repression domain [13]. While Lys858 acetylation has yet to be tested for modulation of RIG-I activity, acetylation of PRRs and HDAC modulation of PRR activities may be a more prominent regulatory feature of the innate immune system than currently appreciated.

Figure 2. Host response to viral DNA is mediated by IFI16. Acetylation-dependent localization of IFI16 allows dual-compartment sensing of viral DNA, promoting pro-inflammatory response and innate immune signaling during herpesvirus infection. (**A**) IFI16 senses HSV-1 DNA within the nucleus. (**B**) IFI16 senses cytoplasmic DNA resulting from transient transfection or viral capsid degradation.



Once PRRs bind their pathogen-derived ligands, innate immune signals are transmitted to neighboring cells via cytokine production and secretion. HDACs have indirectly been implicated in these processes. Animals treated with valproic acid (VPA), a broad-acting HDAC inhibitor, exhibit increased susceptibility to infection by bacterial and fungal pathogens, while being protected from septic shock [73]. In agreement with these observations, VPA-treated macrophages in the presence *E. coli* and *S. aureus* exhibit reduced phagocytosis and subsequent cytokine bursts [74]. Several reports have described attenuated expression of cytokines important in innate immunity signaling (e.g., IFN-β and IFN-γ), pro-inflammatory responses (e.g., TNF-α, IL-6, IL-1β and IL-18) and leukocyte invasion and activation (e.g., MCP-1, G-CSF and CXCL10) following treatment with the pan-HDAC inhibitor, TSA [73,74]. However, dampened cytokine expression does not necessarily correlate with repressive chromatin modifications, as histone H4 acetylation at *Tnf* and *IL6* promoters has been shown to increase following TSA treatment [73]. Thus, it is likely that a significant subset of the immunosuppressive, anti-inflammatory effects of HDAC inhibitors occur at a non-histone level.

3.2. HDAC Association with PML/ND10 Bodies during Herpesvirus Infection

Associated with viral DNA after its nuclear deposition, PML bodies are composed of a set of interferon-inducible proteins that play important roles in host anti-viral defense during herpesvirus infection [84,85]. During HCMV infection, HDACs are recruited to the viral MIEP promoters by Daxx, a component of PML bodies, to repress viral gene expression at early stages of infection [47]. However, this intrinsic immune defense mechanism is counteracted by the activity of viral proteins. Specifically, the HCMV protein pp71 promotes degradation of Daxx, and the interaction of the immediate early viral proteins, IE1 and IE2, with HDAC3 relieves repression of viral transcription [46,47]. This mechanism of evading HDAC-mediated anti-viral response appears to be conserved across species, as the mouse CMV protein mIE1 is also reported to bind mHDAC2, which is recruited to ND10 structures via PML and Daxx [86]. Consistent with a model in which HDAC activity represses viral gene transcription through association with the HCMV MIEP, HDAC inhibition has been demonstrated to rescue IE gene expression [87]. During HSV-1 infection, disruption of ND10 structures is necessary for productive viral replication and is accomplished by ICP0 [88]. Moreover, ICP0 promotes the disruption of HDAC1-CoREST to enhance viral gene expression and replication [31,34]. An *N*-terminal domain of ICP0 shares homology with and binds to a *C*-terminal domain of CoREST immediately adjacent to the HDAC1 binding site [34]. Increased viral gene expression allows the growing population of ICP0 to outcompete HDAC1 in the binding of CoREST. Through this mechanism, ICP0 promotes inhibition of CoREST-associated gene repression by abolishing its interaction with HDAC1. Another viral protein, ICP8, also accumulates adjacent to ND10 structures, where it could function to preferentially bind DNA [28,35]. ICP0-mediated inhibition of HDAC activity is also proposed to facilitate the transition from the expression of viral α genes to β genes [32]. Thus, it is likely that viral protein associations with HDACs and HDAC-containing complexes are temporally regulated during the course of infection to coordinate gene expression for productive viral replication.

3.3. HDAC Non-Histone Substrates in Host Defense

Host response to viral infection is known to be regulated by multiple transcription factors, whose downstream targets include genes associated with apoptotic, immune and pro-inflammatory responses. Several of these transcription factors with critical roles in activation of anti-viral gene programs are known to be regulated by acetylation. For example, the interferon-α receptor (IFNAR) is acetylated at Lys399 following IFN-α binding, directly recruiting interferon regulatory factor 9 (IRF9), along with the signal transducer and activator of transcription 1 (STAT1) and STAT2 [89]. IRF9, STAT1 and STAT2 are all acetylated within their DNA-binding domains, and modification is thought to promote transcriptional activation at interferon-inducible promoters. Similarly, STAT3 dimerization is modulated by the p300-mediated acetylation at Lys685, which promotes nuclear accumulation of STAT3 and transcriptional activation following cytokine-induced signaling [90]. These results suggest that acetylation may be able to positively regulate innate immune signaling pathways, which is in apparent disagreement with global HDAC inhibition studies outlined above. However, recent evidence

suggests that the acetylation of STAT1 serves to terminate IFN-α-induced signaling and that deacetylation of STAT1 by HDAC3 promotes its re-association with IFNAR [91]. Thus, HDAC-dependent reinstatement of the STAT1-IFNAR interaction may account for the discrepancy in models for acetylation-dependent regulation of innate immune signaling. As HDACs are effectors of innate immunity signaling, it is not surprising that multiple virus types would develop strategies to promote the disruption of HDAC activities during infection.

Multiple subunits of NF-κB are also regulated by site-specific acetylation (reviewed in [92]). The functional consequences of post-translational modification of NF-κB include modulation of κB-DNA binding by NF-κB, transcriptional activation of NF-κB and association of NF-κB with its negative regulator, IκBα [93]. Specifically, Lys122 and Lys123 of the p65/RelA subunit of NF-κB were shown to be modified by p300/PKAF and HDAC3 [94]. Further analysis revealed that acetylation of NF-κB p65/RelA negatively regulates its binding to κB-DNA and facilitates its nuclear export. In contrast, NF-κB deacetylation has been linked to its activation. Interestingly, several studies have also demonstrated that deacetylation of p65/RelA by SIRT1 and SIRT2 limits NF-κB activity. This is achieved through either direct inhibition of NF-κB transcriptional activity or promotion of its association with IκBα, ultimately leading to diminished secretion of pro-inflammatory cytokines [95–98]. Thus, the association of NF-κB with different deacetylases may be an important determinant of its activity.

These NF-κB functions are carefully regulated during herpesvirus infection in order to redirect NF-κB activity for increased viral gene expression [99,100]. HSV-1 interferes with NF-κB association with the promoter of IκBα and, instead, recruits NF-κB to the ICP0 promoter to facilitate expression of this viral protein [99]. It is conceivable that the altered activity of NF-κB results, at least in part, from changes in its acetylation status, suggesting a possible mechanism by which NF-κB regulation can be exploited in the design of disease treatments. A novel cancer treatment method has recently been proposed, in which treatment of HSV-1 infected cells with TSA induces acetylation and nuclear accumulation of NF-κB, promoting virus production and decreasing the viability of tumor cells [101]. Emerging evidence suggests that NF-κB acetylation may be involved in host immune responses to multiple viruses [102], further highlighting the importance of understanding the regulation of NF-κB and the activity of the enzymes responsible for modulating its acetylation status.

Another critical non-histone HDAC substrate with roles in immune defense during herpesvirus infection is the tumor suppressor, p53. The pro-apoptotic functions of p53, as well as its ability to transactivate IFN-inducible genes during vesicular stomatitis virus (VSV) infection, are dependent on the acetylation state of Lys379, which is negatively regulated by SIRT1 [103]. Interestingly, during HCMV infection, the viral protein IE2 can downregulate p53 function through inhibition of the acetyltransferase p300/CBP [104]. Sequestration of p300/CBP and the transcription factor IRF-3 by ICP0 has also been observed during HSV-1 infection, resulting in attenuated immune response [105]. It has been proposed that HDAC-1, -2 and -3 can downregulate p53 transcriptional activity through deacetylation of residues within its *C*-terminus [106]. In view of these observations, further investigation of the regulatory functions of HDACs with respect to p53 activity during infection is necessary.

3.4. Roles for Additional Acetylation-Modulating Enzymes during Infection: SIRTs and HATs

Further highlighting the significance of protein acetylation during viral infection, recent studies have started to uncover roles for other acetylation-modulating enzymes. While not the focus of this review, it is worth mentioning that the NAD^+-dependent deacetylases, sirtuins (SIRTs) and histone acetyltransferases (HATs) seem to be intimately linked to viral replication. Studies examining the functions of the seven mammalian sirtuins during the progression of viral infection are still limited. However, SIRT1 has been implicated in the progression of human immunodeficiency virus 1 (HIV-1) [102,107–109], human papillomavirus (HPV) [110] and vesicular stomatitis virus (VSV) [111] infection. SIRT1 has also been proposed to control the reactivation of latent varicella zoster virus (VZV) in neurons through the reduction of intracellular NAD^+ levels [112]. Additionally, canine coronavirus (CCoV-II) infection has been shown to induce the expression of SIRT1, SIRT3 and SIRT4 [113], suggesting that the regulation of individual sirtuins is virus specific. Sirtuins possess diverse cellular functions, including modulation of gene expression, DNA repair and apoptosis. Such pathways are commonly exploited by herpesviruses in order to promote viral replication. Therefore, it is tempting to speculate that sirtuins, like the Zn^{2+}-dependent HDACs, have roles in modulation of herpesvirus lifecycles through protein complex associations and regulation of substrate activities within essential pathways.

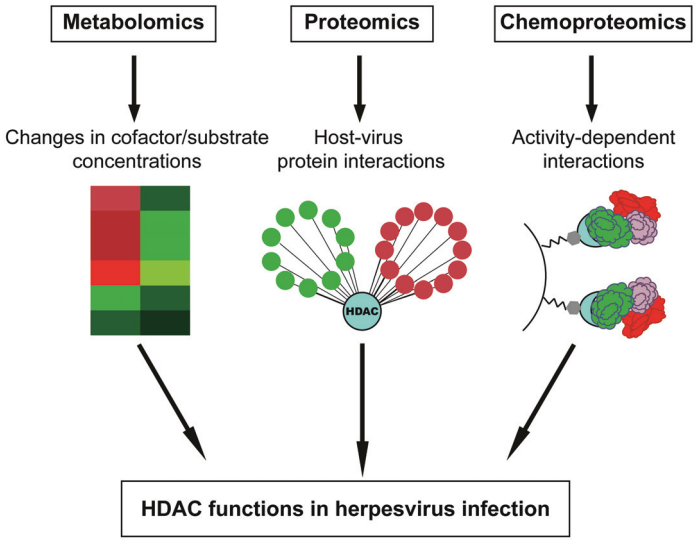
The modulation of HAT complexes and HAT activities during infection has proven to be an equally interesting subject for investigation. While this review focuses on the modulation of HDAC complexes during herpesvirus infection and the roles of HDACs in host defense mechanisms, it is worthwhile to briefly highlight several examples of roles for HATs during infection. During HSV-1 infection, the circadian HAT CLOCK has been shown to be recruited to ND10 bodies and viral replication compartments, where it associates with the viral proteins, ICP4, ICP27 and ICP22, and the transcription factor, TFIID [114,115]. CLOCK activity promotes the expression of α-genes [114] and, along with its regulator and substrate, BMAL1, remodels viral chromatin [115]. Like their deacetylase counterparts, HAT activities are also modulated by phosphorylation, and, indeed, phosphorylation of TIP60 by a conserved serine/threonine kinase encoded by HSV-1, HCMV, EBV and KSHV has been shown to activate TIP60 during herpesvirus infection and to induce DNA damage responses and chromatin remodeling [116]. TIP60 activity is proposed to have a negative effect on establishment of viral latency, instead promoting active lytic replication [116]. During bovine herpesvirus 1 infection, bICP0 interacts with p300 and may serve to inhibit p300-mediated interferon response to viral stress [117]. Altogether, the dynamic changes in the composition and activity of HAT complexes induced by interactions with viral proteins indicate that these enzymes, like HDACs, have critical roles during herpesvirus infection.

In summary, multiple important regulators of host response to viral infection are dynamically regulated by acetylation and deacetylation, indicating that HDACs are essential upstream modulators of host anti-viral responses. Further analysis of HDAC substrates during viral infection will help define acetylation-dependent mechanisms involved in host response to pathogens, providing additional targets for the development of anti-viral therapeutics.

4. Perspective: "Omic" Approaches in Characterizing HDAC Functions in Virus Infection

As changes in the proteome, metabolome and lipidome of infected cells have started to be established as critical markers for infection, mass spectrometry-based techniques have become an invaluable resource for the field of infectious disease research. Integration of such "omic" methodologies promotes development of a systems biology view of viral infection, which offers a multi-dimensional understanding of the diverse virus-mediated changes in intracellular processes. The impact of such approaches is particularly apparent when considering the diverse functions of HDACs. Omic-based studies can provide insights into the molecular mechanisms underlying HDAC functions during viral infection, including elucidation of their histone and non-histone substrates, their participation in protein complexes and their impact on downstream transcriptional targets (Figure 3).

Figure 3. Assessing HDAC functions during infection through integrative omic approaches. A combination of multi-disciplinary approaches, including metabolomics, proteomics and chemoproteomics, can provide information for comprehensive characterization of the roles that HDACs play during herpesvirus infection.



Developments in mass spectrometry and bioinformatics have provided powerful tools for quantifying protein levels and defining interactions and post-translational modifications. Quantitative mass spectrometry incorporating metabolic labeling has been successfully used to assess changes in protein expression levels during pseudorabies virus (PRV) infection, identifying alterations in expression patterns for proteins involved in intracellular transport, translation and host stress response [118]. Similarly, identification of dynamic host-virus protein interactions during infection has contributed significantly to the understanding of both virus-mediated effects on host cell functions and of host

defense mechanisms in response to infection (e.g., [38,119–126]). The use of affinity purification coupled with mass spectrometry (AP-MS) has proven effective for both targeted and global protein interaction studies [127]. Furthermore, fluorescent affinity-tags have allowed integration of knowledge regarding protein interactions and localizations, thereby providing a spatial-temporal view of infection [119,128]. Construction of networks incorporating host-virus and virus-virus protein interactions readily illustrates the functional relationships among individual proteins and protein complexes during infection. Application of these methods has advanced the current understanding of virus-host protein interactions during HCMV [38,121,123] and PRV [126] infections. As discussed earlier in this review, proteomic approaches have helped establish the roles of HDACs during HCMV infection, as exemplified by the finding that the HDAC1-containing NuRD complex is recruited by viral proteins during infection [38,39].

On a larger scale, several recent AP-MS studies have examined global host-virus protein interactions, generating comprehensive interaction networks that further highlight the benefit of applying proteomic approaches to investigating viral infection [120,124,125,129]. Moreover, the resulting large-scale protein interaction networks are useful in the development and optimization of statistical methods for characterizing protein associations, including the SAINT algorithm for determining specificity of protein interactions [130]. Curated protein databases, including the HIV-1 Human Protein Interactions Database and VirusMINT, further integrate knowledge of viral protein interactions [131–134]. The expansion of these databases to include proteomic analyses for other viruses will be invaluable for future studies.

In addition to the identification of protein interactions, proteomic approaches allow for identification of post-translational modifications of both viral and host proteins. For example, a recent proteomic study of HSV-1-infected cells led to the identification of multiple phosphorylation and ubiquitination sites within viral proteins [135]. Future investigation and functional characterization of post-translational modifications, including HDAC-regulated acetylations, will expand the current understanding of mechanisms involved in the regulation of viral gene expression and replication.

Alongside advances in AP-MS methodologies, chemoproteomics approaches have emerged as effective techniques for profiling changes in enzymatic activities and for studying activity-dependent protein interactions during infection. Activity-based protein profiling (ABPP) has been applied to the examination of ubiquitin proteases during herpesvirus infection [136]. Chemoproteomic approaches have also been used to resolve the composition of HDAC complexes and to assess the ability of small molecules to inhibit specific HDACs [26]. The development of probes allowing for site-directed capture of HDACs has enabled comprehensive characterization of HDAC substrates and of additional activity-dependent interactions [137,138]. Application of this method for the quantitative study of HDAC activity-dependent interactions during herpesvirus infection will further elucidate the molecular roles of HDACs during infection.

In conjunction with monitoring proteomic changes during infection, examination of alterations in metabolite stability can provide important insight into specific metabolic pathways employed during viral replication cycles. Developments in mass spectrometry-based techniques have allowed quantification

of changes in cellular metabolism with increased accuracy [139,140]. Metabolomics studies following either HCMV or HSV-1 infection have revealed variations in host cell metabolic patterns that correlate with viral growth kinetics [139,141–144]. These studies demonstrated that herpesviruses can impact metabolic flux through individual pathways [144]. Specifically, HCMV infection induces glycolytic flux to fuel fatty acid biosynthesis, whereas HSV-1 preferentially stimulates production of components required for pyrimidine nucleotide biosynthesis. Interestingly, HSV-1 infection triggers a significant decrease in intracellular NAD^+ levels that is not observed during HCMV infection, suggesting that NAD^+-dependent deacetylases, SIRTs, may be differentially regulated in the presence of these viruses. Similarly, with developments in mass spectrometry-based lipidome studies [145], virus-induced changes in cellular lipids are becoming more apparent following infection with various types of viruses [146–148], including herpesviruses [149,150]. Overall, the identification of distinct cellular pathways and pathway components necessary for the replication and spread of individual viruses will allow for increased target specificity and selectivity in the design of anti-viral therapeutics [151].

As methodologies for proteomic, metabolomic and lipidomic studies, as well as necessary bioinformatics approaches, are constantly being adapted and improved, it is expected that these "omic" technologies will play an increasingly important role in infectious disease research.

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Conflicts of Interest

The authors declare no conflict of interest.

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Communication

Epigenetic Control of Cytomegalovirus Latency and Reactivation

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Abstract: Cytomegalovirus (CMV) gene expression is repressed in latency due to heterochromatinization of viral genomes. In murine CMV (MCMV) latently infected mice, viral genomes are bound to histones with heterochromatic modifications, to enzymes that mediate these modifications, and to adaptor proteins that may recruit co-repressor complexes. Kinetic analyses of repressor binding show that these repressors are recruited at the earliest time of infection, suggesting that latency may be the default state. Kidney transplantation leads to epigenetic reprogramming of latent viral chromatin and reactivation of immediate early gene expression. Inflammatory signaling pathways, which activate transcription factors that regulate the major immediate early promoter (MIEP), likely mediate the switch in viral chromatin.

Keywords: cytomegalovirus; latency; reactivation; epigenetics; chromatin; intrinsic immunity; transplantation

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1. Introduction

Human cytomegalovirus (HCMV) is an important opportunistic pathogen of the beta family of herpesviruses. Transmission of the virus occurs through exposure to infectious body fluids, including saliva, urine, breast milk, semen and blood [1]. Primary infection in immunocompetent hosts is typically subclinical, but infection of immunocompromised hosts, such as immunosuppressed recipients of solid organ or bone marrow transplants, is associated with increased risk of acute and chronic allograft rejection, infection with other opportunistic pathogens, graft failure, and death [2]. Due to the high prevalence of latent CMV, 75% of solid organ transplant patients are either newly infected with CMV, which occurs when a seronegative recipient receives an organ from a seropositive donor (designated D+/R−), or experience reactivation of latent virus present in either the donor organ or the recipient (D−/R+ or D+/R+) [3]. The highest risk of CMV disease is associated with the D+/R− combination [2,3]. Effective anti-viral drugs have reduced the incidence of post-transplant complications due to CMV infection. However, in many cases, these drugs have simply delayed the problem, and their use has been limited by toxicity and the emergence of resistant strains [4]. Immunologically immature hosts are also at risk for CMV disease. Congenital CMV infection, which occurs in utero via the placenta [5], can result in deafness, mental retardation, blindness, microencephaly, cerebral calcification, and sometimes, death [1,6].

Unlike alpha or gamma herpesviruses, which have a highly restricted cellular tropism, CMV infects many cell types *in vivo*, including epithelial, endothelial, smooth muscle, and connective tissue cells, as well as specialized parenchymal cells in various organs [7]. However, CMV is similar to other herpesviruses in many aspects, including virion structure, temporal regulation of gene expression, and strategies for immune evasion [8]. Like all herpesviruses, CMV has the ability to establish lifelong latent infection, in which viral DNA is present, but replicating virus is not detectable, and to reactivate from latency. HCMV latency and reactivation have been very difficult to study *in vivo*, since the virus does not infect other species. However, murine cytomegalovirus (MCMV) has proven to be a valuable model to study several aspects of CMV pathogenesis. The similarities between HCMV and MCMV of relevance here include (*i*) ability to establish latency and to reactivate [9–11]; (*ii*) hierarchical control of viral gene expression, in which the immediate early (*ie*) genes activate expression of the early genes, leading to viral DNA replication, late gene expression, and viral assembly; (*iii*) structure, function, and organization of immediate early genes [9,11,12], and regulation of the major immediate early promoter, which controls major *ie* gene expression [13,14].

2. CMV Latency

2.1. Cellular Sites of Latency

Cellular sites of HCMV and MCMV latency have recently been extensively reviewed and discussed [11]. Although many cell types support productive infection, latent HCMV infection has been documented most convincingly in cells of the myeloid lineage [10]. However, other cell types

may also carry latent virus. Analysis of HCMV latency in cells within organs has been hampered by the difficulty in obtaining human tissue, by the very low frequency of latently infected cells, and the difficulty in determining whether the presence of the virus in a particular cell type is due to latent infection, or to spread of the virus after trauma-induced reactivation in deceased donors. HCMV is efficiently transmitted by solid organ transplantation, suggesting that cells within the organ harbor latent virus. While it is not possible to definitively exclude passenger leukocytes as agents of transmission, there is evidence for HCMV latency in other cell types within organs, including endothelial and epithelial cells [15–17]. One study sought to address the question of endothelial cell latency through analysis of saphenous vein endothelial cells taken from patients undergoing cardiovascular surgery, and concluded that these cells were unlikely to be a major site of latency [18]. However, recent studies underscore the importance of tissue-specific endothelial cell variability in the outcome of herpesvirus infection [19]. The site(s) of HCMV latency is a controversial area in need of further study.

MCMV establishes latency in multiple organs, where endothelial cells of the kidney, liver, and heart have convincingly been shown to be sites of carriage [11,20,21]. Although some studies support the view that macrophage/monocyte lineage cells also harbor latent virus [20,22], other studies do not [11,21,23]. Thus, as with HCMV, the question of the site(s) of MCMV latency has not been definitively settled.

A molecular basis for cell type specific CMV latency, despite promiscuous acute infection, has not been definitively established, but recent studies indicate that the decision between permissive and latent infection may be determined by the balance between activating and repressive factors that control transcription of viral genes upon initial infection, and this may differ among cell types [24].

2.2. Viral Gene Expression Is Repressed in Latency

The major immediate early genes encode transcriptional regulatory proteins, which are required for activation of early gene expression, and, therefore, for all subsequent phases of viral replication. These proteins are encoded by two alternatively spliced transcripts (called IE-1/IE-2 in HCMV and IE-1/IE-3 in MCMV) whose expression is controlled by the major immediate early promoter/enhancer region. In HCMV latently infected CD34+ hematopoietic progenitor cells, the immediate early genes, and most other genes associated with productive infection, are transcriptionally silent [10]. Two genes that may have roles in latency, UL138 and LUNA, are expressed in these cells, but these genes are also expressed in productive infection. Recent studies indicate that UL138 mediates degradation of the MRP1 drug transporter, and may impair generation of an HCMV-specific immune response through reduced migration of infected dendritic cells to draining lymph nodes [25], and that LUNA plays an important role in expression of UL138 in experimental models of latency and in reactivation from latency [26]. Neither of these proteins is thought to play a direct role in repressing viral gene expression in latency.

Expression of genes involved in productive infection is also repressed in mice latently infected with MCMV [11,14,20,27–32]. Although early studies of MCMV latency showed that transcripts from the

immediate early region were sometimes detectable in organs of latently infected mice [29,30,33–35], subsequent studies have made it clear that the number of viral genomes greatly exceeds the number of transcripts. Thus, the vast majority of genomes are transcriptionally silent, but random, focal IE-1 gene expression, without progression to subsequent phases of viral replication, occurs in some cells in latent mice [9,11,27,31,32].

Recent studies have begun to elucidate the molecular mechanisms leading to transcriptional repression of viral genomes in latency [31,32]. Expression of some cellular genes is repressed through methylation of cytosines in CpG dinucleotides [36]. Studies of latent MCMV, as well as HSV, have shown that the DNA is not methylated in latently infected mice [37,38]. Rather, modifications of histones and other epigenetic factors bound directly or indirectly to the genome appear to hold the key to understanding the molecular basis of CMV latency.

2.3. Latent Viral Genomes Are Heterochromatinized

Neither HCMV nor MCMV genomes are associated with histones in the virion [39,40]. As with HSV, CMV DNA is thought to enter the nucleus after transport of DNA-containing capsids along microtubules and translocation of the DNA through the nuclear pore complex [41]. Within 30 min of infection in fibroblasts, HCMV DNA is associated with histones [42]. Studies of HCMV chromatin in latently infected cells have shown that the transcriptionally silent IE promoter is heterochromatinized, while the transcriptionally active LUNA promoter is euchromatic [43,44].

Epigenomic control of MCMV gene expression has been analyzed in kidneys of infected mice. In these cells, viral DNA also becomes associated with histones at very early times post-infection [32]. In latently infected mice, viral genomes are highly enriched in histones relative to cellular genes, suggesting that the DNA is in a highly condensed configuration, which is closed to the transcription apparatus [31,32]. In contrast to acute infection, where histones bound to viral lytic promoters have post-translational modifications consistent with active transcription, histones bound to lytic promoters have heterochromatic modifications, including de-methylated H3K4, de-acetylated H3K9, and mono- and di-methylated H3K9 in latently infected mice [31,32].

Studies of CMV and HSV latency indicate that alpha and beta herpesviruses share many features of chromatin regulation. The DNA is not methylated, and lytic genes are heterochromatinized, while genes expressed in latency have euchromatic histone modifications [45,46]. In contrast, gamma herpesvirus DNA is methylated in latently infected cells, and, although transcriptionally silent, much of the viral chromatin appears to be poised for reactivation in latently infected cells [46–49]. An important caveat to keep in mind, however, is that alpha and beta herpesvirus latency has been studied using *in vivo* models, while gamma herpesvirus latency is studied using transformed cell lines.

2.4. Transcriptional Repressors Are Recruited onto Viral Genomes in Latency

Repression of gene expression is mediated by recruitment of co-repressor complexes, composed of enzymes that catalyze repressive histone modifications, compact the chromatin and prevent nucleosome

remodeling [50,51]. In latently infected mice, the MCMV MIEP is bound to several repressor proteins, including histone de-acetylases (HDACs) 2 and 3, Heterochromatin Protein 1γ (HP-1γ), Death-associated protein (Daxx), Recombination signal binding protein for immunoglobulin kappa J region (Rbpj), also known as CBF-1 and CSL, and its co-repressor, Corepressor Interacting with RBPJ, 1 (CIR), and Ying-Yang1 (YY1) [31,32]. Although the MIEP has been most extensively studied, viral promoters from early (M112) and late (M100) genes are also associated with transcriptional repressors in latent mice.

Chromatinization of HCMV genomes in cell culture models of infection has recently been reviewed [47,52]. The mechanisms by which viral genomes become chromatinized in the initial stages of infection are not understood. Deposition of canonical histones onto cellular DNA to form nucleosomes usually occurs as the DNA replicates in S phase [53]. However, HCMV and MCMV infect highly differentiated parenchymal cells *in vivo*, which are unlikely to be in the process of cell division. Daxx is a histone H3.3 chaperone, which deposits non-canonical histones onto DNA independently of replication [54]. Thus, Daxx may have a role in chromatinization of viral genomes during infection *in vivo*.

Histones bound to latent viral genomes are de-acetylated, and Class I HDACs are bound to viral promoters. Continued HDAC activity is likely required to maintain repression of viral gene expression, since HDAC inhibitors induce reactivation of latent herpesviruses, including HCMV and KSHV, in cell culture models [55,56]. HDACs do not bind directly to DNA, but rather, are recruited through co-repressor complexes containing proteins that interact either with methylated DNA or with transcription factors that bind to specific DNA sequences [57]. Thus, knowledge of the mechanisms by which HDACs are recruited to latent viral genomes is important to understanding how CMV latency is established and maintained. Previous studies indicate that latent MCMV DNA is not methylated [37], and thus, HDAC recruitment is likely mediated by transcription factors. Two proteins that could potentially play this role, YY1 and Rbpj/CBF-1, are bound to the MIEP in latently infected mice, which contains 12 and 10 potential binding sites for YY1 and Rbpj/CBF-1, respectively [32].

Rbpj/CBF-1 is the downstream effector of the Notch signaling pathway [58,59]. In unstimulated cells, Rbpj/CBF-1 binds to the consensus sequence YGTGRGAA (where Y=C or T, and R=A or G), and recruits co-repressors, including, CIR and HDACs to silence expression of Notch-responsive genes. Binding of ligands to membrane-bound Notch results in cleavage of the receptor and translocation of the Notch Intracellular Domain (NICD) to the nucleus, where it induces allosteric changes in Rbpj/CBF-1 that result in loss of co-repressors and formation of an activating complex containing Rbpj/CBF-1, NICD, and Mastermind. Although best known for its roles in development, Rbpj/ CBF-1 regulates many other processes, including innate and adaptive immunity [60–62] and viral latency and reactivation [63,64]. Several Rbpj/ CBF-1 binding sites in the MCMV MIEP overlap with NF-κB binding sites [11]. Thus, Rbpj/CBF-1 could have roles in MCMV latency, through recruitment of co-repressor complexes and competition with the activating transcription factor NF-κB.

In addition, Rbpj/CBF-1 could function as a molecular switch to reactivate viral gene expression in response to activation of Notch-dependent or -independent signaling pathways. In gammaherpesvirus

infection, CBF-1 recruits viral transactivators, EBNA-2 or Rta to activate expression of EBV or KSHV genes, respectively [46,49].

YY1 is a zinc finger DNA-binding protein that has dual roles as a repressor or an activator of gene expression, depending on its associated complexes and the intracellular milieu [65]. YY1 is a negative regulator of the HCMV MIEP and the HIV LTR in non-permissive cells [66,67]. YY1 may repress transcription through competition with activating factors for promoter binding sites, through interference with the function of transcriptional activators, or through recruitment of co-repressor complexes [65]. YY1 has been shown to interact with SAP30, a component of the Sin3A co-repressor complex, and to mediate recruitment of HDACs to promoters via SAP30 [68–70].

YY1 is subject to post-translational modifications, including phosphorylation and acetylation, and its activity, binding site affinity, interaction partners, and cellular localization can be modulated by changes in the cellular environment [69,71–74]. Thus, like Rbpj/CBF-1, YY1 could potentially have roles in both repression of viral gene expression in latency, and in reactivation of the virus through recruitment of different complexes in response to different signaling environments.

In addition to the MIEP, HDACs are bound to MCMV promoters representative of early and late genes in latent mice [32]. The M100 late promoter, but not the M112 early promoter, has one YY1 binding site, which is occupied in latency. Neither of these promoters has binding sites for CBF-1. Binding of repressors to other promoters has not been studied. Thus, further studies are needed to characterize repressors bound to these regions and the mechanisms by which repressors are recruited to all regions of the genome.

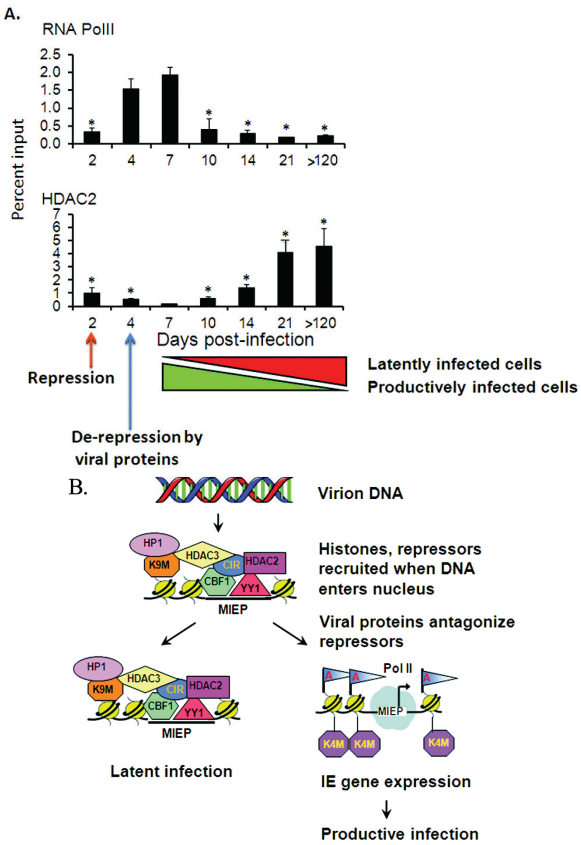
2.5. Latency May Be the Default State in Viral Infection

Cell culture studies of HCMV and HSV-1 infection of permissive cells have shown that viral genomes rapidly associate with ND10 structures upon entry of the DNA into the nucleus [75,76]. ND10s are dynamic nuclear complexes, whose core components include the cellular proteins PML, Daxx, Sp100, ATRX, and HDACs. These complexes are thought to repress viral gene expression as part of an intrinsic host immune response [77]. HCMV, MCMV, HSV-1, and other viruses encode tegument proteins, which enter the cell upon fusion of the viral and host membranes, and immediate early proteins, which are transcribed in the first phase of infection, to counteract this repression and activate viral gene expression [40,75–81]. In addition, viral chromatin undergoes dynamic changes over the course of infection, such that histones bound to HCMV promoters at very early times post-infection have modifications consistent with transcriptional repression, which are replaced by euchromatic modifications as viral gene expression is activated [40].

Unlike cell culture models, the MCMV infection model permits kinetic analysis of binding of repressor proteins during all phases of infection, from acute infection, to convalescence, to latency [32]. These studies show that in kidneys of infected mice, binding of repressors, including HDACs, YY1, Rbpj/CBF-1, CIR, and Daxx, to viral genomes has three phases: binding is initially detected at the earliest phase of infection, prior to activation of viral gene expression and DNA replication, and then falls as RNA polymerase II is recruited to viral promoters and viral gene expression is activated. After

a nadir at Day 7–10, when viral gene expression is most active, the percentage of viral genomes bound to repressors increases during the convalescent phase, and then plateaus (Figure 1A).

Figure 1. (**A**) Chromatin immunoprecipitation analysis of binding of RNA polymerase II and HDAC2 to the murine cytomegalovirus (MCMV) major immediate early promoter (MIEP) over the course of infection [32]. Results similar to those of HDAC2 were observed for binding of other repressors, including HDAC3, YY1, Rbpj, CIR, and Daxx. *, $p < 0.05$ relative to Day 7. Copyright © 2010, American Society for Microbiology; (**B**) Model for establishment of MCMV latency. Viral DNA is not complexed with histones in the virion, but is rapidly heterochromatinized upon entry in the nucleus. Co-repressor complexes may be recruited to MCMV DNA through interaction with transcription factors that bind directly to sequences in the viral genome, including YY1 and Rbpj/CBF-1. We speculate that loss of repressors in MCMV infection is due to inactivation of host cell defenses by viral proteins analogous to the HCMV pp71 tegument protein [77]. Activation of viral gene expression may fail to occur in some cells, and these cells may be the reservoir for latency. Reproduced with permission from [11].



The initial binding and subsequent loss of repressors in infected mice is consistent with host-mediated repression of MCMV gene expression through heterochromatinization of incoming viral genomes and subsequent de-repression by viral proteins, as seen in cell culture models of HCMV infection. However, even at the peak of viral gene expression, some binding of repressors to the genome was detectable [32]. These observations suggest that latency is the default state, and long term carriage of viral genomes results from failure of the virus to overcome the initial host transcriptional repression when the DNA enters the nucleus (Figure 1B). The apparent increase in MCMV genomes bound to repressors during the convalescent phase is likely due to changes in the population of cells, as productively infected cells are cleared by the host immune response, and latently infected cells harboring transcriptionally silent viral genomes predominate (Figure 1A). Eventually, all the productively infected cells are eliminated, and the percentage of genomes bound to repressors plateaus.

Live cell imaging studies have shown that ND10 proteins re-localize to viral genomes immediately upon entry of the DNA into the nucleus [82,83]. The signals that trigger recruitment of these proteins are unknown. Cellular sensors have been identified that detect the presence of pathogen-associated molecular patterns (PAMPs) localized to the cell surface, endosomes, and the cytoplasm, resulting in activation of innate immunity [84]. Entry of histone-free DNA through nuclear pores would be a highly aberrant event in an uninfected cell. It is tempting to speculate that viral DNA passing through the nuclear pore is detected as a PAMP by a protein associated with the nuclear pore complex to activate ND10s and repress viral gene expression through an intrinsic immune response.

3. Reactivation of Latent CMV

3.1. Organ Transplantation Induces Reactivation of Viral Gene Expression

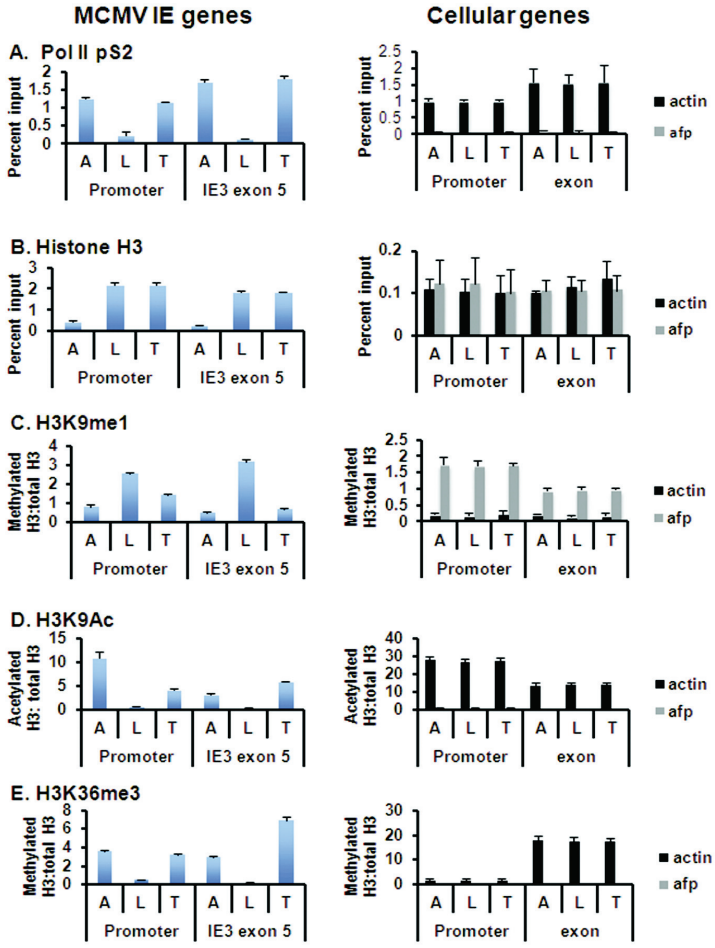
Reactivation of latent CMV is a significant infectious complication of solid organ transplantation. The highest risk of CMV disease is associated with D+/R- transplants [2,3]. Murine renal transplant models using kidneys from MCMV latently infected donor mice have therefore been developed to study the molecular mechanisms of reactivation in the context of D+/R- organ transplantation. Early studies showed that reactivation of *ie* gene expression could be induced within two days by transplanting kidneys from latently infected donor mice into naïve, allogeneic recipients [14]. Reactivation in this model correlated with expression of inflammatory cytokines, including TNF, and activation of transcription factors that bind to the MIEP, including NF-κB and AP-1. Although this model resulted in reactivation of immediate early gene expression, expression of genes representative of later stages of reactivation was not detectable. This was likely due to the very slow rate of CMV replication combined with the rapid rejection that occurs when allogeneic organs are transplanted into immunocompetent mice. Subsequent studies showed that reactivation of infectious virus in the donor kidney, and spread of the virus to recipient organs, could be induced by transplanting latently infected kidneys into immunodeficient recipient mice [85].

3.2. Epigenetic Reprogramming of Viral Chromatin in the ie *Region Is Induced by Transplantation*

Given that viral genomes are heterochromatinized in latency, reactivation of virus likely requires chromatin remodeling. We therefore analyzed changes in epigenetic factors bound to the *ie* region that correlated with transcriptional reactivation of *ie* gene expression induced by kidney transplantation. Kidneys from latently infected BALB/c mice were transplanted into wild type allogeneic C57BL/6 (B6) recipients, and the recipients were sacrificed at Day 2 (Figure 2). To determine the levels of factors bound to the MIEP in kidneys from the same mice prior to the transplant, the contralateral donor kidneys were harvested at the time of the transplant (Day 0) for analysis as latent controls. Due to the very low MCMV DNA copy number in latent mice, chromatin from 30 mice was pooled for analysis at Day 0 or at Day 2 after transplant. Acutely infected mice were analyzed in parallel as positive controls. As an additional control for comparison of different chromatin preparations, we analyzed the promoter and coding regions of two cellular genes, β-actin, a transcriptionally active gene, and α-fetoprotein, which is repressed in kidneys of adult mice (Figure 2, right panels).

We first analyzed binding of RNA polymerase II (Pol II) to the *ie* region. Pol II can bind to promoter regions as pre-initiation complexes, and thus, binding to the promoter does not necessarily indicate active transcription [86]. However, phosphorylation of S2 of the carboxy terminal domain (Pol II pS2) is associated with transcriptional elongation, and thus, active transcription. The MCMV *ie* region encodes two differentially spliced transcripts, IE-1, containing exons 1, 2, 3, and 4, and IE-3, containing exons 1, 2, 3, and 5 [87]. We analyzed binding of Pol II pS2 to both the *ie* promoter region, and to the coding region of IE-3 (exon 5). Our results show that, as expected, high levels of Pol II pS2 were bound to the promoter and to IE-3 exon 5 during acute infection (Figure 2A, lanes A, left panel). These levels were comparable to those seen with the actively transcribed gene, β-actin. Consistent with our previous analyses, binding of Pol II to the MIEP was markedly reduced in latency (Figure 2A, lanes L, left panel). Transplantation of latently infected BALB/c kidneys into allogeneic B6 recipients induced recruitment of Pol II pS2 to both the enhancer and the IE-3 coding region at levels comparable to acute infection (Figure 2A, lanes T, left panel). Analysis of the β-actin and α-fetoprotein genes showed that differences in binding to the MCMV MIEP were not due to differences in the chromatin preparations, and that the binding was specific. These results confirmed our previous finding that transplantation of latently infected kidneys into allogeneic recipients induced transcriptional reactivation of *ie* gene expression within two days after transplant [14]. In that study, IE-1, but not IE-3 expression was detected. This discrepancy is likely due to lower abundance of IE-3 transcripts and lower sensitivity of previous assays.

Figure 2. Chromatin immunoprecipitation analysis of epigenomic changes in the *ie* region induced by allogeneic transplantation. Due to the low copy number, chromatin from 30 mice was pooled for analysis of MCMV DNA in latent and transplanted kidneys. ChIP assays were analyzed in triplicate as previously described [31,32]. Results shown are the mean percent input plus standard deviation (**A** and **B**) or the ratio of modified histone to total histone after normalization to input DNA (**C**–**E**). Ratios are greater than one due to differences in antibody affinity. These results are representative of two experiments. A, acutely infected kidney; L, latently infected kidney; T, transplanted kidney; afp, alpha-fetoprotein.



To determine whether recruitment of Pol II was accompanied by changes in viral chromatin, we analyzed changes in modifications of histones bound to the MIEP. As in our previous studies [31], we found that latent viral DNA is highly enriched in histones relative to cellular genes (Figure 2B). The percentage of viral genomes bound to H3 did not change when latent kidneys were transplanted into

allogeneic recipients. The distribution of various histone modifications along a gene is typically position-dependent, with acetylation most prevalent in the enhancer region [88]. Transplantation of latently infected kidneys caused a loss of the repressive H3K9me1 mark and an increase in the activating mark H3K9Ac bound to both the promoter and coding region of the *ie* genes (Figure 2C,D). As with Pol II binding, the presence of H3K9Ac may reflect paused, rather than productive transcription [86]. However, tri-methylated H3K36 is found preferentially in coding regions, and is therefore a specific marker for elongation of transcription. Transplantation of latently infected kidneys into allogeneic recipients induced an increase in H3K36me3 in both the MIEP and IE-3 coding region (Figure 2E, left panel). No changes were observed in modifications of histones bound to cellular genes, indicating that these differences were specific to viral genes (Figure 2, right panels). These results show that transplantation of latently infected kidneys induces a switch in viral chromatin in the *ie* region, from a repressive heterochromatic state to a euchromatic configuration, leading to expression of both IE-1 and IE-3 transcripts.

3.3. Global Changes in Viral Chromatin Are Likely Required for Reactivation of Virus

The MCMV IE-1 and IE-3 proteins are functionally similar to HCMV IE-1 and IE-2, respectively [89]. Although there is little conservation of amino acid sequence, both HCMV and MCMV IE-1 proteins are thought to counteract host intrinsic immunity through interaction with HDACs and other ND10 components [90–92]. MCMV IE-3 protein shares significant homology with HCMV IE-2 in the C-terminal domain, and like HCMV IE-2, MCMV IE-3 trans-activates early gene promoters, is required to activate expression of β and γ genes during infection, and is essential for viral replication [87,93–95]. Thus, transcriptional reactivation of *ie* gene expression would be expected to lead to activation of transcription and remodeling of other regions of viral chromatin. Due to the difficulty in obtaining sufficient amounts of material for analysis of MCMV DNA, which is present at a copy number of ~1 MCMV genome per 10,000 cellular genomes in latent kidneys [85], only the *ie* region has been analyzed in the allogeneic kidney transplant reactivation model at Day 2. However, previous studies showed that induction of *ie* gene expression was transient in this model, with a peak of expression at Day 2, and that genes representative of later stages of replication were not detectable [14]. Rejection of transplanted organs occurs rapidly in this model, due to recognition of foreign cellular antigens, and it may therefore be necessary to use immunocompromised recipients to observe changes in other regions of viral chromatin.

A new model for reactivation of infectious virus in genetically immunodeficient transplant recipients has recently been described [85]. As in the previous model, activation of transcription factors that bind to the *ie* promoter and reactivation of *ie* gene expression was detectable within two days after transplant (unpublished observations), but unlike the allogeneic transplant model, reactivation of infectious virus becomes detectable in immunodeficient recipients over a period of weeks. This model would therefore be useful for studying changes in viral chromatin downstream of *ie* gene expression.

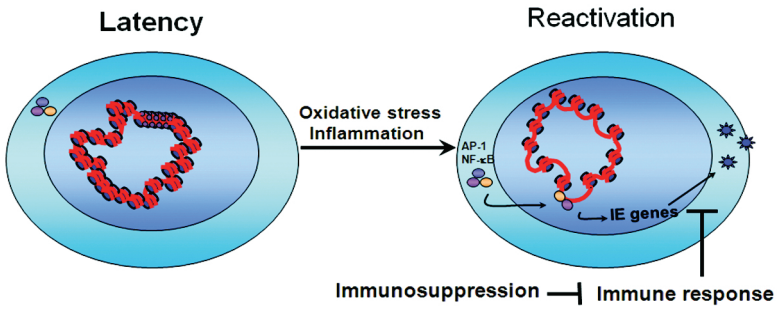
3.4. Mechanisms of Chromatin Remodeling Induced by Transplantation

As mentioned above, YY1 and Rbpj/CBF-1 may have dual roles, both in repression of *ie* expression in latency and in activating *ie* gene expression for productive infection. However, additional activating transcription factors are likely to play a role in reactivating viral gene expression from latency through recruitment of chromatin remodeling complexes. The HCMV and MCMV *ie* enhancers are complex regions with multiple potential binding sites for transcription factors that may activate *ie* gene expression. A comparison of the potential transcription factor binding sites in the enhancers of cytomegaloviruses infecting primate and non-primate species, and a more recent analysis of the MCMV MIEP transcription factor binding sites, have been published [11,96]. Although they differ in the number and arrangement of sites, most CMV species share sites for NF-κB, AP-1, and ATF/CREB in the MIEP, and these sites may have roles in both acute infection and in reactivation from latency. The proteins that bind to these sites are dimeric complexes, which can be derived from multiple family members. Thus, there may be considerable diversity, not only in the transcription factor binding sites that are occupied under given conditions, but also in the composition and function of the complexes bound to these sites. Although there have been some genetic analyses of sites in the HCMV enhancer that regulate *ie* expression [96–102], there has been little analysis of transcription factors that are bound to the genome, either during productive infection or latency. Our preliminary studies indicate that the NF-κB and AP-1 sites are not occupied in kidneys of MCMV latently infected mice, but the NF-κB p65 subunit and the AP-1 junD subunit are recruited to the MIEP when latently infected kidneys are transplanted into allogeneic recipients (unpublished observations). NF-κB p65 is activated by inflammatory cytokines and PAMPs through the canonical pathway, and activates expression of many genes involved in innate and adaptive immunity [103]. JunD is activated by oxidative stress, and induces expression of genes that promote survival and protection from reactive oxygen species induced by oxidative stress [104–107].

Allogeneic transplantation induces both oxidative stress, due to ischemia/reperfusion injury, and an inflammatory response due to allorecognition of foreign antigens. At Day 2 post-transplant, many inflammatory cytokines, antigen-presenting molecules, and co-stimulatory molecules are up-regulated in allogeneic kidney transplants [14,108]. Many of these factors are known to activate signaling pathways that lead to activation of NF-κB and AP-1, and thus, could contribute to reactivation of *ie* gene expression (Figure 3).

CMV infection in transplant recipients is associated with infection with other pathogens, with episodes of acute rejection, and treatment with lymphocyte-depleting regimens that induce release of inflammatory cytokines [2,3]. Several investigators have therefore suggested a link between an inflammatory immune response and reactivation of CMV mediated by activation of transcription factors that bind to the MIEP to initiate viral gene expression [11,13,109–111]. Determining which transcription factors are required for reactivation of CMV from latency, their roles in chromatin remodeling, and the signaling pathways by which these factors are activated, are significant, but important, challenges for the future.

Figure 3. Model for reactivation of CMV from latency. In latently infected cells, episomal viral genomes are heterochromatinized, and transcription factors that drive IE gene expression, such as NF-κB and AP-1, are inactive (NF-κB depicted as cytoplasmic trimeric complex of p65, p50, and the inhibitory subunit, IκB). We propose that inflammatory cytokines and oxidative stress lead to activation of transcription factors that bind to the MIEP and induce chromatin remodeling to activate *ie* gene expression. Reactivation of *ie* gene expression does not lead to reactivation of virus in immunocompetent individuals, but in immunosuppressed individuals, reactivation of IE gene expression leads to production of infectious virus.



4. Conclusions

CMV infection initiates a complex battle for survival between the virus and host. Although CMV encodes a wealth of genes dedicated to subverting host innate and adaptive immune responses [112], the host is generally able to clear cells producing infectious virus in immunocompetent individuals. The end result of this battle is therefore a standoff between the virus and host, in which the virus is able to persist for the life of the host, but does not cause active disease. It is becoming increasingly clear that this latent state is established by epigenetic factors that mediate heterochromatinization of viral genomes to silence viral gene expression. The virus is able to persist indefinitely in these cells because they are not expressing viral proteins, and are therefore invisible to the host immune response. Transcriptional repression may be established at the outset of infection as a result of a third type of immunity, which has been called an intrinsic immune response [79,80], and the reservoir of latently infected cells may arise from failure of the virus to overcome this initial repression in some cells.

Although CMV wins the virus/host battle in one sense, in that it is able to survive in the face of host immunity, replication is the only successful outcome for a virus. Latency is a dead end, and survival of latent virus is dependent on survival of the host. Reactivation of the virus in response to events that threaten the survival of the host, such as infection with another pathogen, would therefore be expected to have an adaptive advantage. Previous studies have shown that reactivation of MCMV *ie* gene expression is rapidly induced by allogeneic transplantation, and by inflammatory mediators, including TNF and LPS [14,30,109]. Reactivation of HCMV can be induced by allogeneic stimulation, dendritic cell maturation and IL-6 [43,113–115]. These stimuli are all associated with an inflammatory immune

response. Collectively, these observations lend credence to the hypothesis that CMV has evolved to escape from hosts where its survival may be compromised.

Here, we have demonstrated that reactivation of latent MCMV gene expression induced by organ transplantation occurs through epigenetic reprogramming, which results in changes in modifications of histones bound to viral DNA and recruitment of activating transcription factors. Although inflammation has been implicated in reactivation, the specific signaling pathways that mediate this response, either in immunocompetent individuals or in transplant recipients, are unknown. The key outstanding questions with respect to epigenetic control of CMV latency and reactivation are (1) how are viral genomes chromatinized in the initial stage of infection; and (2) what are the signaling pathways that lead to chromatin remodeling and transcriptional reactivation of viral gene expression? The answers to these questions may lead to new therapies to eradicate latent virus or to prevent reactivation in immunocompromised hosts.

Acknowledgments

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Conflicts of Interest

The authors declare no conflict of interest.

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Review

The Role of Chromatin in Adenoviral Vector Function

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Abstract: Vectors based on adenovirus (Ad) are one of the most commonly utilized platforms for gene delivery to cells in molecular biology studies and in gene therapy applications. Ad is also the most popular vector system in human clinical gene therapy trials, largely due to its advantageous characteristics such as high cloning capacity (up to 36 kb), ability to infect a wide variety of cell types and tissues, and relative safety due to it remaining episomal in transduced cells. The latest generation of Ad vectors, helper-dependent Ad (hdAd), which are devoid of all viral protein coding sequences, can mediate high-level expression of a transgene for years in a variety of species ranging from rodents to non-human primates. Given the importance of histones and chromatin in modulating gene expression within the host cell, it is not surprising that Ad, a nuclear virus, also utilizes these proteins to protect the genome and modulate virus- or vector-encoded genes. In this review, we will discuss our current understanding of the contribution of chromatin to Ad vector function.

Keywords: adenovirus; chromatin; histones; DNA replication; transcription

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1. Introduction

The biology of human adenovirus (Ad) has been studied in great detail for over 60 years, making Ad one of the best characterized human DNA viruses. These studies, while giving us a great deal of information about DNA replication, control of gene expression, and tumorigenesis, also laid the foundations for the later development of Ads as gene transfer vectors. The first suggestion that Ad could be used as a vector system for expression of foreign genes came in the late 1970s with the identification of spontaneous recombinants between Ad and SV40 which expressed T antigen fused to an Ad structural protein [1,2]. Use of this vector allowed for the purification of large quantities of a T antigen-related protein that retained biological activity and set the stage for further development of Ad as a gene delivery vehicle. A crucial development occurred when cell lines that could complement viruses deleted of the essential early region 1 (E1) were created [3]. This cell line allowed for the development of Ad-based vectors that could not replicate in most cell lines or tissues, but could provide high level expression of an exogenous transgene. Ad vectors now come in a variety of "generations" that differ in their degree of attenuation, from loss of one gene to deletion of all viral protein coding sequences. These vectors can serve a variety of functions ranging from short-term, high-level expression of a transgene in tissue culture to long-term therapeutic gene expression in animal models of human disease.

One common feature of all Ad vectors is that they remain episomal in the transduced cell and, in some animal models, the vector can persist and express a transgene for several years. This finding suggests that the vector may be able to "hide" in the nucleus through adopting a structure similar to the genomic DNA. Thus, the chromatinized viral DNA is likely impacted and influenced by all of the same factors as the native cellular DNA, such as epigenetic regulation. Indeed, recent studies have shown this to be the case, as Ad-based vector DNA associates with histones and is wrapped in nucleosomes within the host cell nucleus. This chromatinized structure strongly influences the level and duration of expression from a vector-encoded transgene. This review will discuss our current understanding of Ad vector chromatinization. For a more detailed review of the dynamic changes in nucleoprotein structure during wildtype Ad (AdWT) infection, please see [4].

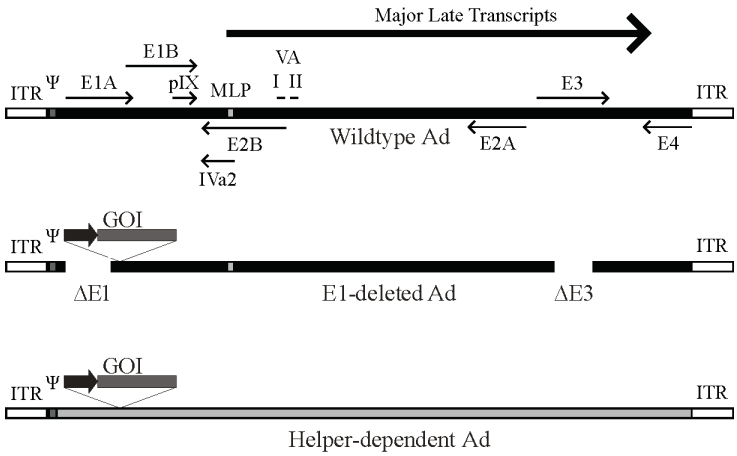
2. Ad Vector Design

Although there are many different generations of Ad vectors which differ in the extent to which the genome is attenuated (reviewed by [5]), the vast majority of studies involving Ad vectors use the simple E1-deleted, first-generation Ad vector (Figure 1). Most first-generation vectors are also deleted of the E3 region, which is not required for virus replication in culture, and its removal increases the cloning capacity for foreign DNA to ~8 kb [6]. Since E1 is absolutely required for virus replication, these vectors do not replicate to any appreciable degree in most cell lines, but can be easily propagated in E1-complementing cell lines such as 293 cells [3]. However, it is important to note that the E1-deleted Ad vectors can undergo limited replication in a number of tissue culture cell lines if they are delivered at a high multiplicity of infection (MOI) [7]. Moreover, first generation Ad delivery to

animals and humans is typically accompanied by the induction of very strong innate and adaptive immune responses [8,9]. These immunogenic responses are at least in part due to leaky expression of viral genes retained in the E1-deleted vectors [10], which ultimately limits the duration of transgene expression to a few days or weeks. First generation Ad vectors are usually ideal for short-term expression in tissue culture or animals, but not ideal for applications requiring long-term (months to years) expression. Second generation Ad vectors have further deletions in the E2 or E4 coding sequences. E2 encodes proteins involved in Ad DNA replication [11], so its removal will prevent replication and reduce the expression of late genes [12]. E4 encodes several proteins involved in a variety of functions that impact on both viral and cellular gene expression and signal transduction [11]; thus, removal of some or all of E4 should significantly attenuate the vector. Deletions of E2 and E4 have been shown in some studies to have beneficial effects in reducing the expression of viral proteins and vector-directed immune responses, thereby extending transgene expression [5]. However, second generation vectors are no longer in common use due to limited evidence of enhanced efficacy over first generation vectors.

Deletion of all viral protein coding sequences results in third-generation Ad vectors, also known as fully-deleted, gutted, or helper-dependent Ad (hdAd) vectors (Figure 1). This latter term arose from the fact that these vectors must be propagated in the presence of a second virus that provides all of the replication and packaging functions *in trans* for growth of the hdAd vector [13]. The hdAd vector needs only the *cis* activating elements required for virus DNA replication (the inverted terminal repeats [ITR], ~100 bp located at both ends) and the packaging sequence (~150 bp, located immediately adjacent to the left ITR). Selection against packaging of the helper virus genome is important to ensure efficient hdAd propagation. Selective packaging can be accomplished using loxP or FRT sites flanking the helper virus DNA packaging element and 293-based cell lines expressing the Cre or FLP recombinase, respectively [13–17]. Such helper viruses can replicate and produce all viral proteins necessary for replication and packaging of the hdAd, but cannot package their own genomes due to loss of the packaging element in the Cre- or FLP-expressing cells. These methods result in relatively pure stocks of hdAd. In numerous studies, we and others showed that hdAd have many desirable characteristics for gene therapy applications, including long term therapeutic gene expression (>2 years) in mice [18–20], rats [21], dogs [22] and baboons [23], and reduced toxicity relative to traditional Ad vectors [24,25]. For a more comprehensive review of hdAd vector function, please see [5,26–28].

Figure 1. Schematic of the adenovirus genome and adenovirus-based vectors. Top panel: A simplified map of the adenovirus (Ad) serotype 5 genome showing the early genes (E1–E4) and the region from which the major late transcript is produced (the L1-L5 transcripts produced from alternative splicing of the major late transcript are not shown). The relative position of pIX, VA RNA I and II and IVa2 are indicated. Also shown are the viral inverted terminal repeats (ITR) located at each end of the genome, the viral packaging element (Ψ) located adjacent to the left ITR, and the position of the major late promoter (MLP). Please note that these features are not drawn to scale. Middle panel: General structure of an early region 1 (E1)-deleted Ad vector. Most E1-deleted vectors are also deleted of the E3 region, which is not required for replication in tissue culture and increases the cloning capacity to approximately 8 kb of foreign DNA. The gene of interest (GOI) is usually introduced to replace the E1 region and is placed under control by a heterologous promoter (dark arrow). Bottom panel: General structure of a helper-dependent Ad vector. hdAd are devoid of all protein coding sequences, and need contain only the viral inverted terminal repeats and packaging element. hdAd also frequently contain non-coding stuffer DNA (shown in gray) to ensure optimal genome size.



3. Ad Virion Structure

Although these Ad vectors lack some (or all) viral coding sequences, the overall virion structure appears identical to AdWT [29,30]. The Ad virion has an icosohedral, non-enveloped capsid structure (~70 to 100 nm in diameter) surrounding a nucleoprotein core containing a linear double-stranded genome that for wildtype Ads is ~30–40 kb. The Ad capsid is composed of 8 polypeptides, named in order of decreasing size [31–34]. Hexon (a trimer of protein II) assemble into a sheet-like structure called the "group-of-nine", which forms the 20 facets of the icosahedon. Protein III clusters into groups of five (known as pentons) at the vertices of the icosahedron, from which extend trimers of

protein IV, known as fibre. These three polypeptides are the major capsid proteins. This general structure is supported by five minor capsid proteins (IIIa, IVa2, VI, VIII, and IX). Within the viral capsid, the viral DNA associates with three basic proteins, VII, V and Mu (μ), which function to neutralize the charge on the DNA, permitting tight packing of the DNA within the virion. Protein VII is similar to cellular protamines, and is the main protein responsible for wrapping and condensing the viral DNA [35]. A shell of protein V is postulated to coat the protein VII-DNA complex [36,37]. Pre-Mu is thought to aid in wrapping and condensing the viral DNA [38]. It is believed that cleavage of pre-Mu by the Ad-encoded protease may function to relax the viral DNA nucleoprotein structure before it enters the nucleus [39]. Since the tightly packed DNA structure is refractory to viral transcription [40,41], the nucleoprotein structure of the Ad DNA must be remodeled to achieve efficient gene expression.

Even though the viral DNA does not directly interact with the outer capsid proteins, it still contributes to the physical stability of the virion, as packaging of subgenomic sized DNA results in decreased Ad virion stability [30,42]. There seems to be a direct relationship: the smaller the genome, the less stable the virion. This observation has significant consequences for the design of Ad-based vectors. In the case of hdAd, which lacks all viral protein coding sequences, the viral genome must be replaced with alternative DNA simply to provide the necessary structural stability to support the mature capsid structure. For hdAd, reducing the genome size to less than ~27 kb causes the DNA to multimerize or rearrange to increase the genome size to between 27 and 36 kb [43]. Thus, if the transgene is large enough (e.g., a genomic loci), it may be sufficient to provide the needed support; however, a small transgene cassette may require the use of a non-coding “stuffer” element to bring the overall genome to an appropriate size. However, the nature of the stuffer DNA can significantly influence the function of the hdAd vector due to different epigenetic modifications, as discussed below [44,45].

4. Ad Infection of a Cell

The most commonly used Ad vectors are based on human Ad serotypes 2 or 5, and many of the details of the infection process have been worked out in great detail. Initially, the Ad fibre protein binds to the Coxsackie-Adenovirus receptor (CAR—a common receptor for Ad5 and Coxsackie B virus) [46,47]. Ad5 can also enter cells using heparin sulfate proteoglycans as an alternative receptor, either through direct binding to the Ad fibre shaft [48], or bridged through interaction of Ad with blood factors such as factor IX, factor X or complement component C4-binding protein [49–51]. Binding is followed by interaction between Ad penton and $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins [52]. Ad is internalized by receptor-mediated endocytosis and escapes from the early endosome [53,54]. The virion is then transported through the cytoplasm to the nucleus along the microtubule network [53], and the capsid is slowly disassembled *en route* [55]. Upon reaching the nuclear pore, the Ad DNA complexed with protein VII is released into the nucleus [55–57], while the rest of the capsid proteins are ultimately degraded. For a more comprehensive discussion of viral entry, please see [58].

5. Ad Vector DNA Remains Primarily Episomal within the Infected Cell Nucleus

All vector systems have at least some ability to integrate into the genome of the host cell. Obviously, retrovirus- and lentivirus-based vectors integrate at very high frequencies in the target cell genome, making them ideal for studies involving rapidly dividing cell types (e.g., stem cells), but this does raise the risk of insertional activation or inactivation of cellular genes [59–61]. For Ad, the spontaneous integration rate is very low. In tissue culture studies, infection of several different cell lines with E1-deleted or hdAd vectors containing a selectable marker led to an integration frequency of 10^{-4} to 10^{-5} per cell (using an initial MOI of 10), and integration appeared to be due to non-homologous recombination [62,63]. A similar frequency was observed for hdAd in mouse hepatocytes *in vivo* [64], suggesting the vast majority of expression from hdAd in animals is due to expression from episomal vectors. Furthermore, in infected mouse hepatocytes *in vivo*, the vectors do not replicate with the cellular DNA and remain monomeric [65]. Traditional wisdom has held that in dividing tissues, non-integrated Ad DNA would be "lost" from the nucleus of transduced cells during cell division, as the virus has no known means of segregating with the host chromosomes. However, *in vivo* studies in which hdAd-transduced mouse hepatocytes were induced to divide through partial hepatectomy showed that Ad has a surprising ability to be retained in daughter cell nuclei after division, suggesting the virus may physically associate with chromosomes in the nucleus through an uncharacterized mechanism [66,67].

6. Early Events within the Infected Cell Nucleus

Much of what we know about the early stages of Ad DNA remodeling comes from studies of AdWT infection, though we assume that many of these events are similar for Ad vectors. Even though many Ad capsid proteins translocate to the periphery of the nucleus, only the VII-DNA complex enters the nucleus through the nuclear pore with the help of histone H1 (H1) [56,68]. This role of H1 appears independent of any known function in eventually condensing the viral DNA in the nucleus. Studies involving UV cross-linking of radiolabeled proteins to the AdWT DNA suggest that protein VII remains stably associated with the Ad DNA throughout the early phase of infection, from at least 2 until 13 hours post infection (hpi) [69]. Several immunofluoresence-based studies using antibody directed to protein VII have shown that protein VII-containing foci can be detected in the nucleus of both AdWT- and Ad vector-infected cells, which represent protein VII-wrapped genomes [70–73]. For AdWT, these foci remain visible for at least 8–10 hpi, but disappear around 12–16 hpi [71]. Treatment of cells with alpha-amanitin, an inhibitor of transcription, prevented loss of VII foci at late times (16 hpi), suggesting that late transcription could be involved in release of VII [70,71]. However, using a similar immunofluorescence-based approach, Karen and Hearing [72] showed that the number of protein VII foci in the nucleus steadily declined from 2 to 14 hpi. DNA replication was not required for loss of the protein VII foci, however blocking viral transcription either pharmacologically (alpha-amanitin) or through use of an E1A-deficient virus blocked the transition, once again suggesting that active viral transcription was required for release of VII from the Ad genome.

Similarly conflicting results regarding the duration and level of association of protein VII with the viral genome have been shown using ChIP-based studies. While some studies have shown that protein VII is stably associated with the Ad DNA during early stages of infection [70,74], other studies showed a gradual decrease in VII association with the viral DNA over time [75–77]. During this same time period, there was a gradual increase in the association with histone H3 (and the other core histones) over most promoter and coding regions, although the binding levels of the histones was less than cellular chromatin [74]. Similar results were observed by Haruki *et al.* [75] who demonstrated the uneven distribution of VII along the Ad genome, with relatively low occupancy at the E1A promoter and relatively high occupancy at the major late promoter (MLP) at 6 hpi. The MLP only becomes active after viral DNA replication has initiated (~8–12 hpi), suggesting protein VII association may be more prolonged for regions of the Ad genome that are activated late in the virus lifecycle. Since it is very likely that the histones were binding directly to the DNA, these observations suggest that at least a portion of protein VII must be removed or remodeled to make space for binding of the histones to the viral DNA. Indeed, protein VII and H3 could be found bound to the same DNA molecules in re-ChIP experiments [74]. Protein VII may actually play a role in regulating early viral transcription, as pre-wrapping plasmids in small quantities of purified protein VII led to higher levels of reporter gene expression compared to naked plasmid in transient transfection assays [74].

In vitro studies have shown that the protein VII-condensed DNA structure does not allow for efficient transcription [40,41], suggesting that the complex must be remodeled before efficient gene expression can occur. Again based on *in vitro* studies, three proteins have been identified that can remodel the VII-wrapped Ad genome: template activating factor Iβ (TAF-Iβ) (also known as SET [40]), TAF-II (NAP-1 [78]) and TAF-III (B23/nucleophosmin [79]). Of the three proteins, TAF-1β is the best characterized with respect to Ad DNA remodeling [70,75,80]. TAF-1β binds the VII-wrapped DNA complex, which "opens" the viral DNA to allow access to nucleases and, presumably, transcriptional activators [41]. Whether remodeling by TAF-1β involves simple translocation or actual disassociation of VII from the DNA has yet to be determined. The observation that TAF-1β knockdown in cells has only a minor effect on Ad early gene expression suggests that accessory proteins may also be required for efficient transcription from the Ad template or that redundant mechanisms exist for remodeling [74].

Whether active transcription of the Ad DNA is required to mediate VII disassociation is also a subject of debate. Although some studies showed that VII appeared to remain associated with the viral DNA for longer periods of time when transcription elongation was inhibited [71,72], other studies did not see a difference [74,76]. It has been suggested that protein VII can recruit E1A to the viral DNA, and that E1A-mediated activation of transcription may lead to removal of protein VII [71,81]. Consistent with this, infection with an E1A-deficient virus prevented loss of the protein VII foci [71]. Again based on immunofluorescence analysis, release of protein VII could be achieved in the absence of E1A or other viral proteins, if the vector contained a strong heterologous promoter, such as the CMV promoter which is not reliant on E1A for expression [71]. Since only part of the AdWT genome is actively transcribed during early times of infection, it may be that protein VII is released from

transcribed regions, but remains associated with the "late" regions until these regions are subsequently transcribed. This suggestion may also be consistent with the immunofluorescence-based studies, as this technique cannot easily distinguish between full- and partial-occupancy of VII on the viral DNA.

7. Ad Vector DNA Associates with Cellular Histones in the Infected Cell Nucleus

Recent studies have established that Ad and its derivative vectors (E1-deleted, replication defective Ad or hdAd) associate with cellular histones early during infection [45,74,76,77]. Histones are detected on the Ad DNA within one hour of infection, and protein VII and histones can be found associated simultaneously with the same viral DNA fragment within the cell [74]. In general, deposition of cellular histones can occur through either a replication-coupled or replication-independent mechanism, and the specific histone variants and chaperones involved vary for each mechanism [82]. Histone H3.1, which is expressed strictly during S-phase, is deposited on newly synthesized cellular DNA in a replication-coupled mechanism by the Chromatin Assembly Factor I (CAF-1) complex [83]. Conversely, the replacement histone variant H3.3, which differs from H3.1 by only 5 amino acids and is expressed throughout the cell cycle, is deposited through a replication-independent mechanism [82]. The histone chaperone involved in the deposition of H3.3 varies depending on the region of the chromosome. Within actively transcribed genes, the chaperone HIRA mediates H3.3 deposition, whereas on telomeres and pericentric DNA repeats, the H3.3 chaperone DAXX assists in the deposition [84–86]. Recent studies have suggested that the rapid deposition of H3.3 mediated by HIRA may be an evolved mechanism to protect "naked" DNA from damage [87].

In ChIP-based studies, hdAd, E1-deleted Ad [76] and wtAd [77] DNA associates with H3.3 as early as four hours post-infection, which suggests a replication-independent mechanism is responsible for the assembly of chromatin on Ad DNA. Recent work by Komatsu *et al.* [77] showing that knocking down of CAF-1 does not affect histone deposition on the Ad genome supports this idea [77]. Knockdown of HIRA reduced association of the hdAd DNA with H3, and also reduced Ad-mediated transgene expression [76], suggesting that assembly into chromatin is required for optimal gene expression. Given that H3.3 is also deposited on incoming Herpes Simplex Virus-1 DNA [88], the cell may utilize a common mechanism for processing incoming DNA originating from nuclear viruses.

AdWT DNA associates with all core histones (H2A-H2B and H3-H4) as early as one hour post-infection [74,77]. Ad vector DNA is also found associated with all these nucleosomal proteins, and the DNA displays a classic nucleosomal laddering pattern upon micrococcal nuclease digestion [76], suggesting that the Ad DNA is wrapped into physiologically-spaced nucleosomes in the infected cell nucleus. Taken together, these studies suggest that assembly of Ad vector DNA into chromatin through deposition of histones and remodeling into nucleosomes are accomplished through a replication-independent mechanism, and this event is important in establishing optimal transgene expression.

8. Epigenetic Regulation of Ad Vectors

Assembly of hdAd DNA into chromatin is necessary for efficient expression of vector-encoded genes, and this event very likely contributes to their significant stability and efficacy *in vitro* and *in vivo* [27,65]. One of the advantages of hdAd is their large cloning capacity, which permits the use of large upstream regulator sequences, or even whole genomic loci, to permit tissue-specific gene expression [18,89,90]. Chromatin plays an important role in gene regulation [91,92], and proper placement of nucleosomes relative to the transcription start site of a gene is crucial for promoter fidelity [93,94]. The ability to incorporate large regulatory regions into hdAd coupled with its swift assembly into physiologically-spaced nucleosomes likely contributes to the maintenance of a faithful expression profile from these control elements when contained in hdAd.

As noted above, hdAd vector DNA must be designed to an optimal size in order to ensure physical stability of the capsid and genetic stability of the genome [30,42,43]. Thus, for small transgenes, additional non-coding "stuffer" DNA must be included in the vector in order to obtain an optimal size. However, the nature of this stuffer DNA can have a significant effect on function of the vector. A hdAd containing 22 kb of eukaryotic stuffer DNA expressed its transgene ~10-fold higher *in vitro* and *in vivo* compared to a vector with an identical expression cassette but containing stuffer DNA derived from prokaryotic DNA [44]. Neither of these vectors was subjected to CpG methylation and both vectors associated with histones to a similar degree. No evidence of heterochromatic methylation marks (*i.e.*, H3K9me2, H3K27me3) were observed, however hdAd-prok chromatin was markedly under-acetylated (a mark of transcriptionally inactive chromatin) [45]. This effect could be blocked through the use of a DNA insulator element or administration of trichostatin A (a histone deacetylase inhibitor), suggesting that a repressive chromatin structure assembled on the prokaryotic stuffer DNA and spread to the transgene. Cellular proteins Sp100 and DAXX were implicated in mediating this event [45], which is perhaps not surprising given that AdWT has evolved a specific mechanism to antagonize DAXX activity [95–97]. Indeed, a recent study has suggested that protein VI from the incoming virion may function to attenuate the action of DAXX, thus facilitating initiation of early gene expression [98].

A recent study has shown that virus-mediated activation of innate immune signaling can culminate in epigenetic down-regulation of expression from Ad vectors. Infection of MyD88$^{-/-}$ mouse embryonic fibroblasts with a hdAd vector resulted in a higher level of transgene expression and was associated with an increased ratio of acetylated-H3K9 to methylated-H3K9 (*i.e.*, a tendency towards "active" chromatin configuration), compared to hdAd-infected wildtype mouse embryonic fibroblasts [99]. This observation suggests that engagement of toll-like receptors (such as TLR9 [100]) by Ad vector infection, and subsequent activation of MyD88-mediated signaling cascades, can lead to epigenetic silencing of the vector DNA. Thus, this study uncovered an innate pathway designed to epigenetically regulate expression of genes located on invading DNA.

Ad-encoded proteins can also actively modulate the activity of certain cellular chromatin modifying complexes. For example, the early region 4 open reading frame 4 (E4orf4) protein was shown to bind

AcfI and modulate the activity of the ACF chromatin remodeling complex [101]. E4orf4 appears to recruit protein phosphatase 2A (PP2A) to the ACF complex (and thus to chromatin), where PP2A may dephosphorylate uncharacterized substrates and alter local chromatin structure. It is thought that this interaction may contribute to E4orf4-induced caspase-independent, non-classical apoptosis at late times of AdWT infection [101–103]. Since first-generation Ad vectors retain the E4 region, it is therefore possible that expression of E4orf4 may in part contribute to acute or chronic toxicity noted for E1-deleted vectors [5].

9. Conclusions and Future Perspectives

Ad vectors are one of the most commonly used systems to deliver genes to mammalian cells. In the nucleus, the viral DNA must conform to the rules of the host environment, and many studies have shown that histones and proper chromatin configuration are as important to Ad as it is to the host DNA. Histones deposited on the Ad DNA can adopt an “active” euchromatic state or an “inactive” heterochromatic state. In the case of more advanced generations of Ad vectors, such as the hdAd, designing the vector such that it resembles a segment of the chromosome (e.g., using genomic loci-based expression constructs or large, non-coding stuffer elements derived from the human genome) allows these vectors to retain high-level expression for many years in mice, rats, dogs and non-human primates. Improving our understanding of the mechanism of assembly of Ad DNA into chromatin, and the cellular proteins involved in modulating this nucleoprotein structure, will undoubtedly improve our ability to design the next generation of Ad vector exhibiting true tissue-specific transgene regulation, optimal safety, and expression. Similarly, uncovering aspects of Ad vector design that permit the vector to remain undetected in the host cell nucleus should improve the function and persistence of the vector DNA within the transduced cell. For AdWT, there are a few obvious areas that require further study, such as whether protein VII is truly maintained and/or required on certain regions of the Ad genome during the early phase of viral replication, and how its association is affected by active transcription. Since the wildtype virus must transition from association with protein VII to histones and back to protein VII for packaging of the DNA into progeny virions, the cellular protein(s) that facilitate these events have yet to be determined. Finally, studying how viruses and vectors have adapted to function with the context of a mammalian nucleus will undoubtedly provide novel insight into how chromatin structure impacts host cell DNA function.

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Conflicts of Interest

The authors declare no conflict of interest.

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Review

The Chromatin Landscape of Kaposi's Sarcoma-Associated Herpesvirus

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Abstract: Kaposi's sarcoma-associated herpesvirus is an oncogenic γ-herpesvirus that causes latent infection in humans. In cells, the viral genome adopts a highly organized chromatin structure, which is controlled by a wide variety of cellular and viral chromatin regulatory factors. In the past few years, interrogation of the chromatinized KSHV genome by whole genome-analyzing tools revealed that the complex chromatin landscape spanning the viral genome in infected cells has important regulatory roles during the viral life cycle. This review summarizes the most recent findings regarding the role of histone modifications, histone modifying enzymes, DNA methylation, microRNAs, non-coding RNAs and the nuclear organization of the KSHV epigenome in the regulation of latent and lytic viral gene expression programs as well as their connection to KSHV-associated pathogenesis.

Keywords: Kaposi's sarcoma-associated herpesvirus; KSHV; KS; viral chromatin; polycomb; EZH2; minichromosome; histone modifications; MLL/Set1; JMJD2A

1. Introduction

It is estimated that 15% of human cancers are caused by viral infections. Among the seven currently known human oncogenic viruses, two of them belong to the herpesvirus family: Kaposi's

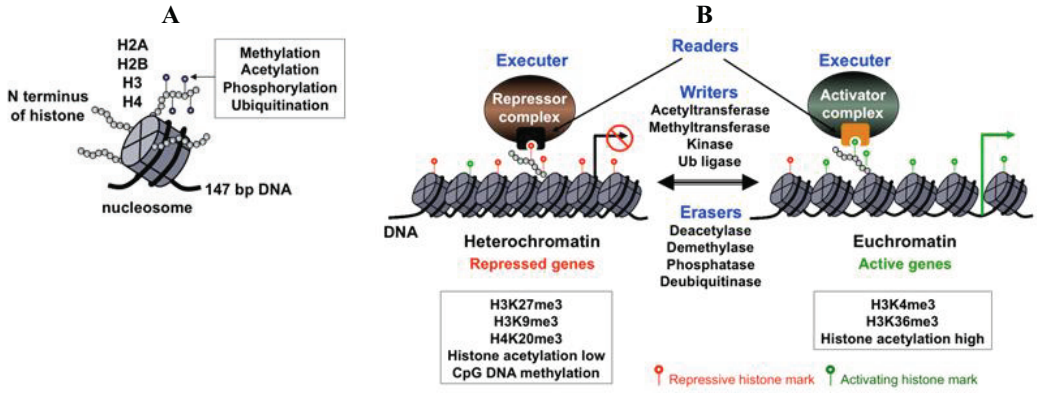
sarcoma-associated herpesvirus (KSHV or Human Herpesvirus 8, HHV-8) and Epstein-Barr virus (EBV or Human Herpesvirus 4, HHV-4). In 1994, KSHV was first discovered in Kaposi's sarcoma patients, who commonly present with cutaneous, neoplastic lesions of endothelial cell origin. Subsequently, it was also linked to the development of two B cell lymphomas: primary effusion lymphoma (PEL) and Multicentric Castleman's disease [1,2]. Like other herpesviruses, KSHV establishes persistent infection in humans and alternates between two different life cycle phases: latency and lytic reactivation. In immunocompetent individuals KSHV establishes latency in $CD19^+$ B cells, where a highly restricted viral gene expression program is thought to be the primary means by which latently infected cells escape detection by the host immune system. On the other hand, immune suppression along with other environmental and physiological factors, including oxidative stress, inflammatory cytokines, hypoxia or infection by other pathogens, each can favorably contribute to the physiological conditions required for KSHV to transition from latency to viral reactivation and virus production [3–8].

The 160-175-kb KSHV genome is composed of a single, 140.5-kb long unique coding region (LUR), which is flanked by 20-35-kb long, GC-rich terminal repeats (TRs) [9]. The LUR encodes at least 86 protein-coding genes, 12 microRNAs and several non-coding and antisense RNAs. The majority of latent genes are located between ORF69 and K14 in the KSHV genome and their expression is driven by only a few promoters. On the other hand, lytic genes are spread across the entire KSHV genome and the expression of each is likely regulated by over 100 different promoters. Latent genes are constitutively expressed in KSHV-infected cells, regardless of whether the infection is in a latent or lytic phase. On the other hand, lytic gene expression is repressed during latency and is only activated upon lytic reactivation, during which the three classes of lytic genes, immediate early (IE), early (E) and late (L) are induced, respectively [10]. While a relatively clear picture of the life cycle-dependent gene expression patterns of KSHV has emerged, we still do not understand the mechanism(s) that allow latent genes to escape the transcriptional repression affecting the rest of the viral genes during latency and the regulatory processes that control the genome-wide repression and temporally-ordered transcription of lytic genes. The fact that lytic reactivation of KSHV can be induced by treating latently infected cells with chemicals that affect chromatin regulatory factors such as histone deacetylases (HDACs), DNA methyltransferases (DNMTs) and histone acetyltransferases (HATs) argues that chromatin and chromatin-associated factors must be involved in the control of viral gene expression [11–13]. Indeed, whereas the KSHV genome is linear and histone-free in the viral capsid, after infection, the viral DNA becomes a closed circular episome that associates with cellular histones and persists in the nucleus as a non-integrated minichromosome [14,15]. Micrococcal nuclease mapping studies and chromatin immunoprecipitations of various histones and chromatin regulatory proteins have demonstrated that KSHV gene expression is controlled by the dynamic modulation of nucleosomal structure, a finding reminiscent of the regulation of cellular genes on the host chromosome (see below).

The fundamental building block of chromatin is the nucleosome, which consists of 147 bp of DNA, which is wrapped around a histone octamer composed of two of each of the histones: H2A, H2B, H3

and H4 (Figure 1A). The N-terminal tail of histones protrudes from the nucleosomes and as such, is subject to various posttranslational modifications, including acetylation, methylation, phosphorylation and ubiquitination (Figure 1A). Depending on whether a gene is destined for activation or silencing, different histone modifying enzyme complexes are recruited to the gene promoter to generate specific posttranslational modifications on histones called histone marks, which function as key modulators of gene expression (Figure 1B) [16]. These histone marks can then be recognized by specific nuclear proteins that use histone mark recognizing modules [17]. These histone mark-readers can recruit additional transcription factors that either inhibit or activate transcription (Figure 1B). In addition to the histone marks, gene transcription can also be modulated by other structural features of chromatin, including the distribution of histone variants, the positioning of nucleosomes and the presence of cytosine 5-methylation of the CpG islands in the gene regulatory regions [18,19]. The nucleosome array of the DNA genome constitutes the primary structure of chromatin, which is further organized into a three-dimensional complex structure through expansive looping of the chromatinized DNA and large-scale compartmentalization in the nucleus [20]. This higher-order organization of the chromatin can influence genes within large chromosomal domains or co-regulate genes that are otherwise far from each other on the chromosome.

Figure 1. Chromatin components and their effect on gene expression. (**A**) The basic unit of chromatin is the nucleosome, which consists of 147 bp DNA wrapped around histones H2A, H2B, H3 and H4. The N terminus of histones is subject to different posttranslational modifications; (**B**) The levels and types of histone marks are dynamically regulated by antagonistic histone modifying enzymes (writers and erasers). Histone marks are recognized and interpreted by specific nuclear proteins (readers), resulting in the recruitment of either repressor or activator transcription regulatory complexes onto the target promoters. Some examples of repressive and activating chromatin modifications are listed in the boxed text.



Chromatin immunoprecipitation combined with microarray technology (ChIP-on-chip), chromatin immunoprecipitation coupled with massively parallel DNA sequencing (ChIP-seq) and chromatin conformation capture assays have proved to be powerful techniques for the genome-wide mapping of histone marks, transcription factors and chromatin regulatory factors on the cellular genome and have provided a large amount data about the structural elements of the chromatin and their functional consequence for transcription. More and more studies have been emerging that also apply whole genome-analyzing tools for the interrogation of the epigenetic regulation of the KSHV genome, revealing an unexpectedly complex picture about the chromatin landscape of the viral genome in infected cells. In this review, we will mainly focus on the most recent studies that have investigated the regulation of chromatin on KSHV, including the relevant cellular and viral chromatin regulatory factors. Also, we will highlight some unanswered questions that require further investigation to better understand the fundamental chromatin-regulatory pathways involved in the control of the different stages of the KSHV lifecycle.

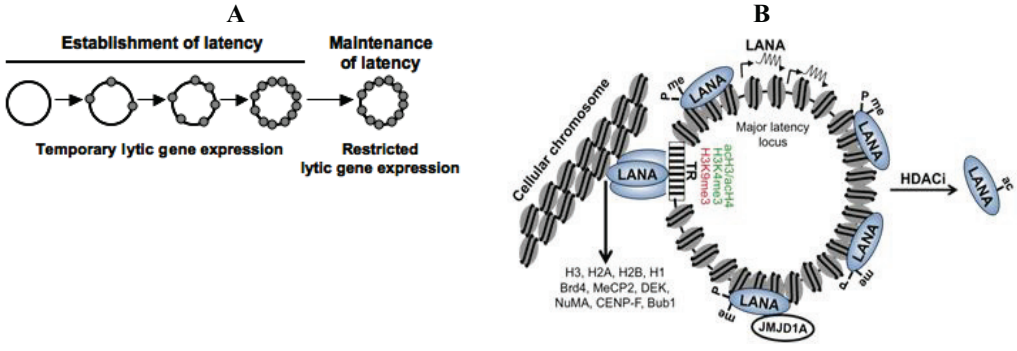
2. *De Novo* Infection and the Requirements for the Establishment of Latency

KSHV can infect a variety of cell types, including endothelial, fibroblast and epithelial cells, and establishes predominantly latent infections in cultured cells. However, without continuous drug selection, KSHV episomes get lost from most proliferating cells and only a subpopulation of cells can stably maintain the viral genome. Grundhoff and Ganem showed that the establishment of latency in these cells is not due to genetic mutations in the host or viral genome, but presumably epigenetic changes on the viral episome [21]. Such epigenetic alterations could involve the nucleosome structure, DNA methylation or histone modifications depositing onto the KSHV genome in the nucleus. Although we are getting a more and more detailed picture of the chromatin architecture of the latent KSHV genome, we still do not understand the processes by which naïve, histone-free KSHV genomes get chromatinized following *de novo* infection (Figure 2A).

Establishment of latency requires repression of lytic genes and continuous expression of latent genes after *de novo* infection. This picture became more complicated when it was shown that a limited number of lytic genes with immunomodulatory and antiapoptotic functions are temporarily expressed after infection, followed by a decline in their transcription as latent gene expression remains constant (Figure 2A) [22]. Interestingly, during this transient period of lytic gene expression, one of the lytic genes that is expressed is ORF50, which encodes an IE protein called Replication and Transcription Activator (RTA). RTA is a viral transcription factor that functions as the master switch between latency and the lytic gene expression programs and is both necessary and sufficient for the initiation of lytic replication as it can activate several lytic promoters and the replication of viral DNA (reviewed in [23]). In addition, RTA binds to several chromatin and transcription regulatory factors, including the histone acetyltransferase CBP, the chromatin remodeling factor SWI/SNF2 and the Mediator, all of which are involved in gene activation [24]. Interestingly, despite the induction of RTA during *de novo* infection, its expression does not lead to full-blown lytic replication in this setting, possibly due to the lack of sustained RTA expression. While the mechanisms responsible for the down-regulation of RTA

are unknown, they likely involve the rapid inhibition of the RTA promoter as well as the lack of the activation of specific signaling pathways. In agreement with this, infection of telomerase-immortalized retinal pigment epithelial cells with a recombinant KSHV clone that expresses RTA from the constitutively active cellular phosphoglycerate kinase promoter results in constitutive lytic replication [25]. Thus, it appears that the RTA promoter is inherently prone to become transcriptionally inactive following *de novo* infection, a process that likely involves certain RTA promoter elements that orchestrate the recruitment of transcription repressor complexes and heterochromatin-associated epigenetic marks such as DNA methylation and repressive histone modifications. Indeed, several repressive chromatin-associated factors have been identified on the RTA promoter during latency (see details below) but the molecular events involved in their initial recruitment upon *de novo* infection are still not known.

Figure 2. Chromatinization of the KSHV episome and the role of the latent KSHV protein, LANA. (**A**) The KSHV genome is linear and histone-free in the viral capsid and becomes a closed circular episome following *de novo* infection. Subsequently, the viral DNA is organized into a nucleosome structure and it persists in the nucleus as a non-integrated minichromosome; (**B**) LANA is a constitutively expressed gene that is encoded within the major latency-associated locus of the KSHV genome. LANA binds to terminal repeat (TR) region of the viral genome and tethers the viral genome to the host chromosome by interacting with histones or other components of the cellular chromatin such as Brd4, MeCP2…*etc*. LANA also binds to several sites within the viral genome and it is involved in the recruitment of the H3K9me1/2 histone demethylase JMJD1A and repression of lytic genes. The posttranslational modifications of LANA play a critical role in the association of LANA with the KSHV genome and also regulate its activity in transcription regulation. During latency, LANA in maintained in an arginine methylated state resulting in its binding to the KSHV genome. Upon HDAC inhibitor (HDACi) treatment, LANA gets acetylated, which leads to its dissociation from the KSHV genome and the concomitant induction of lytic genes.



Since latent genes are rapidly induced after *de novo* infection and their expression persists during latency, it is plausible that latent proteins are involved in the repression of lytic genes. One of the latent proteins is the latency-associated nuclear antigen or LANA, which is a nuclear protein that binds to several sites on the KSHV genome, most notably the TR region (Figure 2B) [26–28]. It was shown that IE gene expression significantly increased in 293T cells following transfection of a LANA-deletion mutant of KSHV, suggesting that LANA plays a role in the downregulation of lytic genes during the establishment of latency [29]. Another group reported that HEK293 cells carrying a LANA-deletion KSHV mutant show increased expression of lytic genes, demonstrating that LANA can contribute to latency by repressing the expression of lytic genes [30]. The transcription repression activity of LANA can be attributed to its cellular binding partners, several of which are transcription repressors, including the heterochromatin protein HP1α, methyl-CpG-binding protein MeCP2, the H3K9me3 histone methyltransferase SUV39H1, histone deacetylase co-repressor mSin3 complex and DNA methyltransferases (DNMTs) [31–36]. LANA also interacts with and inhibits the enzymatic activity of the histone acetyltransferase CBP, resulting in repression of CBP target genes [37]. The functional consequences of the interaction of LANA with these chromatin regulatory proteins have been studied mainly in transcription reporter assays and not in the context of KSHV-infected cells. Therefore, it is currently unclear which of these protein-protein interactions has a role in the regulation of the viral chromatin and in repression of lytic KSHV genes.

Other important functions of LANA in the promotion of latency are the recruitment of cellular DNA replication factors onto TR and the tethering of the viral genome to the host chromosome during mitosis [14,38–40]. These LANA functions ensure that the replication of the KSHV genome occurs concurrently with that of the host genome and that viral episomes are disseminated to both daughter cells following mitosis. In accordance with this, a KSHV mutant lacking LANA was rapidly lost from cells after multiple cell divisions [41]. LANA interacts with histones H2A, H2B, H1 and cellular chromatin-associated factors such as Brd2, Brd4, MeCP2, DEK, nuclear mitotic apparatus protein (NuMA), centromeric protein F (CENP-F) and the kinetochore protein Bub1, all of which may be involved in the binding of LANA to the host chromosome [39,42–47]. Interestingly, LANA can associate with heterochromatin through binding to MeCP2 and this interaction might be involved in the downregulation of lytic genes during *de novo* infection, perhaps through tethering of the viral episome to a transcriptionally silenced host chromosomal regions [31,47]. Despite the ample number of cellular chromatin factors that bind to LANA, knowledge of the functional consequence of these interactions is limited, particularly in the setting of the LANA-mediated repression of lytic gene expression that occurs following *de novo* infection.

Posttranslational modifications of LANA also influence its function in the repression of lytic genes during latency by modulating its binding to the viral episome (Figure 2B). One group showed that arginine methylation in the histone-binding domain of LANA by the Protein arginine methyltransferase 1 (PRMT1) is associated with strong binding of LANA to the KSHV genome and repression of lytic genes [27]. Another group reported that treatment of KSHV-infected cells with histone deacetylase inhibitors causes the acetylation of LANA, which in turn results in the dissociation

of LANA from the RTA promoter and the upregulation of RTA transcription [29]. Phosphorylation of LANA by Pim-1 and Pim-3 kinases can also counteract the transcription repressor activity of LANA on lytic genes [48]. Similarly, the phosphorylation of the histone-binding domain of LANA by different kinases can modulate association of LANA with the cellular chromatin [49]. These studies clearly show that the posttranslational modifications of the latent protein, LANA, play a critical role in the LANA-mediated inhibition of lytic genes during viral latency and presumably in the establishment of latency following *de novo* infection as well.

3. Regulation of the Chromatin Structure of KSHV during Latency and Lytic Reactivation

During latency the KSHV genome exists as a circular episome in the nucleus and has a nucleosome structure similar to the bulk cellular chromatin (Figure 2A) [12,15]. Since chromatin restricts the accessibility of transcription factors to promoters, modification of the chromatin architecture has a pivotal role in the control of gene expression. Histone modifying enzymes and ATP-dependent chromatin remodeling complexes each play a role in chromatin remodeling through the covalent modification of histones and the repositioning of nucleosomes, respectively. There is evidence that both classes of chromatin regulatory proteins are involved in the regulation of KSHV gene expression. However, only a few studies have investigated the effect of these chromatin regulatory factors on the entire KSHV genome. Most of what we know about the chromatin structure of KSHV is based on studies performed with PEL cells that carry KSHV in latency and can be readily reactivated upon stress stimuli. Here, we summarize the most recent results regarding the role of histone modifications, histone modifying enzymes, DNA methylation, miRNAs, non-coding RNAs and the nuclear organization of the KSHV epigenome in the regulation of latent and lyitc viral gene expression programs.

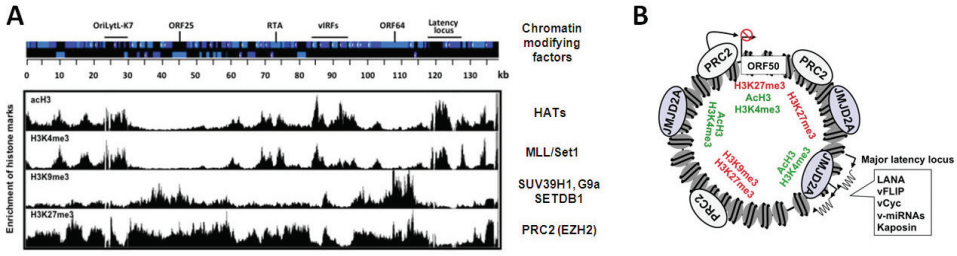
3.1. Activating and Repressive Histone Modifications on the KSHV Genome

Since lytic genes are repressed during latency it was presumed that they are associated with heterochromatin that is poised for rapid and ordered transition to a transcriptionally permissive state. The histone modifying enzyme complexes involved in the regulation of specific genes can be determined by the identification of their corresponding histone modifications associated with the genes. Two studies have used chromatin immunoprecipitation assays in conjunction with high-resolution KSHV-specific tiling microarrays (ChIP-on-chip) to shed light on the nature of the chromatin that is associated with the KSHV episomes in infected cells [50,51]. Specifically, these techniques were used to analyze chromatin from a KSHV-infected B cell lymphoma cell line called BCBL1 and a latently infected adherent cell line called SLKp, providing the first genome-wide views of the KSHV epigenetic landscape, including the occupancy of total histone H3 and several of its modified forms: the activating marks, H3K4me3 and acetylated H3K9/K14 (acH3) as well as the repressive H3K9me3 and H3K27me3 histone modifications. While H3 turned out to be uniformly distributed, the histone modifications show distinct patterns along the KSHV genome (Figure 3A). Specifically, in certain regions of the KSHV genome, the genomic localization of H3K4me3 overlaps with acH3 and the

majority of H3K27me3 co-localizes with H3K9me3 on the viral episome. In contrast, other regions of the genome showed mutually exclusive enrichment of activating and repressive histone marks. Latent genes have H3K4me3/acH3-rich chromatin during latency and reactivation, a finding that is consistent with the presence of transcriptionally active RNA polymerase II (RNAPII) on the latent promoters and their constitutively active transcription (Figure 3A) [50,52]. Interestingly, the chromatin of IE and the majority of E genes also displays high level of H3K4me3 and acH3 during latency, despite the absence of IE and E gene expression. During latency, the promoter region of the IE genes, RTA and ORF48, shows a characteristic bivalent chromatin, defined by the concomitant presence of H3K4me3 and H3K27me3 [50,51]. Although bivalent chromatin is not permissive for gene expression, it is associated with genes that are in a poised state of transcription activation, as is the case for IE and E genes. In contrast to the IE/E gene-rich genomic regions of KSHV, the parts of the viral genome that encode many of the late genes are mainly associated with the repressive H3K9me3 and H3K27me3 and these chromatin marks are presumably responsible for the inhibition of late gene expression during latency and also during the early phase of reactivation. High expression of late genes only occurs upon viral DNA replication and is accompanied by the dissociation of heterochromatin from the KSHV genome [50]. These observations imply that the activation of late gene expression may be triggered by the de-chromatinization of the KSHV genome upon viral DNA replication. In addition, recent studies with MHV68 showed that deletion of certain viral genes can abrogate the induction of late genes, without affecting viral DNA replication, suggesting that viral proteins with transcription regulatory activity are also involved in the regulation of late genes [53–55].

Strikingly, while H3K9me3 is restricted to two loci that encode mainly late genes, H3K27me3 is widespread across the entire KSHV genome, with the exception of the latency locus (Figure 3A) [50,51]. In addition, lytic reactivation leads to the decline of H3K27me3 on lytic genes, while the level of H3K9me3 remains constant [50]. In keeping with this, the H3K27me3 histone methyltransferase of Polycomb repressive complex 2 (PRC2), called EZH2, binds to the KSHV genome and colocalizes with H3K27me3 during latency. Upon reactivation, EZH2 dissociation from the KSHV genome is initiated and correlates with the concomitant decline of H3K27me3, increase of H3K4me3/acH3, and induction of IE and E gene expression [50]. A number of additional experiments were used to examine how viral gene expression is affected by the presence of EZH2 and its corresponding histone mark, H3K27me3 on the KSHV episome. The inhibition of EZH2 expression with either shRNA knockdown or the chemical compound, DZNep triggered the induction of lytic genes in latently infected PEL cells [50]. Conversely, the transient expression of enzymatically active H3K27me3 demethylases, such as UTX or JMJD3, could induce lytic genes in latently infected cells, while the H3K9me3 demethylase JMJD2A failed to do so [50,51]. These data suggest that the PRC2 complex maintains a H3K27me3-enriched heterochromatin on lytic genes to repress their expression during latency (Figure 3B). Moreover, viral reactivation triggers the dissociation of PRC2 from the KSHV genome and the concomitant deposition of activating histone modifications and RNAPII on viral promoters, resulting in the induction of the IE and E genes [50–52].

Figure 3. The chromatin landscape of KSHV during latency. (**A**) Schematic of the KSHV genome and the genome-wide distribution of different histone marks along the viral genome. There are distinct chromatin domains on the viral episome, which are characterized by different histone modification patterns indicating the targeted recruitment of specific cellular chromatin modifying enzyme complexes to different sites of the viral genome. The cellular chromatin modifying factors associated with each histone mark are listed to the right. The position of the latency locus and examples of an IE gene (RTA), some early genes (viral interferon regulatory factors or vIRFs and the OriLytL-K7 locus) and L genes (ORF25 and ORF64) are indicated at the top; (**B**) The PRC2 complex co-localizes with H3K27me3-rich chromatin domains, while the binding of the H3K9me3 histone demethylase JMJD2A overlaps with the H3K4me3/acH3-enriched chromatin regions that have a low level of H3K9me3. PRC2 is involved in the inhibition of ORF50 (RTA) expression during latency.



3.2. The Polycomb Connections

Polycomb group (PcG) proteins are cellular transcription repressors, which form two enzymatically distinct complexes called PRC2 and PRC1, both of which have been shown to inhibit transcription [56]. A number of different transcriptional silencing mechanisms contribute to PcG-mediated inhibition of transcription, including induction of chromatin condensation, inhibition of transcription initiation, blocking transcription elongation and recruitment of H3K4me3 histone demethylases to the target promoters [57–60]. PcG proteins are required for the inhibition of number of genes involved in development, cell proliferation and differentiation [56]. Importantly, there are several examples of human cancers in which increased expression of PcG proteins, specific point mutations or translocations of PcG gene loci have been observed [61]. Increased expression of specific PcG proteins have been shown to contribute transformation, indicating that PcG proteins can have oncogenic properties [62]. The PRC2 complex includes several subunits: EED, SUZ12, RbAp46/48, as well as EZH2, which catalyzes the di- and trimethylation of H3K27. In Drosophila, PRC2 has been shown to bind to target promoters through polycomb responsive DNA elements (PREs) [56]. In mammals, on the other hand, the recruitment of PRC2 to its target sites appears to be mediated mainly by various non-coding RNAs and distinct transcription factors [56]. The association of PRC2 with the chromatin is often extended beyond the target promoters in mammalian cells and overlaps with the distribution of

H3K27me3 along the target genes. This was explained by the finding that PRC2 could be recruited to H3K27me3 via EED, a PRC2 subunit that can recognize and bind to this histone mark [63,64]. Similarly, the localization of the PRC2 components, EZH2 and SUZ12, on the KSHV episome is not restricted to specific sites but spread across the entire genome [50]. While we have a clear picture of PRC2 occupancy during the latent and early lytic phases of the KSHV lifecycle, the events that enable the initial recruitment of PRC2 to the KSHV genome following *de novo* infection and its maintenance on the latent genome are still not understood.

It has been shown that most polycomb target genes are repressed by both PRC2 and PRC1 complexes. Recently, a comprehensive proteomic and genomic analysis revealed that there are six major PRC1 complexes comprising a distinct set of proteins [65]. However, all six of these PRC1 complexes harbor a common subunit called RING1A/B, which are E3 mono-ubiquitin ligases that generate H2AK119ub. In most cases the binding of PRC1 to target genes depends on the activity of PRC2 and the presence of its corresponding histone mark, H3K27me3, a histone modification that is recognized by PRC1 and used for its recruitment. Nevertheless, it should be noted that PRC1 can also be recruited to number of target sites that lack H3K27me3 [66]. Indeed, the subunit composition of the individual PRC1 complexes affects their genomic localizations, indicating that different recruitment mechanisms exist for each PRC1 complex [65]. Specific components of both PRC2 and PRC1 have been found to interact with lytic promoters of Herpes simplex virus type 1 (HSV-1) during latency, suggesting that not only PRC2, but also PRC1 might be involved in the control the heterochromatin of KSHV [67,68]. However, whether PRC1 also binds to the KSHV genome and plays a role in the repression of lytic genes has not yet been addressed.

Another interesting aspect of KSHV epigenetic regulation is the process by which PcG protein-mediated repression of lytic gene expression is reversed during reactivation. Importantly, the lysine 27 residue on histone H3 (H3K27) can either be acetylated (H3K27ac) or mono-, di-, or trimethylated. H3K27ac is associated with the activation of genes and is catalyzed by the histone acetyltransferases CBP/p300 in mammals [69]. The induction of lytic genes upon the overexpression of H3K27me3 demethylases UTX and JMJD3 in latently infected cells suggests that the modulation of posttranslational modifications of H3K27 must be critical for the regulation of lytic genes [50,51]. RTA, which binds to and activates number of lytic promoters during reactivation, has been shown to interact with CBP [24]. Furthermore, several RTA responsive promoters can be found in the viral genomic regions where H3K27me3 declines during reactivation, suggesting that RTA may recruit CBP to these sites, resulting in a transition from H3K27me3 to H3K27ac. In addition, the mixed-lineage leukemia (MLL) protein-containing MLL/Set1 complexes mediate H3K4me3 and, like RTA, increase at the same sites where H3K27me3 declines on the KSHV genome during lytic reactivation [50]. Moreover, MLL/Set1 complexes have been shown to interact with CBP as well as the H3K27me3 demethylase, UTX [70–72]. Therefore, it is possible that the H3K27me3 decline on IE and E genes during reactivation is the consequence of the recruitment of protein complexes to the viral promoters that can catalyze both demethylation and acetylation of H3K27 to induce lytic gene expression. In fact, a recent study showed that, a highly abundant KSHV non-coding RNA called polyadenylated nuclear

(PAN) RNA can interact with the RTA promoter and recruit the H3K27me3 demethylases, JMJD3 and UTX, as well as the H3K4me3 histone methyltransferase, MLL2 [73]. Since PAN RNA expression is dependent on IE genes, especially RTA, these data suggest that PAN RNA-mediated recruitment of chromatin factors to the RTA promoter may be part of a positive feedback regulatory mechanism that perpetuates RTA expression during later stages of reactivation. However, given that the expression of RTA is induced prior to that of PAN RNA (RTA is an IE gene and PAN RNA is an E gene), PAN RNA is not likely to be involved in the initial de-repression of PRC2-mediated inhibition of the RTA promoter during physiologically relevant reactivation conditions, *i.e.*, in the absence of exogenous PAN RNA expression. Thus, the means by which repressive histone marks are initially removed from the RTA promoter is still not understood.

The treatment of latently infected cells with histone deacetylase (HDAC) inhibitors can also trigger the induction of RTA and leads to the dissociation of EZH2 and the decline of H3K27me3 on the RTA promoter [74]. Several HDACs have been implicated in the repression of PcG target genes and may play a role in deacetylation of H3K27ac, allowing its trimethylation [75,76]. In fact, EED was found to interact with HDAC1, 2 and 3 in a yeast two-hybrid screen, suggesting a possible connection between PRC2 and HDACs [76]. Importantly, the HDAC inhibitor, trichostatin A (TSA), can induce the activation of PcG-silenced genes, including the expression of KSHV lytic genes [12,76]. Thus, HDACs can also be involved in the maintenance of PRC2-mediated lytic gene repression by promoting H3K27 deacetylation, thereby allowing PRC2 to catalyze the trimethylation of H3K27. Further experiments are required to see whether there are any specific HDACs that have a role in the regulation of PRC2-maintained KSHV latency.

3.3. Regulation of the Heterochromatin Mark H3K9me3 on the KSHV Genome

H3K9me3 occupies two specific regions of the KSHV genome, both of which encode primarily late genes (Figure 3A) [50,51,77]. Although H3K9me3 does not seem to be a major repressive histone mark for lytic genes, several studies found that H3K9me3 and its associated chromatin regulatory factors have important regulatory roles during the KSHV life cycle. Several histone methyltransferases (HMTs) can catalyze the methylation of H3K9, including G9a, SUV39H1 and SETDB1 [78]. SUV39H1 and the H3K9me3-binding protein HP1 have been shown to interact with LANA, resulting in their recruitment to the TR and some lytic promoters of KSHV, where they are involved in heterochromatinization during latency [33]. Since LANA ChIP-seq and ChIP-on-chip experiments revealed a number of LANA-binding sites on the KSHV genome, it would be important to know whether SUV39H1 can also be recruited to these LANA-binding sites and thereby facilitate H3K9me3 [26,27]. In addition, other H3K9me3 HMTs can also be recruited to the KSHV episome by cellular factors. Indeed, Hsing-Jien Kung's group has found that the cellular transcription repressor, KAP-1, is a novel regulator for KSHV latency, which is known to interact with SETDB1, a H3K9me3 HMT [79]. KAP-1 was found to be associated with a significant number of lytic promoters during latency, which becomes dissociated upon reactivation. Interestingly, KAP-1 is a substrate of the viral kinase encoded by ORF36 and the binding of KAP-1 to the KSHV genome is modulated by

ORF36-dependent phosphorylation [79]. The phosphorylation of KAP-1 causes the decline of its sumoylation, which decreases the ability of KAP-1 to bind to chromatin and repress genes. Importantly, the knockdown of KAP-1 by shRNA resulted in a 5-fold increase of RTA-mediated reactivation. Thus, KAP-1 could be one of the cellular transcription factors responsible for the recruitment of a H3K9me3 methyltransferase onto the KSHV genome, which is also involved in the inhibition of lytic genes during latency [79].

Two elegant studies have recently reported the genome-wide binding and functions of the H3K9 histone demethylases, JMJD2A and KDM3A/JMJD1A, on the latent KSHV genome, shedding light on the reasons behind the restricted localization pattern of H3K9me3 on the KSHV genome (Figures 2B and 3B) [77,80]. One of the studies showed that shRNA knockdown of JMJD2A resulted in attenuated lytic reactivation, while overexpression of an enzymatically active form of JMJD2A facilitated the induction of lytic genes [77]. Strikingly, the binding sites of JMJD2A on the viral episome inversely correlate with the presence of H3K9me3 and overlap with the H3K4me3/acH3-enriched genomic regions [77]. These results indicate that binding of JMJD2A to the IE and E genes during latency may be involved in preventing H3K9me3-marked heterochromatin formation on their promoters, thereby facilitating H3K9 acetylation and priming of the IE and E genes for robust induction upon reactivation. Based on the survey of several viral proteins, an IE/E protein called K-bZIP (K8) was found to interact with JMJD2A [77]. *In vitro* demethylase assays revealed that K-bZIP can inhibit the demethylase activity of JMJD2A. Moreover, it was shown that K-bZIP can recognize the H3K9me3 moiety and thereby interfere with the binding of JMJD2A with H3K9me3. Furthermore, K-bZIP could also block the demethylation of H3K9me3 caused by overexpressed JMJD2A in 293T cells. Despite of the robust expression of K-bZIP H3K9me3 level does not change significantly on the KSHV chromatin during reactivation. Thus, the effect of K-bZIP on H3K9me3 must be limited to a subset of K-bZIP target genes in infected cells. In fact, K-bZIP expression in KSHV-negative cells causes a global repression of host genes and increased H3K9me3-marked heterochromatin formation on cellular genes, including host immune-related genes [77,81].

Izumiya's group has recently reported that LANA forms a complex with KDM3A/JMJD1A, a cellular H3K9me1/2 histone demethylase, in KSHV infected cells [80]. The genome-wide occupancy of LANA and JMJD1A on the KSHV genome showed a high degree of overlap and was inversely correlated with the level of H3K9me2. Furthermore, depletion of LANA expression or overexpression of a JMJD1A-binding deficient LANA mutant in cells decreased the binding of JMJD1A to the viral episome, indicating that LANA recruits JMJD1A to the KSHV genome during latency. *In vitro* histone H3 peptide pull-down assays using purified LANA showed that LANA can interact with H3, H3K9me1 or H3K9me3 but not H3K9me2. Finally, the shRNA knockdown of JMJD1A resulted in decreased lytic reactivation, suggesting that JMJD1A is involved in the maintenance of H3K9 methylation-free chromatin on latent and a specific subset of lytic genes [80]. These studies altogether have shown that the regulation of H3K9me3 on the KSHV genome is indeed important for the proper induction of the lytic gene expression program [77,79,80].

3.4. H3K4me3 and the MLL/Set1 Family

Enrichment of H3K4me3 is typically present at the 5' end of transcriptionally induced genes. The first histone methyltransferase complex that can catalyze mono-, di- and trimethylation of H3K4 was identified in yeast and is called COMPASS (complex of proteins associated with Set1) [82,83]. The enzymatic subunit of COMPASS is Set1, which has at least 6 homologs in human, Set1A, Set1B, MLL1, MLL2, MLL3 and MLL4, all of which form different complexes and have distinct genomic localization [84]. In addition to their shared common subunits (Ash2, RbpBp5, Wdr5 and Dpy30), they differ in their enzymatic subunits and also have unique components. For instance, the tumor suppressor protein, Menin can only be found in complex with MLL1 or MLL2, while UTX, the Pax transactivation domain-interacting protein (PTIP), PTIP-associated protein 1 and nuclear receptor coactivator NCOA6 are exclusive components of the MLL3 and MLL4 complexes. In addition, Wdr82 is restricted to Set1A/Set1B complexes [84]. Whereas the Set1A/B complexes are the major H3K4 methylases responsible for the bulk H3K4me3 in mammalian cells, MLL1-4 play an important regulatory role for specific subsets of genes [85,86]. Mutations and random translocations of MLL genes are frequent occurrences in hematological malignancies like acute myeloid and lymphoid leukemia [87].

Regulation of H3K4me3 levels is important for controlling gene expression, particularly during lytic reactivation of KSHV, when lytic gene expression is rapidly induced. H3K4me3 is present on certain viral promoters during latency and is increased following reactivation (Figure 3) [50,51]. However, further studies are needed to identify the cellular factors that modulate H3K4me3 levels during the KSHV life cycle. It has been reported that while the binding of the core subunits of MLL/Set1 complexes, Ash2 and Wdr5, can increase on lytic promoters following reactivation, this was not observed on the latent LANA promoter [88]. Also, recruitment of Set1A could be detected on lytic promoters during reactivation but whether it is the sole H3K4me3 methylase responsible for the increase of H3K4me3 on lytic promoters has yet to be determined [88]. In addition, another group showed that MLL2 can interact with the RTA promoter through the viral non-coding PAN RNA, which raises the question whether distinct MLL/Set1 complexes can be targeted to different viral promoters [73]. Future studies using MLL/Set1 component-specific shRNAs as well as applying ChIP assays to test which of the unique subunits of MLL/Set1 complexes bind onto the KSHV episome will help to identify which of the MLL/Set1 complexes is responsible for the regulation of H3K4me3 on the KSHV genome during latency and following lytic reactivation.

There are a number of examples known of crosstalk between different histone modifications. For instance, H2B monoubiquitination at lysine 120 by the RAD6/BRE1 ubiquitin ligase complex is required for the methylation of H3K4 in human cells [89]. Furthermore, it was shown that the RNA polymerase II-associated factor (PAF) complex mediates the recruitment of RAD/BRE1 to the transcription machinery, where the monoubiquitination of H2B promotes Set1-mediated methylation of H3K4 in human cells [89]. Thus, it would be interesting to determine whether differential regulation of H2B ubiquitination is involved in the regulation of H3K4me3 levels on the KSHV episome.

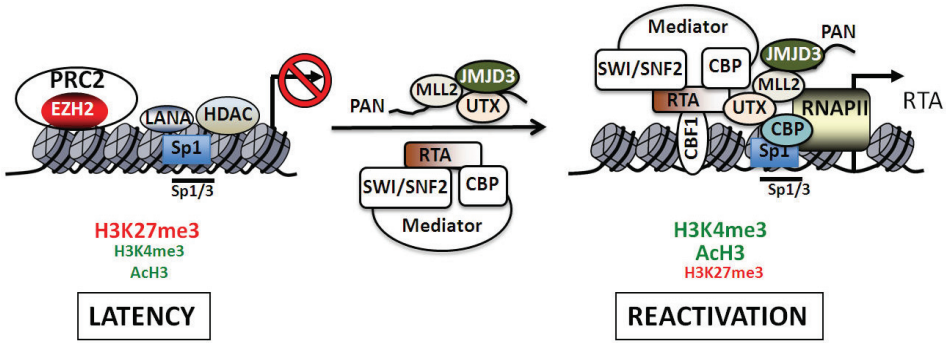
3.5. Histone Acetylation and Deacetylation on the KSHV Genome

Acetylation and deacetylation of lysine residues of histone tails have antagonistic effect on gene transcription. While acetylation of histones by histone acetyltransferases (HATs) unravels chromatin and activates transcription, histone deacetylases (HDACs) induce chromatin condensation and gene silencing [90,91]. Shortly after the discovery of KSHV, it was found that lytic genes of KSHV can be induced from latency by treating latently infected cells with various HDAC inhibitors, highlighting the importance of histone acetylation in the regulation of lytic gene expression. Histone acetylation can occur at more than 20 different lysine residues positioned within histones. To date, 19 different HATs are known and each one is part of distinct transcription activator complexes. The majority of HATs can acetylate histones at multiple positions and likewise, the same lysine residue can be acetylated by various HATs. This redundancy makes it difficult to pinpoint the HAT(s) involved in a given histone acetylation on the KSHV chromatin (Figure 3A). Two ChIP-on-chip studies revealed that H3K9/K14ac are enriched on several lytic promoters during latency and are further increased upon lytic reactivation [50,51]. Using luciferase reporter assays, several cellular transcription factors that interact with HATs or HDACs (e.g., C/EBPα, AP1, Oct-1, STAT3 and RBP-Jκ) have been implicated in the regulation of specific lytic promoters such as RTA [23,92,93]. Nevertheless, comprehensive studies of histone acetylation on the KSHV epigenome during latency and reactivation are needed in order to provide more detailed insight into the role of histone acetylation and ultimately pave the way toward the identification of the HATs involved in viral gene regulation during the KSHV life cycle.

The interplay between HATs and HDACs in the regulation of RTA transcription was examined in detail. Lieberman's group analyzed the chromatin structure of the core promoter of RTA in the presence or absence of the HDAC inhibitors (HDACi) such as sodium butyrate (NaB) and trichostatin A (TSA) [12]. They found that the RTA promoter is highly inducible by these HDACs and a NaB responsive element was mapped to a short GC-box DNA sequence that binds Sp1 and Sp3. Micrococcal nuclease (MNase) mapping and restriction endonuclease accessibility assays revealed that there is a stably positioned nucleosome at the transcriptional initiation site of RTA during latency, which rapidly changes upon NaB treatment [12]. Furthermore, it was also observed that HDAC1, 5 and 7 bound to the RTA promoter, resulting in hypoacetylation of the viral chromatin during latency, while reactivation resulted in the hyperacetylation of histones H3 and H4 concomitantly with the recruitment of components of the SWI/SNF2 ATP-dependent chromatin-remodeling complex onto the RTA promoter (Figure 4). Ectopic expression of the CREB-binding HAT, CBP, resulted in 10-fold induction of the RTA promoter as determined by luciferase reporter assay, whereas expression of other HATs such as p300, PCAF, GCN5 and TIP60 had no such effect. The CBP-mediated promoter stimulation depends on the Sp1/3-binding site, suggesting that CBP can be recruited to the RTA promoter via interaction with Sp1/3 during reactivation, resulting in the acetylation of both H3 and H4 [12]. In agreement with this, others also showed that NaB treatment could induce an Sp1-binding site-dependent recruitment of CBP and p300 onto the RTA promoter. Moreover, the increased Sp1-binding on the viral promoter is transient during reactivation [94]. The role of CBP and SWI/SNF2

in the regulation of the RTA promoter was further detailed by Gwack and his co-workers [24]. They showed that the C-terminal activation domain of RTA forms a complex with the Mediator, SWI/SNF2 and CBP in cells. During reactivation RTA recruits these cellular factors onto the RTA promoter and other RTA-responsive lytic promoters resulting in their transcriptional activation [24]. These studies altogether show that reactivation of lytic genes requires the recruitment of HATs by cellular and viral transcription factors to the viral promoters, resulting in the remodeling of the chromatin structure of the virus (Figure 4).

Figure 4. Regulation of the RTA promoter during latency and reactivation. The H3K27me3 histone methyltransferase complex PRC2 as well as specific histone deacetylases (HDAC1, 5 and 7) are critical transcriptional repressors that are present on the RTA promoter during latency. The bivalent chromatin of the RTA promoter is indicated by the presence of both activating (H3K4me3 and acH3) and repressive (H3K27me3) histone marks. After reactivation RTA binds to its own promoter via CBF-1 (also called RBP-Jκ) and recruits several cellular transcription factors such as the Mediator, the ATP-dependent chromatin remodeling complex SWI/SNF2 and the histone acetyltransferase CBP. The induction of transcription is accompanied by the rapid recruitment of RNA polymerase II (RNAPII) and increased binding of Sp1 to the promoter. The KSHV non-coding PAN RNA can also interact with the RTA promoter and recruit the H3K4me3 histone methyltransferase MLL2 as well as the H3K27me3 histone demthylases UTX and JMJD3. The Sp1/3-binding sites play an important role in the HDAC inhibitor-mediated reactivation of the RTA promoter. While these sites contribute to the LANA-mediated repression of the RTA promoter during latency, they are also involved in recruitment of CBP, which can catalyze the hyperacetylation of histones on the viral promoter during reactivation.



Histone acetylation and nucleosome positioning also play a critical role in the regulation of the chromatin structure of the origin of latent replication (Ori-P), which is embedded in the GC-rich TR region of the KSHV genome. Ori-P contains two LANA-binding sites, where LANA binds and recruits the cellular DNA replication proteins, ORC2 and MCM, to facilitate the replication of the viral genome concomitantly with the replication of the host genome during mitosis [38,40]. It was shown

that Ori-P is enriched with hyperacetylated histones H3 and H4 and that this enrichment is due to the activity of LANA, which recruits HATs such as HBO1 or CBP, to the Ori-P (Figure 2B) [40]. In addition, the bromodomain-containing cellular protein, Brd2, which binds to acetylated histones and is a known LANA-binding protein, is also recruited to the Ori-P, and may also be involved in the maintenance of the hyperacetylation of histones in Ori-P. Nucleosome mapping assays revealed a highly ordered nucleosome array in Ori-P that becomes disorganized in a cell cycle-dependent manner [40]. The current model is that the maintenance of a hyperacetylated chromatin in Ori-P facilitates the assembly of the DNA replication complex.

3.6. DNA Methylation of the KSHV Genome

In mammalian cells, DNA methylation occurs mainly on cytidine residues in the context of CpG dinucleotides. Methylated CpGs are often found in clusters called CpG islands [95]. Hypermethylated CpG islands in the 5' regulatory regions of genes are associated with gene silencing. Methylation of DNA inhibits gene expression either by impeding the binding of transcription factors to the promoter or by the action of methyl-CpG-binding proteins (e.g., MeCP2), which bind to methylated CpG islands and recruit HDACs and other transcription repressors to the gene to turn off its expression [96]. The maintenance of DNA methylation during cell division is mediated by DNA methyltransferase 1 (DNMT1), which copies the DNA methylation pattern to the daughter strands during DNA replication, while DNMT3a and DNMT3b are *de novo* DNA methyltransferases [97].

It has been shown that the treatment of PEL cells with the DNA methyltransferase inhibitor 5-AzaC can induce lytic reactivation of KSHV, suggesting that DNA methylation of lytic promoters is involved in the suppression of lytic genes during latency [11]. Recently, the global DNA methylation pattern of KSHV was determined in different latently infected cell lines by using immunoprecipitation of methylated DNA (MeDIP) in conjunction with KSHV specific high-resolution tiling microarray [51]. These experiments showed that KSHV is subject to extensive DNA methylation during latency. Like the H3K27me3 pattern on the latent KSHV genome, DNA methylation was excluded from the transcriptionally active latency-associated locus, while most of the lytic genes were associated with DNA hypermethylation. Surprisingly, the RTA promoter was not methylated in most of the cell lines, suggesting that DNA methylation is unlikely to be involved in the inhibition of RTA expression during latency [51]. In addition, while the latency-specific histone modification patterns were rapidly deposited on the viral episome following *de novo* infection, DNA methylation patterns were established comparatively slower, indicating that DNA methylation does not play a role in establishment of latency. On the other hand, DNA methylation may reinforce the inhibition of lytic genes at late timepoints of infection [51].

3.7. Nuclear Organization of the KSHV Genome

It is known that the KSHV episome is tethered to the cellular chromosome by LANA, which interacts with histones and several components of the cellular chromatin. One question that remains is

whether tethering of the KSHV genome is random or prone to localize on specific regions of the host chromosome. Infected cells harbor approximately 30–80 copies of the KSHV genome and ChIP-seq analysis has revealed at least 256 LANA-binding sites on the cellular genome, suggesting that not all LANA proteins may be involved in tethering the viral genome [26]. An interesting hypothesis is that the viral genome could be tethered to heterochromatin-enriched nuclear regions during latency and may relocate to a transcriptionally favorable nuclear compartment upon reactivation. Microscope analysis of the nuclear distribution of LANA in latently infected B cells showed that LANA preferentially associates with the border of heterochromatin, inviting speculation that close proximity to heterochromatin-rich regions of the host cell may be critical for the formation and maintenance of heterochromatin on the KSHV genome [98].

The structural organization of chromatin domains in the cellular genome plays an important role in the regulation cellular gene expression. Similarly, the chromatinized KSHV episome is also compartmentalized into different chromatin domains, which can interact with each other by a looping mechanism mediated by cellular factors that bind to the KSHV genome [99]. The maintenance of viral latency requires the separation of the latent genes from the lytic genes-encoding part of the KSHV genome so that the latent genes can be continuously expressed, while the lytic genes are repressed during latency. Lieberman's group found that CTCF and the cohesion complex, which are known chromatin boundary factors separating active and inactive chromatin domains, bound to several sites of the KSHV genome and they are involved in the transcription regulation of both latent and IE genes during latency [100,101]. One of the CTCF-binding sites (CBS) can be found in the intron of the latent gene, LANA. Deletion of CBS disrupted cohesin binding to the KSHV genome and caused viral episome instability and increased expression of lytic genes [100]. In addition, chromatin conformation capture (3C) assays provided evidence for the first time that the latent LANA promoter physically interacts with the lytic RTA promoter during latency, which is mediated by the CTCF/cohesin complex in the intron of LANA [99]. In agreement with this, CBS mutation, siRNA depletion of CTCF or the cohesin complex component RAD21 diminished the interaction between these latent and lytic promoters and deregulated the latent gene expression program. This viral chromosome looping was also disrupted during lytic reactivation suggesting that the CTCF/cohesin-mediated looping in the viral genome is dynamic and involved in the regulation of latent and lytic gene expression [99]. The CTCF/cohesin complex in the intron of LANA also plays a critical role in the regulation of transcription elongation and nucleosome organization in the latency locus [102,103].

Every KSHV infected cell carries multiple copies of KSHV genomes, which raises the question whether they all have the same nucleosome structure during latency, which change simultaneously during reactivation or they show diversity. To answer this question a recent study used single-molecule footprinting assays called MAPit (Methyltransferase Accessibility Protocol for individual templates), which allows the detection of multiple chromatin states at selected loci within a cell population [104]. The chromatin architecture of the promoter of the constitutively expressed latent gene, LANA, the promoter of the IE gene, RTA and the promoter of the early gene, K2 was investigated. Their analysis showed diverse chromatin at each of these promoters, which ranged from closed to open

conformations. Interestingly, the induction of lytic gene expression program resulted in the remodeling of the viral chromatin only on a fraction of the viral episomes. These results indicate that KSHV genomes possess diverse chromatin conformations in infected cells during both latency and reactivation. Furthermore, local chromatin condensations caused by epigenetic drift can restrict the expression of any viral genes irrespectively of what gene expression classes (IE, E, L or latent) they belong to [104].

4. Reprogramming of the Host Transcriptome during KSHV Infection

In vitro experiments have shown that KSHV infection of endothelial cells results in transcriptional reprogramming such that infected lymphatic endothelial cells are driven toward a blood vessel endothelial cell phenotype and vice versa [105,106]. In addition, KSHV infection can also induce the transcriptional reprogramming of lymphatic endothelial cells to mesenchymal cells [107]. These observations imply that viral factors may be involved by either deregulating the expression of cell type-specific transcription factors or modulating their functions, which results in altered cellular gene expression profile. Both LANA and viral miRNAs, for example, have been implicated to play a role in changing the expression of specific cellular genes encoding master transcription factors [106,108]. These viral factors can use different ways to regulate cellular genes. For example, LANA recruits DNA methyltransferases to specific cellular promoters such as that of H-cadherin and TGF-β type II receptor, resulting in hypermethylation and transcriptional repression of these promoters [32,109]. Because LANA has been shown to interact with a wide variety of chromatin-associated regulatory proteins, it can affect large number of cellular genes. Recent ChIP-seq analysis revealed 256 LANA-binding sites on the cellular genome, several of which are linked to p53-, TNF- or IFN-γ regulated genes [26].

KSHV encodes 12 miRNA genes that produce 25 mature miRNAs. These are small non-coding gene regulatory RNAs that can bind to mRNAs resulting in translational block or their degradation to repress genes [110]. A recent study showed that the KSHV miRNA miR-K12-11 shares significant sequence homology with the cellular miRNA called miR-155, which is often overexpressed in human tumors [111]. Strikingly, when miR-K12-11 was expressed in bone marrow cells, it caused the downregulation of Jarid2, a component of the PRC2 complex [111]. Thus, this viral miRNA can potentially deregulate the expression of PRC2 target genes such as that are involved in cell cycle control, for example, which can contribute to the development of KSHV-associated malignancies. Another KSHV miRNA, miR-K12-4-5p targets the retinoblastoma-like protein 2 (Rbl2), which is a repressor of the *de novo* DNA methyltransefarses DNMT3a and DNMT3b genes [112]. Expression of miR-K12-4-5p reduces Rbl2 expression resulting in the increased expression of DNMTs that can globally affect cellular gene expression in infected cells. PAN RNA of KSHV that is abundantly expressed in the nucleus during reactivation has been reported to downregulate the expression of many immunomodulatory genes [113]. PAN RNA can bind to gene promoters and recruit different histone modifying enzymes, which can be involved in the PAN RNA-mediated cellular gene regulation observed in infected cells [73,113].

Viral proteins often targets cellular histone modifying enzymes to relocate them on the cellular genome, modulate their enzymatic activity or their expression, which can affect the expression of a large number of cellular genes, all of which serve the needs of the virus for efficient infection of the host. Several KSHV proteins such as LANA, K8 and vIRF1 can bind to the histone acetyltransferase CBP/p300 and inhibit CBP/p300-mediated cellular transcription [37,114,115]. In contrast, RTA can use CBP to robustly activate gene transcription but it is still unclear how widely RTA uses CBP in activation of cellular genes [24]. K8 also interacts with the H3K9me3 histone demethylase JMJD2A and blocks its activity, which could play a role in the observed K8-mediated global cellular gene repression [77]. LANA forms a complex with the H3K9me1/2 histone demethylase JMJD1A in infected cells, which raises the question whether LANA uses JMJD1A to activate any of its cellular target genes [80].

EZH2, the H3K27me3 histone methyltransferase of the PRC2 complex has been shown to be overexpressed in KSHV-infected cells in Kaposi's sarcoma (KS), suggesting that KSHV could be involved in EZH2 upregulation [116]. Indeed, KSHV infection of endothelial cells *in vitro* can induce the expression of EZH2, and this is mainly mediated by the KSHV latent proteins LANA and vFLIP via the upregulation of the NF-κB pathway [116]. Importantly, the increased expression of EZH2 turned out to be essential for KSHV-induced angiogenesis in KSHV-infected cells [116].

KSHV infection of B cells in human can lead to the development of the KSHV-associated primary effusion lymphoma (PEL), which is characterized by the disruption of the B-cell specific transcriptional program. Protein expression of several transcription factors that are essential for B-cell development is significantly altered in PEL cells compared to uninfected B-cells [117]. While PEL cells constitutively express IRF4, the protein level of other B-cell transcription factors such as Pax5, Oct-2, PU.1 and IRF-8 was completely abolished [117]. The viral factors involved in the reprogramming of the B-cell-specific transcription factor network have not yet been identified.

5. Outlook

ChIP-on-chip analysis of the KSHV episome revealed distinct chromatin domains on the viral genome, which are characterized by different histone modification and DNA methylation patterns indicative of targeted recruitment of specific cellular chromatin modifying enzyme complexes (Figure 3). The benefit of such compartmentalization of the KSHV genome might be the use of common transcription regulatory mechanisms for expression of genes that have related functions during the lifecycle of the virus. Strikingly, the IE and most of E genes are marked by activating histone marks while the majority of late genes, which are transcribed only following viral DNA replication, are associated with heterochromatin that remains unaltered during the early phase of reactivation [50,51]. These observations suggest that the type of chromatin structure associated with each viral gene can play a role in the regulation of their expression. Two recent papers have reported that H2AX, an isoform of the canonical histone H2A and the phosphorylation of histone H3 at serine 10 residue are also components of the viral chromatin landscape and regulate the persistence of viral episome in infected cells as well as the reactivation of the lytic gene expression program, respectively [118,119].

These studies also demonstrate that we only see the tip of the iceberg regarding to the components and the organization of the KSHV chromatin in infected cells.

Currently, at least 130 different posttranslational modification sites have been identified on histones and there are at least 150 histone-modifying enzymes known [120]. Because of the unique DNA sequence elements, the close proximity of genes and overlapping gene regulatory regions in the KSHV genome, KSHV may utilize unique mechanisms to regulate its genome. Also, the modulation of the function of cellular chromatin modifying enzymes by viral proteins allows the reprogramming of the cellular transcriptome in a way that becomes beneficial for persistent infection as well as the development of KSHV-associated malignancies. It has been demonstrated that drugs targeting of histone modifying enzymes is a viable strategy for controlling HSV-1 and Human cytomegalovirus (HCMV) infections [121–123]. Therefore, identifying the relevant cellular and viral factors involved in the chromatin control of KSHV infection may bring new opportunities for pharmacological control of KSHV infection and KSHV-associated diseases.

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Conflicts of Interest

The authors declare no conflict of interest.

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Commentary

Interpreting the Epstein-Barr Virus (EBV) Epigenome Using High-Throughput Data

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Abstract: The Epstein-Barr virus (EBV) double-stranded DNA genome is subject to extensive epigenetic regulation. Large consortiums and individual labs have generated a vast number of genome-wide data sets on human lymphoblastoid and other cell lines latently infected with EBV. Analysis of these data sets reveals important new information on the properties of the host and viral chromosome structure organization and epigenetic modifications. We discuss the mapping of these data sets and the subsequent insights into the chromatin structure and transcription factor binding patterns on latent EBV genomes. Colocalization of multiple histone modifications and transcription factors at regulatory loci are considered in the context of the biology and regulation of EBV.

Keywords: Epstein-Barr virus; gammaherpesvirus; chromatin; histone modification; CTCF; OriP

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1. Introduction

Epstein-Barr virus (EBV) is a human gammaherpesvirus that establishes long-term latent infection in B-lymphocytes [2,3]. The latent infection is associated with various B-cell malignancies, including Burkitt's lymphoma, Hodgkin's disease and lymphoproliferative diseases, following immunosuppression. EBV infection can efficiently immortalize naive resting B-cells and establish long-term quasi-homogenous lymphoblastoid cell lines (LCLs). In LCLs, the majority of viral genomes adopt a gene expression program, referred to as type III latency, which represents the most permissive form of latent infection [4]. In type III latency, the complete set of viral genes required for B-cell proliferation and survival are expressed, while the viral genes required for lytic replication and virion production are repressed. The viral genomes are maintained as multicopy circular mini-chromosomes that reside in the nuclear compartment. Viral gene expression is regulated by a combination of host and viral regulatory factors, and latent replication is limited to once per cell cycle in concert with host chromosomes [5]. While most cells maintain the viral genome in a type III latent state, a percentage of cells in the population can undergo spontaneous lytic replication, and the extent of this lytic replication depends on the LCL and culture conditions [6].

To appreciate the relevance of the EBV epigenome, it is first necessary to highlight some of the major properties of the EBV genome during latency. The type III latency-associated gene expression program in LCLs consists of nine protein coding genes, 21 microRNAs and several non-coding RNAs. The protein coding genes include the Epstein-Barr Nuclear Antigens (EBNAs) EBNA-LP, 1, 2, 3a, 3b and 3c, as well as the Latency Membrane Proteins, LMP1, LMP2a and LMP2b. Two small non-coding RNAs, EBER1 and EBER2, are generated by RNA polymerase III. The miRNAs are generated from two different host transcripts from the BHRF1 or BART regions of the genome [7]. The latent genome is circularized through the joining of the terminal repeats (TRs), which generates the template for the LMP2a and LMP2b transcripts. The viral episome is maintained through the interaction of the EBNA1 proteins with the viral origin of plasmid replication (OriP), which consists of a family of repeats (FR) and a dyad symmetry (DS) element. The FR is required for maintenance through a mechanism that involves tethering to metaphase chromosomes and the DS functions as an efficient origin of bidirectional DNA replication. EBNA1 also binds to the Q promoter (Qp), which functions as an alternative promoter for expressing the EBNA1 transcript only. OriP can also function as an EBNA1-dependent transcriptional enhancer of the C promoter (Cp), which controls the transcription of a large multicistronic transcript encoding the EBNA-LP, -2, -3a, -3b, -3c and -1 genes. LMP1 transcription can initiate from the TR or from regions near the TR, and its poly A site resides in the first intron of the LMP2 transcripts that are transcribed in the opposite orientation from the complementary DNA strand of LMP1. Lytic origins of DNA replication remain mostly inactive in LCLs, but contain promoters for non-coding RNAs and miRNAs that can be generated at high levels during latency. How these genetic elements are coordinately regulated may be partly revealed through analysis of the viral epigenome.

2. Assaying the EBV Epigenome

Epigenetically regulated loci in the EBV genome can be elucidated by high-throughput sequencing data in latently infected human cell lines. Large data sets generated by labs around the world are deposited in standardized databases, such as the NCBI sequence read archive (SRA) and gene expression omnibus (GEO). The raw data can be downloaded and reanalyzed with respect to EBV by aligning the reads to EBV and subtracting any reads that map to the human genome [1]. We have developed a simple open access browser for viewing ENCODE ChIP-seq data sets mapped to the EBV genome (http://ebv.wistar.upenn.edu). The data deposited to this site include raw alignments, coverage tracks and use original accessions as filenames to ensure reproducible analysis.

Due to the small size of the EBV genome relative to the human genome, the alignment can be performed orders of magnitude faster by common tools, such as bowtie and bwa [8,9]. Interestingly, the average number of reads mapping to the viral genome tend to be an order of magnitude more than what would be expected from a randomly selected equally sized portion of the human genome given the estimated episome copy number. This suggests that the viral chromatin may be more soluble and/or amenable to sonication and enzyme digestion chromatin fragmentation.

The EBV genome contains several loci that should be interpreted with caution when using sequencing data. Regions that are seemingly depleted may in fact be regions whose copy number was overestimated (and thus, over-normalized, e.g., terminal or W repeats) or has orthologous regions in the human genome and, therefore, is unmappable (e.g., the simple repeat elements in EBNA1 and EBNA2). Furthermore, an initial challenge in any large data study is segregating the data into what is robust, spurious or artifactual. In the case of ChIP, the traditional controls of sonicated genomic DNA ("input") and non-specific IgG ChIP provide information on two independent background noises. The EBV genome has no regions that appear enriched in the input controls, indicating that the genome is fairly uniform with no genomic regions being more easily sonicated than others. However, the FR repeats are enriched in several IgG ChIP controls, which suggests that FR lacks antibody specificity and is likely to be some form of "sticky" chromatin, possibly due to its potential role as a nuclear matrix attachment region [10]. Even though FR immunoprecipates upon non-specific IgG interrogation, it is possible that this non-specific interaction occurs *in vivo*, with many proteins genuinely binding the chromatin. However, these two scenarios cannot be disambiguated using current technologies.

Since meta-analyzing genomics experiments for EBV comes with the same caveats as analyzing data for human, it is highly recommended that all experiments be first mapped to the human genome. Quality statistics should be generated from the typically millions of mapping reads and thousands of relevant human loci instead of the typically <20 sites in the viral episome. Quality metrics include cross-strand correlation, which is an effective measure for fragment length and enrichment relative to genomic background [11] and enrichment estimates via percentage reads in peaks.

Experiment reproducibility should also be examined in both the human and EBV genomes. However, even examining biological replicates across only the EBV genome can generate an estimate

of reproducibility. For instance, NF-kB experiments give widely varying peak results. In this case, only the best replicates can be selected from the human aligned data. In some cases, even when an experiment is highly reproducible in human, a low number of reads mapping to the viral episome makes one or multiple replicates unreliable; however, this has mostly ceased being a problem with the advent of deeper sequencers.

One complication of analyzing the EBV epigenome through publically available data is that most LCLs are transformed using the B95.8 genome, which contains a large deletion in the Bam HI A region that encompasses the duplicated lytic origin (OriLyt right or DSr) and many BamHI A non-coding RNAs and miRNAs [7,12]. Even though the B95.8 strain is more commonly used, all recent studies, including our own, map to the complete EBV reference genome (NC_007605). Consequently, many of the data sets fail to map to the BamHI A region and lack information on the chromatin structure and transcripts generated from this region. Nevertheless, much information exists for the remaining regulatory elements of the B95.8 genome, which functions efficiently in B-cell immortalization and maintenance of latent infection.

3. Tour of the EBV Epigenome

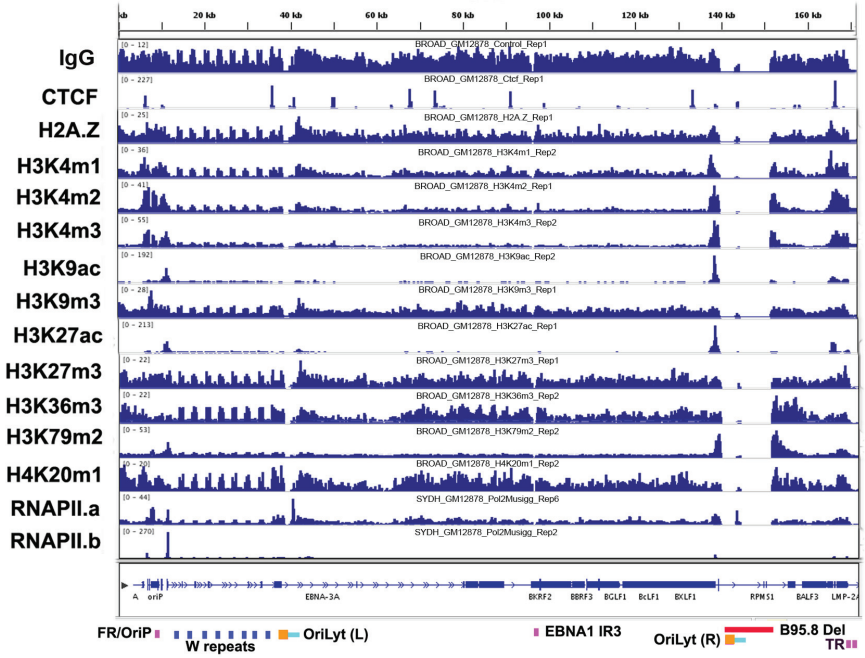
3.1. Overview of Chromatin Structure

In latently infected cells, the EBV genome is chromatinized with density similar to that of the host genome. Nucleosome position and histone tail modifications are strong indicators of chromatin structure and gene regulation. Micrococcal nuclease I (MNase I) and DNase I mapping studies can be used to assess the overall chromatin density and structure of viral genetic loci. For EBV, the majority of the genome is occupied by nucleosomes with varying degrees of static positioning or phasing, consistent with the dynamic nature of the viral genomes in LCL populations [13].

3.2. Histone Modifications

Mapping histone modifications across the EBV genome reveals enrichment of several marks at specific genome positions (Figure 1B). Enrichment can be assessed based on background input or IgG control DNA, as well as relative to average values for all histone modification specific ChIP assays and MNase I nucleosome density mapping [14]. Using these guidelines, histone modifications, H3K4m1, H3K4m2 and H3K4m3, are similarly enriched at the EBER-OriP-Cp locus (~7,000–13,000), the BART transcript promoter region (BARTp) (~138,500) and the LMP2-LMP1 promoter locus (~165,400–169,600). These regions represent the major sites of RNA polymerase II and III loading for type III latency transcripts. This is consistent with the established role of H3K4 methylation in transcription enhancer and promoter function, as well as sites of DNA replication [15]. Acetylated histones (H3K9ac and H3K27ac) are enriched at much sharper peaks that correlate well with sites of transcription initiation at Cp (~11,537), the BARTp (~138,563), LMP1p (~169,246) and LMP2a promoter region (~165,319).

Figure 1. Chromatin overview of the Epstein-Barr virus (EBV) epigenome. ChIP-seq in lymphoblastoid cell lines (LCLs) are mapped to the wild-type HHV4 genome for CTCF, histone variant, H2A.Z, histone modifications and RNAPII. Many colocalized modifications are sites of type III latent gene transcription, such as Cp, BARTp and the LMP locus. RNAPII tracks illustrate the heterogeneous and dynamic nature of recruitment across the viral genome.



Histone modifications associated with facultative (H3K27m3) or constitutive (H3K9m3) heterochromatin appear generally low throughout the EBV genome. This may be due to the type III latency program in which most of the latent genome is transcribed. It may also be due to the partial or abortive lytic gene expression observed in some LCLs. In contrast, the latent KSHV genome has several broad peaks of H3K9m3 and bivalent K3K27m3 and H3K4m3 marks at lytic switch regulatory regions [16]. For EBV, only modestly enriched peaks for H3K27 and H3K9 trimethylation and no apparent bivalent control regions exist. The enrichment of H3K9m3 at the FR region of OriP, while very likely to be the result of sticky chromatin, since this locus also IPs with IgG, is potentially intriguing, because of colocalization with the Origin Recognition Complex (ORC), which has been implicated in H3K9 heterochromatin formation, as well as in replication origin function [17,18]. H3K27m3 is modestly elevated at the BHRF1 promoter control region (~41,852), which may regulate aspects of EBV miRNA production. H3K79m2 is found elevated at the 5' end of the Cp generated EBNA2 transcript (~11,292) and the BARTp generated BART transcripts (~139,054–155,254). H3K79m2 is conferred by the Dot1 methyltransferase, and recent studies have implicated Dot1 and

H3K79m2 in pluripotent stem cell reprogramming [19]. Additionally, Dot1 and H3K79m2 have been implicated in controlling DNA damage response during DNA replication and colocalizing with BAT3 transcription factors [20]. Each of these potential functions are worthy of further investigation at EBV regulatory elements.

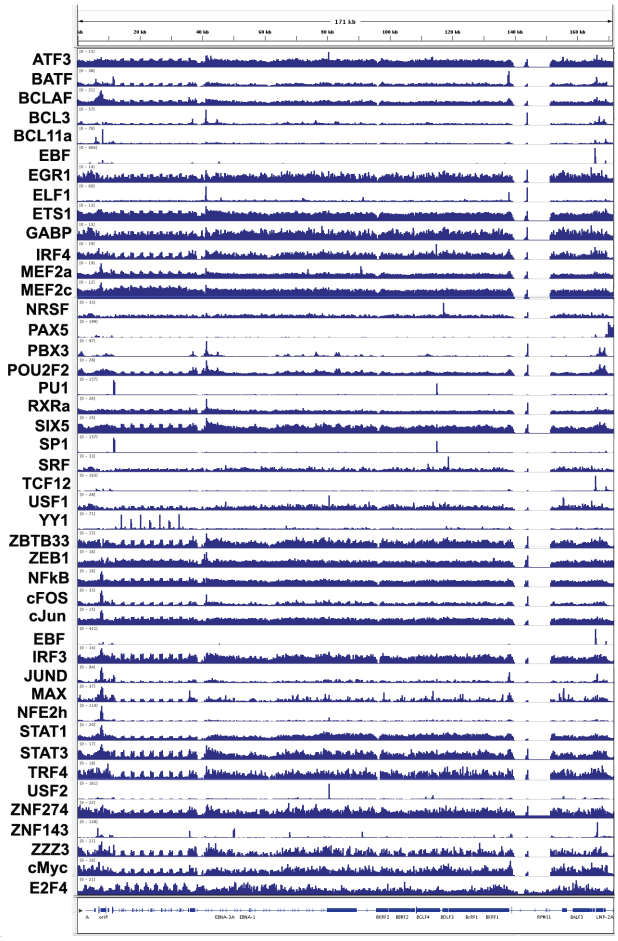
3.3. CTCF and Cohesin Binding Sites

CTCF is an eleven zinc finger DNA binding protein that has been implicated in chromatin boundary function, enhancer blocking and DNA-loop formation [21,22]. CTCF ChIP-seq reveals at least 19 sites of significant enrichment in multiple replicates, each of which contains a strong CTCF binding motif. These include binding sites at ~6,559 (5' EBER-1 promoter), ~10,494 (5' of Cp), ~36,000 (5' EBNA2 ORF), ~40,792 (OriLyt between divergent promoter of BHLF1 and BHRF1), ~49,973 (5' Qp), ~67,812 (BMRF1 ORF), ~73,845 (BSLF1/BMLF1 ORF), ~91,290 (BZLF1p), 99,028 (BKRF3 ORF); 133,524 (BVRF1 ORF), 139,033 (BART intron), 143,866 (BARF1 transcript) and 166,446 (LMP2 first intron/LMP1 poly A). It is remarkable that most of these CTCF sites can be assigned to important regulatory regions of the genome. However, it is impossible to assign a single function to CTCF that explains the binding to each of these sites. Surprisingly, many CTCF binding sites are proximal to RNA polymerase regulatory elements, which is in contrast to the host genome, where the vast majority of CTCF sites are located at positions far from transcription initiation. This finding is consistent with other gammaherpesvirus studies, including those with Kaposi's sarcoma-associated herpesvirus (KSHV) multicistronic LANA-vCyclin-vFLIP transcript, that suggest CTCF regulates RNA polymerase programming [23,24]. It is also likely that some of these CTCF sites represent DNA loop junctions and inter-chromosomal linkages, as was found for the CTCF-mediated interactions between the OriP and Qp [25] or OriP and LMP1/2 region [1]. It is also worth noting that CTCF peak heights vary substantially, suggesting that some sites may be stronger or perhaps only bound to a subset of episomes.

3.4. Transcription Factor Binding Sites

EBV gene expression is regulated by mechanisms similar, if not identical, to host cell genes. Therefore, it is not surprising that many cellular transcription factors bind at multiple locations across the EBV genome (Figure 2). Transcription factor binding at known viral promoter regulatory elements is expected, and many of these interactions have been described previously. For example, PU.1 and Sp1 co-regulate Cp/Wp and LMP1 and are found colocalized at these loci and at an unanticipated site within a cluster of lytic genes (e.g., BGLF1 ORF) not expressed during latent infection. The function of PU.1/Sp1 binding at this site in latently infected LCLs is not obvious. YY1 has also been implicated in regulation of Wp and is highly enriched in at least one, possibly all, W repeats. Given the role of YY1 in polycomb-mediated chromatin regulation [26], it is tempting to speculate that the function of YY1 in these repeats is related to H3K27m3 formation and higher order chromatin organization at these internal repeats.

Figure 2. Transcription factor occupancy on the EBV epigenome. ChIP-seq tracks for various transcription factors (as indicated) were mapped from B95-8 LCLs. There is extensive colocalization at multiple loci across the genome.



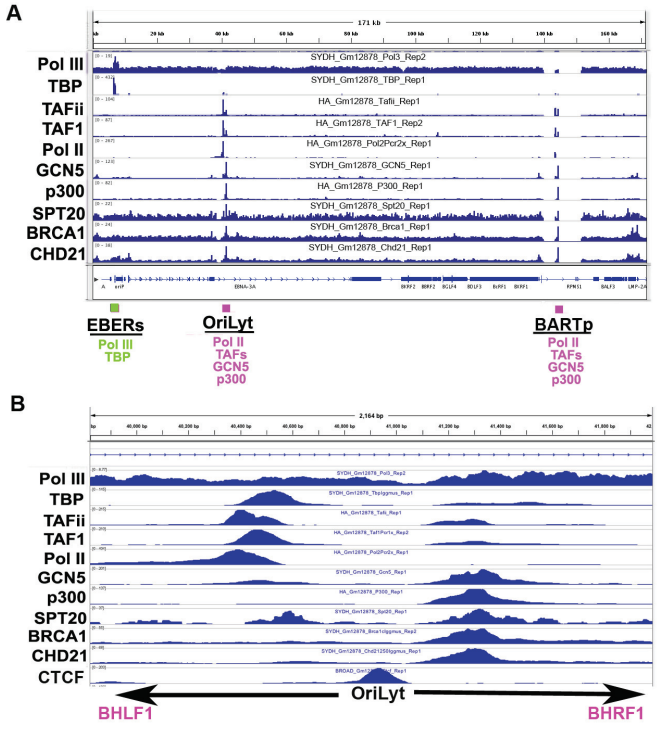
Transcription factor co-occupancy is observed at several key regulatory elements of EBV. The combination of factors at each of these sites may provide interesting new insights into signaling pathways and interactive transcription factor networks. For instance, BATF, JunD, Max and TRF4 are enriched at Cp; OriLyt-L (BHLF1/BHRF1 divergent promoter) contains binding sites for BCL3, ELF1, PBX3, POU2F2, RXRa and cFOS; and the LMP2 promoter binds TCF12, EBF, ZNF143 and JUND. Several cellular factors were found to bind to regions of the viral episome with no known regulatory functions. For example, ATF3, USF1 and USF2 show strong colocalization at ~80,655, which falls within the first internal repeat of the EBNA3A transcript. Another example is SRF and NRSF binding at 112,407, which falls near the putative promoter elements of the capsid protein

BGLF3 promoter, a lytic protein not likely to be expressed during latent infection. The colocalization of this particular subset of factors at these genetic regulatory elements suggests a partitioning of factor functions and warrants further investigation.

3.5. Co-Activator Binding Sites

Examination of non-sequence specific transcription co-activators reveals a remarkable enrichment at the OriLyt (L) or OriLyt (R) control elements (Figure 3). In particular, enrichment of GCN5, p300, BRCA1 and CHD21 occurs at the BHRF1 promoter in OriLyt, while TAFs and Pol II are enriched at the divergently transcribed BHLF1 promoter. A CTCF binding site sits between these two different regulatory elements, possibly functioning as a latent/lytic insulator. Also remarkable is that TBP was highly enriched at the EBERs, colocalizing with RNA Pol III.

Figure 3. RNA polymerases and transcriptional co-factor occupancy on the EBV genome. ChIP-seq tracks for RNA Pol III, TBP, TAFii, TAF1, RNA Pol II, GCN5, p300, SPT20, BRCA1 and CHD21 for B95-8 LCLs. EBERS oriLyt (R) and BART promoter are indicated below. B) Zoom of the OriLyt region of EBV.



3.6. Origin of Latent Replication

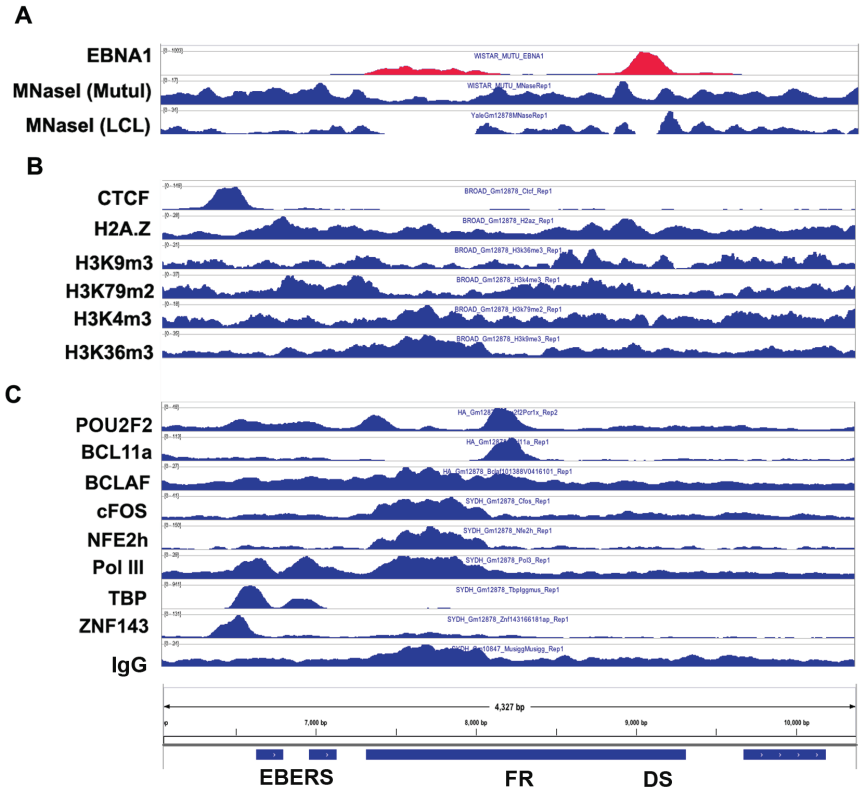
The origin of latent replication (OriP) is the episomal maintenance element that can serve as an origin of DNA replication and tethers the viral genome to host metaphase chromosomes during mitosis [27,28]. As such, it is possible that some of the factors that associate with OriP may reflect close interactions of OriP with host chromosomal proteins or histone modifications. As mentioned above, OriP can function as a transcriptional enhancer for Cp and LMP1/LMP2, and recent studies have implicated CTCF and cohesins in loop formation between OriP and these promoters [1,25]. CTCF binding sites appear to bracket the entire EBER-OriP region, potentially forming a functional DNA loop for OriP enhancer mobilization and insulation of other gene activation. Examination of the epigenomic features of OriP reveals a nucleosome-free region overlapping EBNA1 binding sites at FR and DS, but a strong positioning and phasing of nucleosomes at positions flanking DS and FR (Figure 4A). Strong nucleosome position flanking DS was reported previously using conventional methods [29]. The epigenetic modification of these nucleosomes are not clear, since H3K4m3 was reported to be elevated, but has significantly higher peaks at regions 5' to FR and overlapping EBER transcripts. H2A.Z appears to be enriched at these positions, but the relative enrichment is modest.

As mentioned above, several transcription factors colocalize at OriP. Previous studies have shown that Oct2 (Pou2F2) can bind to FR, and ENCODE ChIP-seq shows strong enrichment of Pou2F2 at FR (Figure 4B). A number of other factors show ChIP-seq signal at FR (e.g., BCLAF, BCL11a, NFE2h, cFos, RNA Pol III and others); however, this region is also elevated in non-specific IgG immunoprecipitation. BCL11a showed a more discrete peak that overlapped with the Oct2 binding site, suggesting that this may reflect-specific binding. As noted above, the relative enrichment of IgG at the FR complicates interpretation of ChIP-Seq data and may reflect important physical features of OriP, including nuclear matrix attachment [10].

3.7. DNA Methylation

DNA methylation contributes to the balance of limiting gene expression and avoiding immune detection during latency, while establishing a landscape that can be overcome during lytic reactivation to express the >70 gene products involved in replication. The EBV genome packaged in virions is unmethylated and gradually becomes methylated by host factors during initial cellular infection. Genome-wide analysis of methylated CpG levels in the EBV genome has revealed that the origin of plasmid replication OriP, the Cp and the Qp promoters, and the region for the noncoding RNA EBERS lack significant levels of DNA methylation in lymphoblastoid cell lines during latency [30,31]. Interestingly, CTCF demarcates the boundaries of unmethylated high CpG frequency regions in EBV, and at least in the case of the Qp and Cp, the loss of CTCF binding alters the functionality of these regions [31,32]. Highly methylated loci inhibit the transition from the latent to lytic phase; however, the Zta transcription factor selectively activates methylated promoters of lytic genes, including genes encoding for the viral helicase, the DNA polymerase and the DNA polymerase processivity factor [33, 34].

Figure 4. Histone modifications and transcription factor occupancy at OriP. (**A**) MNase I seq analysis for MutuI (type I latent lymphoma cell line) and MutuLCL (type III latent LCL using the same viral strain as MutuI), showing nucleosome depletion at the EBNA1 binding sites in DS. (**B**) ChIP-seq for CTCF, H2A.Z, histone modifications and EBNA1 binding at OriP region. (**C**) ChIP-seq tracks for transcription factors CTCF, POU2F2, BCL11a, BCLAF, cFOS, NFE2h, RNA Pol III, TBP, ZNF143 and EBNA1 at the OriP region.



3.8. Negative Results

Through meta-analysis, it is possible to discover and observe many phenomena; however, just as notable are the phenomena that are not observed. While negative results are typically not formally reported, they can be accrued in unbiased databases of experiments mapped to the viral genome [1]. For instance, of the 68 transcriptional regulators previously examined, only 26 have had reproducible binding sites in the EBV genome. While some of the 42 TFs may bind the viral genome, possibly with lower affinity, it seems more likely that the majority of host transcriptional regulators do not physically interact with the viral episome. Furthermore, repressive histone modifications, such as H3K27me3, are

largely absent from the viral genome (or at the very least, had no spatial enrichment, meaning that if H3K27me3 is present on viral nucleosomes, the modification lacks spatial regulatory specificity).

Previously identified regulatory interactions are largely confirmed in high throughput analyses. However, a small subset of experiments yielded surprising negative results. For instance, NF-kB binding at LMP1p was either weak or non-existent. Additionally, ZEB1 binding to Zp could not be confirmed. In both of these examples, it was crucial that positive controls in the viral and host genomes were provided. Importantly, the ChIP-seq methods are only semi-quantitative, and observed peaks of significant interest need to be validated by conventional ChIP and qPCR methods, which have been performed at only a small subset of these sites.

4. Conclusions

The EBV epigenome, as revealed by data mining, reflects only a small fraction of the protein interactions and histone modifications that define the viral chromosome. It is certainly not a complete nor comprehensive characterization of the proteins and modifications that regulate the EBV genome in all its dynamic complexity. Nevertheless, the insights gained from this "tip of the iceberg" glimpse of the EBV epigenome suggest that this discovery approach can reveal many new and previously unanticipated regulatory features of viral-host interactions, gene regulation and chromosome organization. Many of the observations discussed in this review need to be experimentally validated and further characterized to fully assess their functional significance. However, these observations indicate that "omics" dissection of viral and host gene regulation can generate new concepts and hypotheses and a deeper understanding of how the viral and cellular genomes persist during latent infection.

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Conflicts of Interest

The authors declare no conflict of interest.

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Review

Bromodomain Proteins in HIV Infection

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Abstract: Bromodomains are conserved protein modules of ~110 amino acids that bind acetylated lysine residues in histone and non-histone proteins. Bromodomains are present in many chromatin-associated transcriptional regulators and have been linked to diverse aspects of the HIV life cycle, including transcription and integration. Here, we review the role of bromodomain-containing proteins in HIV infection. We begin with a focus on acetylated viral factors, followed by a discussion of structural and biological studies defining the involvement of bromodomain proteins in the HIV life cycle. We end with an overview of promising new studies of bromodomain inhibitory compounds for the treatment of HIV latency.

Keywords: bromodomains; HIV; PCAF; PBAF; p300; CBP; TRIM28; BRD2; BRD4

1. The Bromodomain Protein Family

Reversible modifications of nucleosome components are increasingly acknowledged for their regulatory potential [1]. One such modification is acetylation, which involves the transfer of an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ε-amino group of lysine side chains on histone and non-histone proteins [2,3]. This modification is catalyzed by lysine acetyltransferases (KATs, also

known as HATs), whose action is reversed by lysine deacetylases (KDACS, also known as HDACs) or sirtuins (SIRTs). Histone acetylation is a well-studied modification that antagonizes the nucleosomal DNA-protein interaction, promoting chromatin accessibility and transcriptional activation [4]. However, aside from causing physiochemical changes in the nucleosome core, acetylation also generates novel and unique interaction interfaces for the assembly of macromolecular complexes important for a variety of cellular processes.

The bromodomain is a conserved protein module of ~110 amino acids that recognizes and binds ε-*N*-acetylated lysine residues in histone and non-histone proteins [5,6]. Recognition of acetyl-lysine residues by bromodomain-containing proteins is at least partly responsible for the functional consequences linked to protein acetylation. The first reference to a bromodomain can be traced to the Drosophila gene *brahma* (*brm*) [7], and the human bromodomain family to date includes 46 distinct proteins and 61 unique bromodomains (Figure 1A) [8]. Select transcriptional regulators (*i.e.*, BRD4, TAF1, TIF1), chromatin-modifying enzymes (*i.e.*, p300, PCAF, MLL), and nucleosome remodelers (*i.e.*, SMARAC2, PB1, BAZ1B) contain bromodomains [6].

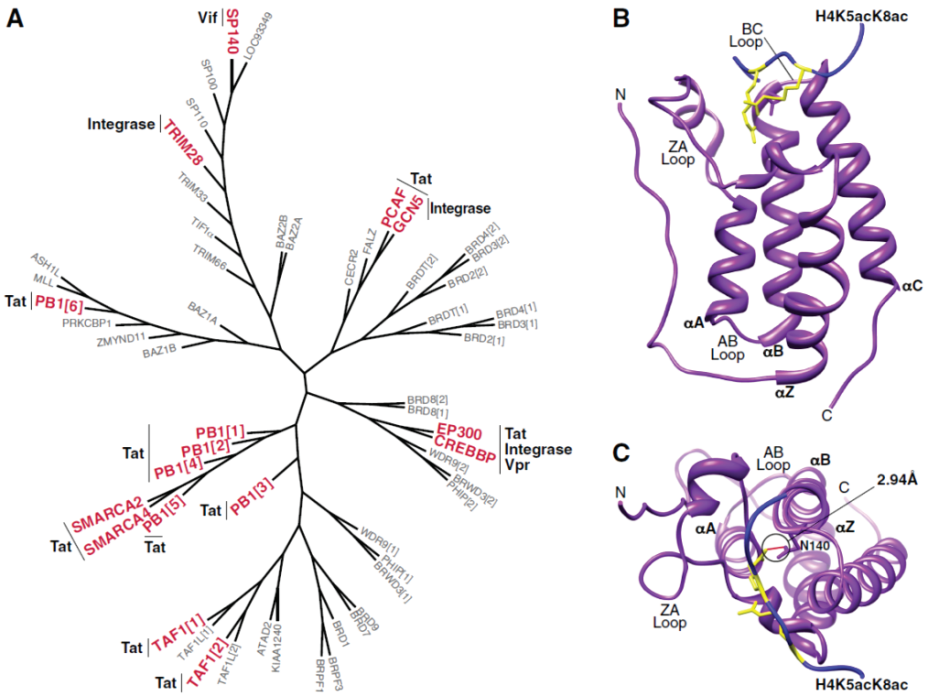
Structurally, bromodomains are comprised of four left-handed α-helices (αZ, αA, αB, and αC) connected by two loops (ZA and BC loops) (Figure 1B) [5]. This structure forms a deep hydrophobic cavity that serves as the acetyl-lysine recognition site [8]. While the helical regions are moderately conserved among different bromodomains, the loop regions are highly variable in both length and sequence composition [8]. This loop variability guides the substrate specificity observed among members of the bromodomain family. Co-crystal structures of bromodomains with various acetylated peptides demonstrate that the neutralized acetyl-lysine residue forms a hydrogen bond with an asparagine residue found in most bromodomains (Figure 1C). Some bromodomains exhibit higher affinity for multiply acetylated substrates, while the affinities of others are regulated by additional post-translational modifications of the ligand, such as phosphorylation [8,9].

2. HIV Infection and Reversible Acetylation

Reversible acetylation of histone and non-histone proteins plays a key role in HIV transcription and is a lead target in preclinical and clinical efforts to reverse HIV latency [10–14]. Upon integration into the human genome, the HIV proviral cDNA is organized into higher-order chromatin and becomes subject to regulation by host chromatin-modifying enzymes, including acetyltransferases and deacetylases [15]. Indeed, it has been shown that following stimulation with phorbol esters, distinct lysines in histone H3 (H3K9 and H3K14) become rapidly acetylated within a single nucleosome (nuc-1) located immediately downstream of the viral transcription start site [16]. However, the HIV provirus differs from cellular genes because it encodes a viral protein called transactivator of transcription (Tat). Tat is essential for HIV replication. It relieves a powerful block to the elongation of HIV transcripts by cooperatively binding to (1) an RNA stem-loop structure called TAR present at the 5' end of all nascent viral transcripts and (2) the positive transcription elongation factor b (P-TEFb), which together with other elongation factors, including PAF1c, forms a “super-elongation complex” [17,18].

Additionally, Tat is known to recruit several acetyltransferases to the HIV LTR, thus enhancing HIV transcription in the context of chromatin. These include KAT3B/EP300/p300 [19], KAT3A/CREBBP/CBP [19], KAT2A/GCN5 [16], and KAT2B/PCAF [16]. Conversely, several HDACs have been shown to bind to the HIV promoter located in the 5' long terminal repeat (LTR) through interactions with cellular transcription factors, including YY1 [20], LSF [20], NF-κB [21], AP-4 [22], CBF-1 [23], c-Myc and Sp1 [24]. HDAC inhibitors, which are known to activate HIV from latency in cell culture models [25], are being clinically tested for their potential to reactivate HIV from transcriptional latency [10].

Figure 1. The human bromodomain family. (**A**) Phylogenetic tree of 57 human bromodomains. Those bromodomain-containing proteins that have been shown to interact with HIV proteins are denoted in red with the corresponding viral factor indicated alongside. Phylogenetic trees were generated using Seaview v4.4.1 with individual bromodomain sequences obtained from [23]. (**B**) Structure of the first bromodomain of BRD4 (purple) in complex with a diacetylated histone peptide (blue). Histone acetyllysine residues are shown in yellow. Structural representations in (B,**C**) were rendered using Chimera (UCSF) with PDB: 3UVW (**C**) Top view of interaction between first bromodomain of BRD4 and a diacetylated histone peptide. The hydrogen bond between the canonical bromodomain asparagine residue (N140 in BRD4) and the histone acetyllysine residue is shown in red with an estimated length of 2.94A.



Tat itself is subject to reversible acetylation. Tat is acetylated by KAT2B/PCAF at lysine 28 within a characteristic cysteine-rich region required for its interaction with P-TEFb [26]. Tat is also acetylated by KAT3B/EP300/p300 and the close KAT2B/PCAF homologue KAT2A/GCN5 at lysines 50 and 51 located in its basic RNA-binding domain [26–28]. Both acetylation events positively support Tat's transcriptional activity [29,30] and are reversed by the deacetylase activities of HDAC6 and SIRT1 [31,32]. In addition to Tat, HIV integrase, a DNA-binding protein that catalyzes 3' processing and strand transfer of the viral genome, is acetylated by KAT3B/EP300/p300 at lysines 264, 266 and 273 [33]. These residues are also subject to acetylation by KAT2A/GCN5, in addition to lysine 258 [34]. Integrase acetylation increases the affinity of the enzyme for DNA and also enhances strand-transfer catalysis *in vitro*. Lastly, Vpr, a viral protein implicated in nuclear translocation of the HIV pre-integration complex, HIV-mediated G_2/M arrest, and transcription of viral and cellular promoters, interacts with KAT3B/EP300/p300 [35]. Mutational analysis suggests that the Vpr-p300 interaction occurs independently of the p300 bromodomain [35], and it is unclear whether Vpr is acetylated.

3. Interactions between Bromodomain-Containing Proteins and HIV Proteins

3.1. Acetylation-Dependent Interactions

3.1.1. p300/CBP-Associated Factor (PCAF)

KAT2B/PCAF (p300/CBP-associated factor) is a histone acetyltransferase that contains a *N*-acetyltransferase domain and a *C*-terminal bromodomain. KAT2B/PCAF also participates in the reversible acetylation of various non-histone proteins, including p53, β-catenin, retinoblastoma protein (Rb), and several transcriptional regulators, such as the general transcription factors $TF_{II}E\beta$ and $TF_{II}F$ and the sequence-specific transcription factors E2F1, c-Myc, and MyoD (reviewed in [36]) [37]. In HIV infection, KAT2B/PCAF acetylates Tat at lysine 28 [26,38]. Acetylation of Tat on lysine 28 facilitates recruitment of P-TEFb kinase, resulting in the efficient phosphorylation of the heptad repeats in the carboxyl terminus of cellular RNA polymerase II and thus promoting HIV transcript elongation [26,29,38]. KAT2B/PCAF is recruited to Tat through an acetylation-dependent mechanism (Figure 2). Acetylated K50 in Tat acts as a specific binding partner of the KAT2B/PCAF bromodomain, an interaction that was examined by NMR spectroscopy at the structural level and characteristically involves additional residues in Tat that interact with the KAT2B/PCAF bromodomain in an acetylated K50-dependent manner [39,40]. KAT2B/PCAF binding to Tat positively supports HIV transcription either through enhanced lysine 28 acetylation in Tat, or enhanced local histone acetylation during HIV transcriptional elongation. Consequently, mutations in the KAT2B/PCAF bromodomain that suppress interactions with acetylated Tat or treatment with small molecules that specifically bind the KAT2B/PCAF bromodomain effectively suppress Tat transactivation, supporting the concept that this interaction could serve as a specific target for anti-HIV transcription therapeutics [39,41].

3.1.2. SWItch/Sucrose Non-Fermentable (SWI/SNF)

Members of the SWI/SNF family form evolutionarily conserved chromatin-remodeling complexes that utilize the energy of ATP hydrolysis to induce nucleosome remodeling. Two important and distinct SWI/SNF complexes have been described in humans: BAF and PBAF. These complexes contain either Brg-1 (also known at SMARCA4) or BRM (also known at SMARCA2) as the major catalytic subunit together with several accessory proteins. BAF is distinguished by the presence of the BAF250a/b subunit, while PBAF contains BAF180 (also known as Polybromo or PB1), BAF200, and BRD7 subunits. BAF has been shown to repress HIV transcription by positioning the nuc-1 nucleosome immediately downstream of the transcription start site in a manner that encumbers processive transcription [42]. Such repressive remodeling events may be crucial for the maintenance of HIV latency in the absence of Tat. Conversely, the PBAF complex is required for robust Tat-mediated transactivation of HIV expression [43,44]. Both shared constituents of BAF and PBAF, Brg-1 and BRM, interact with Tat. These interactions are regulated by acetylation of Tat, with K50 acetylation enhancing the Tat-Brg1 interaction [45] and inhibiting the Tat-BRM interaction [46]. The interaction between acetylated Tat and Brg-1 was mapped to the Brg-1 bromodomain, while the Tat-BRM interaction was shown to be bromodomain-independent [46]. Tat displays enhanced interaction with the PBAF-specific constituent BAF200 in a K50/K51-dependent manner [47]. In addition, Tat acetylated at K50/K51 interacts with PBAF through the BAF180 subunit, an interaction that facilitates Tat-mediated transactivation (Figure 2) [42]. It is likely, though not experimentally confirmed, that acetylated Tat interacts with one or more of the six bromodomains present in BAF180. These findings evoke a model in which Tat acetylation at K50/K51 functions to switch the repressive BAF complex with the activating PBAF complex through specific interactions with several bromodomain-containing proteins in the PBAF complex, including Brg-1 and BAF180.

3.1.3. Bromodomain-Containing Protein 4 (BRD4)

BRD4 is a mitotic chromosome-associated protein that serves as an important regulator of post-mitotic transcription by recruiting various transcriptional regulators to acetylated chromatin. BRD4 is also required for maintaining a proper higher-order chromatin structure [48–50]. BRD4 is a member of the bromodomain and extraterminal domain (BET) family of bromodomain proteins. Members of the BET family are distinguished by the presence of two functional domains—tandem bromodomains and a so-called extraterminal domain, the latter of which may serve to mediate protein-protein interactions [49]. BRD4 contains a third functional domain termed the P-TEFb-interacting domain (PID) [51]. The PID serves to recruit and activate the Tat cofactor P-TEFb, a heterodimer composed of cyclin T1 and CDK9 that when complexed with the HEXIM1 inhibitor is part of an inactive ribonucleoprotein complex found in HeLa cells and other tumor cell lines [52,53]. While BRD4 is an important factor recruiting P-TEFb to the HIV promoter in the absence of Tat, Tat and the BRD4 PID compete for P-TEFb binding, making BRD4 a negative factor in Tat transactivation [51,54]. In

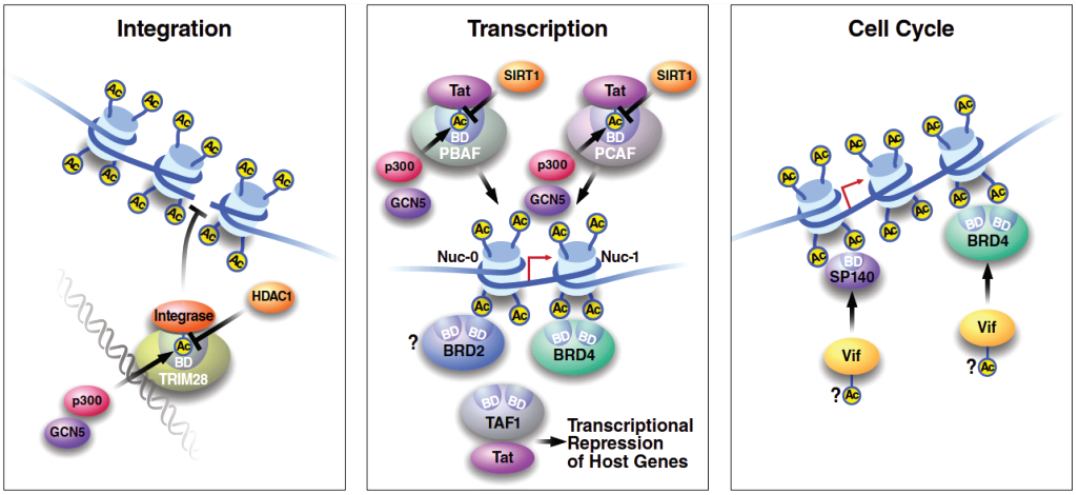
addition, BRD4 negatively regulates HIV transcription by inducing an inhibitory phosphorylation event on CDK9 [55].

The cyclin T1 subunit of P-TEFb is acetylated at four defined residues by KAT3B/p300 [56]. Three of the four acetylated residues (K380, K386, K390) bind the second bromodomain of BRD4 defining a second acetylation-dependent P-TEFb interaction site in BRD4 besides the PID. Interestingly, while this acetylation-dependent interaction is required for basal HIV LTR activity and cellular gene expression, it is not necessary for Tat-mediated transactivation of HIV transcription, supporting the model that Tat- and BRD4-mediated activities at the HIV promoter are mutually exclusive [51,54]. Vollmuth *et al.* determined the crystal structures of the two bromodomains of BRD4 and showed that acetylated K390 weakly bound to the second bromodomain of BRD4 [57]. This binding is markedly enhanced when K380 and K386 are also acetylated [52], supporting an emerging model that bromodomains of BET proteins have a preference for binding to multiply acetylated proteins.

3.1.4. Tripartite Motif-Containing Protein 28 (TRIM28)

TRIM28 was originally identified as an interaction partner of members of the family of Krüppel-associated box (KRAB) domain-containing zinc finger transcription factors. It is also named KRAB-associated protein 1 (KAP1), KRAB-A-interacting protein 1 (KRIP1), and transcription intermediary factor (TIF) 1β. Its protein architecture includes an *N*-terminal tripartite motif (TRIM) that acts as a protein-protein and oligomerization interface, and contains a RBCC (Ring finger, two B-box zinc fingers, and a coiled coil) domain, a central heterochromatin protein 1 (HP1)-binding domain, a TIF1 signature sequence (TSS) domain, and a *C*-terminal plant homeodomain (PHD) and bromodomain. The TRIM28 bromodomain is a 100-amino acid stretch consisting of four α-helices that, similar to other bromodomain-containing proteins, has a conserved hydrophobic core and recognizes the backbone of histone tails [58]. Structural analysis of the tandem PHD and bromodomain (PB) of TRIM28 between amino acids 624 and 812 revealed that both domains function as a cooperative unit to facilitate lysine sumoylation, which is required for TRIM28 co-repressor activity in gene silencing [58]. In a yeast two-hybrid screen, TRIM28 was identified as an interaction partner of acetylated HIV-1 integrase [59]. TRIM28 *in vitro* and *in vivo* preferentially binds integrase when acetylated at K264, K266 and K273, rather than the unmodified protein, implicating that this interaction is mediated by the TRIM28 bromodomain. Integrase K264, K266 and K273 are targeted for acetylation by KAT3B/p300, which is a prerequisite for the interaction of TRIM28 with integrase and leads to subsequent recruitment of HDAC1. As a consequence, TRIM28 inhibits HIV-1 integration through integrase deacetylation by HDAC1 (Figure 2) [60].

Figure 2. Role of bromodomain proteins in HIV infection. Bromodomain proteins implicated in HIV transcription, HIV integration, and cell cycle progression are schematized. Briefly, acetylated integrase displays enhanced enzymatic activity, yet generates an interaction interface for the TRIM28 bromodomain that in turn recruits the HDAC1 deacetylase, negatively impacting HIV integration. With respect to viral transcription, Tat acetylated at K50/51 interacts with BAF180 and Brg-1 within the PBAF complex to support viral transcription. Acetylated Tat also interacts with p300/CBP-Associated Factor (PCAF) to induce local acetylation of histones and potentially other factors at the site of viral transcription. BRD4 is present at the HIV long terminal repeat (LTR), yet is hypothesized as an intracellular competitor of Tat, while the role of BRD2 in HIV transcription is unknown. Tat also interacts with TAF1 to repress select cellular promoters. BRD4 and SP140 are both cell cycle regulators that interact with Vif, yet the bromodomain-dependence and functional significance of these interactions remain unclear.



3.2. Other Interactions with Bromodomain-Containing Proteins

3.2.1. Transcription Initiation Factor TFIID, Subunit 1 (TAF1)

TAF1, also referred to as $TAF_{II}250$, is the largest component of transcription factor TFIID that is composed of TATA-binding protein (TBP) and a variety of TBP-associated factors. TAF1 contains *N*- and *C*-terminal serine/threonine kinase domains, but can also function as an acetyltransferase and an ubiquitin-activating/conjugating enzyme. It contains two tandem bromodomains, both located in the *C*-terminus of the protein. Structurally, the two bromodomains form two side-by-side, four-helix bundles, each with an acetyl lysine binding pocket at its center, recognized by diacetylated histone H4 peptides [61]. When Weissman *et al.* identified TAF1 as a Tat interaction partner, the interaction was mapped to amino acids 80 to 83 in Tat and a site in TAF1 that overlaps the acetyltransferase domain,

inhibiting TAF1 acetyltransferase activity [62]. TAF1 was not required for Tat transactivation of the HIV LTR; instead, the interaction with TAF1 was linked to Tat-mediated transcriptional repression, *i.e.*, of the MHC class I promoter (Figure 2) [62]. These findings could be explained by a model in which the interaction with Tat inhibits TAF1 acetyltransferase activity, thereby recruiting an inactive Tat/TAF1 complex to actively transcribing gene promoters containing hyperacetylated histone H4.

3.2.2. Nuclear Body Protein SP140

SP140, also referred to as lymphoid-restricted homolog of Sp100 (LYSp100) is a component of a subset of nuclear bodies in lymphoid cells. It is involved in the pathogenesis of acute promyelocytic leukemia and has been shown to be an autoantigen in primary biliary cirrhosis [63–65]. Its protein architecture includes a *N*-terminal homogeneous staining region (HSR) domain, a central SAND domain which mediates DNA binding, a PHD-type zinc finger, and a *C*-terminal bromodomain. In a yeast two-hybrid screen, SP140 was identified as an interaction partner of the HIV Vif protein [66]. Vif enhances the infectivity of HIV virions released from so-called non-permissive cells by inducing the degradation of an antiviral restriction factor APOBEC3G [67]. SP140 was found specifically in non-permissive cells, and HIV-1 infection induced its dispersal from nuclear bodies into cytosolic colocalization with Vif [66]. SP140 interacts with the N-terminal and central regions of Vif (amino acids 1–112) in the yeast two-hybrid screen, and the SP140 prey cDNAs that were isolated encoded the *C*-terminal region between amino acids 527 and 836, which includes the SAND domain, the PHD-type zinc finger, and the bromodomain [66]. Further studies are needed to determine whether an acetyl-lysine-bromodomain interaction is involved in the interaction between Vif and SP140.

3.2.3. BRD2

BRD2, formerly named RING3 (really interesting new gene 3) or Fshrg1 (female sterile homeotic related gene 1), is a nuclear serine/threonine kinase possessing chromatin binding and transcription activity. Along with BRD4, BRD2 is a member of the BET family, but lacks a *C*-terminal PID domain.

However, BRD2 was found to coimmunoprecipitate with CDK9/Cyclin T1 or Cyclin T2 [68]. BRD2 also functions as a Tat-independent suppressor of HIV transcription in latent cells [69]. In cell lines containing latent HIV, lentiviral shRNA-mediated depletion of BRD2 resulted in activation of the HIV LTR, and this effect was independent of Tat. The fact that BRD2 is known to bind co-repressor complexes including HDACs [70] supports a model whereby BRD2, by recruiting repressor complexes to the latent HIV LTR, directly suppresses HIV transcription. It remains to be seen whether BRD2, like BRD4, interacts with P-TEFb in an acetylation-dependent manner.

3.2.4. BRD4

In addition to its role in HIV transcription, BRD4 has been implicated in Vif-mediated cell-cycle progression. By mass spectrometry, Wang *et al.* identified BRD4 and CDK9 as Vif interactors required for Vif-mediated acceleration of cell-cycle transition from the G_1-to-S phase [71]. BRD4 also

regulates the G_2-to-M transition and stimulates cell-cycle progression from G_1 to S through recruitment of P-TEFb to chromosomes and stimulation of G_1 gene expression during late mitosis [72,73]. It is unknown whether the Vif-BRD4 interaction involves the BRD4 bromodomains.

4. Bromodomain Inhibitors and HIV Infection

The characteristic architecture of the bromodomain-acetyl-lysine interface represents a potential target for the development of small-molecule inhibitors. In initial attempts to identify bromodomain inhibitors, NMR-based screens of commercial compound libraries were used to identify compounds that inhibit Tat transactivation at the Tat-PCAF interface [41]. Two lead compounds were discovered using this approach, both with relatively low IC_{50} values for the Tat-PCAF interaction *in vitro*. Recently, several high-affinity binding molecules for bromodomains of the BET family were described [74–78]. JQ1, a thienodiazepine derived from a BRD4 ligand developed by Mitsubishi Pharmaceuticals [79], was shown to bind the first bromodomain of BRD4 with high affinity and target the second bromodomain of BRD4, and those of BRD2, BRD3, and BRDT [75]. MS417, a BET inhibitor derived from JQ1, specifically targets the interaction between BRD4 and the acetylated p65/RelA subunit of the transcription factor NF-κB and has potent anti-inflammatory effects in a mouse model of HIV-associated kidney disease [78]. I-BET, a synthetic "histone mimic" identified using an ApoA1 reporter system, also functions as a potent anti-inflammatory agent that suppresses expression of pro-inflammatory genes in activated macrophages and confers protection against lipopolysaccharide-induced endotoxic shock and bacteria-induced sepsis [76]. These and other compounds are excellent tools to study BET protein function, but have also shown impressive preclinical promise for the treatment of the NUT midline carcinoma (JQ1, [75]), specific types of leukemia (JQ1 [80], I-BET151 [77]) inflammation (I-BET [76], MS417 [78]), and viral infections, including HIV.

Current antiretroviral therapy only suppresses viral replication, requiring life-long adherence to continuously limit viral loads, but does not eradicate virus from most infected people. Therefore, novel treatments that eliminate persistent viral reservoirs and thereby cure patients are needed. One approach is to reactivate proviral genomes in latently infected cells in order to "purge" viral reservoirs, either through the immune system or through the cytopathic effects associated with viral reactivation. HDAC inhibitors, including valproic acid and vorinostat (SAHA), show great promise as anti-latency therapeutics and have already begun to be tested in clinical trials [10,11,81,82]. Recently, a flood of publications demonstrated that BET bromodomain inhibitors reactivate HIV from latency in cell lines and primary T-cell models (summarized in Table 1). Notably, cells treated with drugs like JQ1 show little synergy with vorinostat, indicating that both drugs target similar pathways in HIV reactivation. However, it is unclear whether any of the current *ex vivo* models faithfully recapitulates the *in vivo* situation of latently infected cells; further studies are needed to evaluate the clinical potential of BET inhibitors in primary T cells.

Table 1. Selected reported bromodomain inhibitors tested in models of HIV latency.

Compound	Model of HIV latency	Effect	Reference
JQ1	Ach2	reactivation	[83]
JQ1	U1	reactivation	
JQ1	J-Lat 10.6	reactivation	
JQ1	Acutely infected primary $CD4^+$ cells	reactivation	
JQ1	JΔK	reactivation	[84]
JQ1	J-Lat A2	reactivation	[85]
JQ1	Jurkat 1G5	reactivation	
JQ1	HeLa NH1 and NH2	reactivation	
JQ1	HeLa-T4	reactivation	[86]
JQ1	Primary $CD4^+$ T cells	reactivation	
JQ1	Primary $CD4^+$ T cells	inhibition	
JQ1 + Prostratin or PMA	J-Lat 6.3	reactivation	
JQ1 + Prostratin or PMA	J-Lat 8.4	reactivation	
JQ1 + Prostratin or PMA	J-Lat 9.2	reactivation	
JQ1 + Prostratin or PMA	J-Lat 15.4	reactivation	
JQ1	J-Lat A2	reactivation	[87]
JQ1	J-Lat A72	reactivation	
JQ1	infected primary Bc12-transduced $CD4^+$ T cells	reactivation	
JQ1	infected primary nonpolarized T helper cells	no reactivation	
I-BET	infected primary Bc12-transduced $CD4^+$ T cells	reactivation	
I-BET	infected primary nonpolarized T helper cells	no reactivation	
I-Bet151	J-Lat A2	reactivation	
I-Bet151	J-Lat A72	reactivation	
I-Bet151	infected primary Bc12-transduced $CD4^+$ T cells	reactivation	
I-Bet151	infected primary nonpolarized T helper cells	no reactivation	
MS417	J-Lat A2	reactivation	
MS417	J-Lat A72	reactivation	
MS417	infected primary Bc12-transduced $CD4^+$ T cells	reactivation	
MS417	infected primary nonpolarized T helper cells	no reactivation	

5. Concluding Remarks

The acetyl-lysine-bromodomain interface, first therapeutically explored in HIV infection, represents an important regulatory axis that controls many aspects of HIV infection, including viral integration, Tat transactivation, HIV latency, cell-cycle progression of infected host cells, and virally induced inflammation. Furthermore, it is likely that new molecular functions for bromodomain-containing proteins in HIV infection await discovery. We expect that the use of existing and the development of novel bromodomain inhibitors will facilitate both the study and the treatment of HIV infection.

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Conflicts of Interest

The authors declare no conflict of interest.

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Review

Chromatin, Non-Coding RNAs, and the Expression of HIV

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Abstract: HIV is a chronic viral infection affecting an estimated 34 million people worldwide. Current therapies employ the use of a cocktail of antiretroviral medications to reduce the spread and effects of HIV, however complete eradication from an individual currently remains unattainable. Viral latency and regulation of gene expression is a key consideration when developing effective treatments. While our understanding of these processes remains incomplete new developments suggest that non-coding RNA (ncRNA) mediated regulation may provide an avenue to controlling both viral expression and latency. Here we discuss the importance of known regulatory mechanisms and suggest directions for further study, in particular the use ncRNAs in controlling HIV expression.

Keywords: HIV; non-coding RNA; chromatin remodeling; latency; reactivation

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1. Introduction

Upon entering a cell, HIV integrates into the genome of the host and essentially functions as an endogenous gene. The ability of HIV to remain dormant, in a relatively quiescent state in the infected cell remains the major barrier in providing an effective cure. Highly active antiretroviral therapy (HAART) is the current standard of care, and although viral load may be reduced to extremely low if not undetectable levels, it is unable to deplete viral reservoirs, and thus the HIV infection remains a

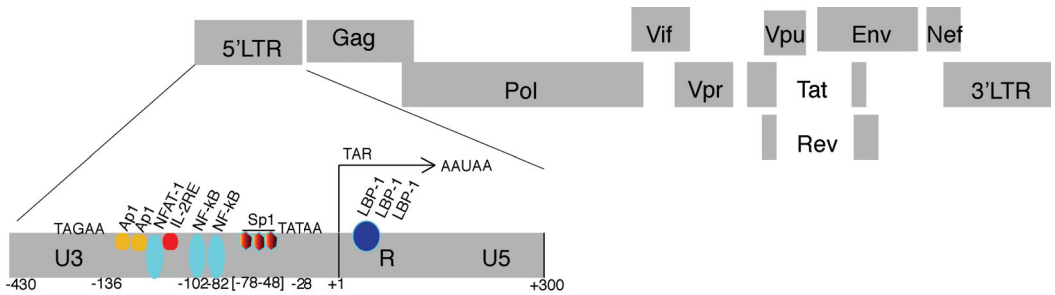
lifelong infection. The HIV-1 provirus employs numerous mechanisms to regulate its patterns of gene expression that are vital to the maintenance of latency in cells and therefore its survival. Currently, most of the literature surrounding HIV gene regulation focuses on the role of the long terminal repeat (LTR), and the interactions between of Tat and TAR. This view may be incomplete as new insights suggest a role for long non-coding RNAs (lncRNAs) in the regulation of gene expression patterns in many organisms however their role in HIV is still yet to be elucidated. These ncRNAs have the potential to impact viral lifecycles and pathogenesis in cells and therefore a more holistic understanding of the mechanisms of HIV viral latency and reactivation are paramount if therapeutics are to be developed to eradicate HIV.

2. Regulation of HIV-1 Chromatin and Latency

2.1. The HIV LTR

HIV-1 gene expression is regulated primarily at a transcriptional level by a series of *cis*-acting elements and *trans*-regulatory components located within the LTR at both the 5' and 3' ends of the provirus. The HIV-1 5' LTR comprises of three distinct regions, U3, R and U5, each with different elements involved in transcriptional regulation. The U3 region contains three functional domains, which regulate HIV-1 positive sense transcription: a core promoter region, core enhancer region and a modulatory region (Reviewed in [1]). The promoter region contains numerous transcription factor-binding sites, most notably, a TATA box and three Sp1 binding sites as well as an initiator element [2–4]. Following the promoter region is the core enhancer domain, which contains two NF-κB sites. Interaction between the Sp1 and NF-κB proteins is essential for positive regulation of HIV-1 transcription as the binding of NF-κB alone to the enhancer region is not sufficient for transcriptional activation [5] (Figure 1).

Figure 1. The LTR promoter of HIV. The genomic organization of HIV-1 is shown with lines emphasizing/delineating the 5' LTR/promoter. The transcription factor AP1, NFAT-1, IL-2RE, NF-kB, Sp1 and LBP-1 binding sites, TATA box, upstream weak transcriptional start TAGAA, Tat activating region (TAR), and the AAUAA poly-adenylation sites downstream of TAR in the LTR are shown. Note not all transcription factor binding sites are shown.



Many other cellular proteins have also been suggested to have sequence-specific interactions with the modulatory region, contributing to both positive and negative regulation of HIV-1 transcription (Reviewed in [1]). The R region, downstream of the U3 region, contains the transactivation response element (TAR); a viral stem-loop RNA structure which has been well documented to recruit and bind the Tat protein for efficient transcriptional elongation ([6,7], reviewed in [8]). CBP and p300 are coactivating proteins, which acetylate Tat, permitting its association with TAR RNA [9–12]. The Tat/TAR association synergistically enhances transcriptional efficiency of proviral RNA. The U5 region has also been shown to contain important motifs, which are involved in transactivation of the HIV-1 provirus [13,14]. The capacity of the HIV-1 LTR to mediate complex DNA-protein interactions, which positively and negatively regulate transcription, reveals its importance in the HIV viral lifecycle.

2.2. HIV Chromatin Regulation

Post-integration into the host genome, the provirus undergoes packaging into chromatin. Histones are highly conserved proteins, which form the foundation of chromatin structure. Histone proteins associate with DNA to form highly organised and compact structures. This structural unit is termed the nucleosome; and its tight arrangement will act as a barrier for DNA-binding factors such as transcription factors. Limited access to specific proviral sequences impacts the ability of the virus to express genes. Although histones have historically been thought of only as structural elements of the chromatin fibres, it has become evident that they are crucial players in the regulation of gene transcription and have the ability to alter the course of transcription regulation machinery through their histone code [15]. Covalent chemical modifications of the N-terminus of histone tails form the basis of their regulatory capacity and the histone code. These epigenetic modifications such as acetylation, phosphorylation, methylation, ubiquitination and ribosylation are key markers on the protruding histone tails, which may alter chromatin structure and regulation of gene expression (Reviewed in [16]).

The remodeling and modification of chromatin architecture has since been suggested to be an important factor in the transcriptional regulation of HIV-1 [17,18]. Upon HIV-1 integration into the host genome, the deposition of two nucleosomes, nuc-1 and nuc-0 occurs at two defined regions of the proviral LTR [19]. The deposition of nuc-1 plays an integral role in the suppression of HIV-1 gene expression, ultimately implicating chromatin modification as a key aspect of viral gene repression and latency [20].

Histone deacetylases (HDACs) have been associated with HIV-1 transcriptional silencing and viral latency. Cellular transcription factors, such as YY1, LSF and more recently c-Myc, Sp1 and AP-4, have been shown to be involved in the recruitment of HDAC1 to the viral LTR, repressing the HIV-1 promoter and maintaining viral latency [21–23]. These changes result in an impaired ability of RNA polymerase II to localise to the HIV-1 promoter and initiate transcription [24]. Following HDAC1 recruitment to the LTR, nucleosome deacetylation and subsequent transcriptional silencing of proviral transcriptional expression have been observed [25].

Another epigenetic modification, DNA methylation, has also been identified as a contributing mechanism for viral latency. HIV DNA may become methylated within the viral LTR, which has been associated with transcriptional silencing and the inhibition of the methylation has been shown to result in the reactivation of previously latent HIV [26–28]. DNA methylation however, does not seem to be the main silencing mechanism as the effect appears to be reversible and partially methylated promoters may still be reactivated [26].

Likewise, other epigenetic modifications to nucleosomes may play a role in transcriptional activation of HIV-1. It has been shown that the acetylation of histones is correlated with transcriptional activation of the HIV-1 promoter [20]. Acetylation primarily occurs at the lysine residues of the histone tails, which reduces the positive charge of the tails, lowering their affinity for DNA and ultimately causing partial or full disassociation with the DNA [29]. Histone acetyltransferases (HATs) have an opposite action to HDACs and have been known to interact with and be recruited to the viral promoter by the viral protein Tat [9,30,31]. Acetylation of the H3 and H4 histones has been shown to play a strikingly significant role in the onset of viral mRNA transcription in HIV-1 infected cells. In conditions under which the HIV-1 viral promoter is transcriptionally inactive, its activation by Tat correlates with acetylation of H3 and H4 on nuc-0 prior to the onset of viral mRNA production [32]. Furthermore, HAT activity at the promoter sites of genes has been shown to enhance gene transcription although alone it is not able to induce expression of viral expression (Reviewed in [33,34]).

Essentially, the ability of HIV-1 to alter chromatin structure and epigenetic modifications through cellular and viral transcriptional regulation mechanisms ultimately determines the path of transcriptional activation or viral latency in HIV.

3. NcRNAs and Their Role in Genetic Regulation

It has long been accepted that the genome is regulated by a series of aforementioned epigenetic modifications known as the histone code and HIV also appears to be under this mode of gene regulation. The mystery about the concise choreography and execution of the histone code continues to be enigmatic as we delve further into the nuances of the genome. Previous estimates suggested that up to ninety eight per cent of the genome was ‘junk’, however recent observations suggest that this is not the case. It turns out that these so-called ‘junk regions’ of the genome are active and may in fact be assiduous writers of the histone code controlling transcriptional and epigenetic states, among other things. The association of these ncRNAs with chromatin remodeling complexes shapes the future of their bound genes.

3.1. LncRNAs, Xist and PTENpg1

In humans, lncRNAs have been found to play a substantial role in chromatin remodeling and gene silencing in both normal cellular function as well as the development of diseases. The most eloquent example of this is the inactivation of the entire X chromosome during cell development. In mammalian females, one copy of the X chromosome is transcriptionally silent for the life of the cell. This is to

ensure that the correct ‘dosage’ of expression of the chromosome is achieved with respect to males, who only possess one copy [35]. The silencing of the entire chromosome is mediated by a sophisticated long intergenic noncoding RNA (lincRNA) known as Xist, which is a multiexonic, spliced RNA that possesses its own 5' cap and polyA tail [35–38]. PRC2 is a large complex with histone methyltransferase activity and is required for the extensive formation of the transcriptionally silent heterochromatin by trimethylation of histone H3 on lysine 27 (Reviewed in [39,40]). The PRC2 complex has been shown to bind RepA; and in turn is bound with Xist to target PRC2 histone methyltransferase activity on the future silent X chromosome [41]. This is the most well recognized example of how a lncRNA can facilitate extensive and essential changes to chromatin and dramatically alter gene expression.

Non-coding RNAs are not only vital for normal development but can also play a role in the development of human diseases such as cancer, Alzheimer’s disease and α-thalassemia [42,43]. The tumor suppressor gene, PTEN, is post-transcriptionally regulated by a lncRNA termed PTEN pseudogene 1 (PTENpg1) [44]. PTENpg1 in turn is modulated by a PTENpg1 encoded asRNA, which exists as one of two isoforms, α and β [45]. The α isoform has been shown to epigenetically silence PTEN transcription while the β isoform interacts with the sense pseudogene to alter the stability and localization of the transcript [45]. The sophistication of directive regulation by lncRNAs allows them to exert substantial control over gene expression and that the equilibrium by which they exist is fundamental for normal cellular functioning.

3.2. MicroRNAs (miRNAs) and Small Interfering RNAs (siRNAs); The Role of Dicer and Drosha

LncRNAs are not the only ncRNAs that have a regulatory effect on the genome. MiRNAs and siRNAs are the workhorses of RNA-directed RNA-processing. The action they exert on processing machinery is termed RNA interference (RNAi). miRNAs are small sequences of about 20–24 nucleotides which regulate gene expression by binding specific cellular mRNA transcripts and directing them to degradation (Reviewed in [46]). The nuclear enzyme, Drosha, and the cytoplasmic enzyme, Dicer are responsible for the maturation of miRNAs in a multistep process (Reviewed in [47]). After the maturation of the miRNA, other components of the RNA induced silencing complex (RISC) are recruited, most of these being nucleases, and degrade target RNA (Reviewed in [48]). The suggested method of targeting particular transcripts comes from the miRNA sequence homology with the mRNA that is to targeted and ultimately degraded [49]. MiRNAs have been found in many species of plants, animals and even in a number of viruses, including the Epstein-Barr Virus (EBV) [50–58]. Since viral genomes are extremely compact, utilization of both the positive and negative sense strands seems to be the ideal way to capitalize on their limited regulatory and coding capacity space.

3.3. Mechanisms of RNA-Directed Epigenetic Modification

It is thought that the main mechanism for ncRNA directed epigenetic modifications occurs both in *cis* and in *trans* through specific RNA:DNA and likely RNA:RNA interactions (in the case of *trans*).

Cis-acting RNAs are localised to their point of transcription and act directly on surrounding genes on the same chromosome. Acting in *cis*, it is likely that ncRNAs are transcribed with a histone-modifying protein-binding site, such as the histone methyltransferase PRC2. The PRC2 protein is recruited to the lncRNA while the RNA is still tethered to its transcription site by RNA polymerase II. Transcription factors such as YY1 are then recruited which clamp the hanging protein:ncRNA complex to the chromatin (Reviewed in [59]). The chromatin-modifying protein is then free to induce epigenetic changes such as histone methylation. It has been shown that up to 20% of lincRNAs associate with the histone methyltransferase PRC2 [60].

Trans-acting RNAs differ to this as they are not tethered to their site of synthesis but rather are able to move freely and act on genes that are on different chromosomes, some distance away [45]. The ncRNAs are able to recruit histone-modifying proteins and act as a scaffold by which these proteins can associate and form complexes [61]. The mechanism by which *trans*-acting ncRNAs target certain genetic regions to be epigenetically altered is debatable. While there are examples of targeting through sequence homology, whereby the ncRNA forms a DNA:RNA hybrid with the complementary target gene, this may not always be the case. The lncRNA HOTAIR has been shown to interact with up to 850 genome wide target [62] suggesting it is not sequence-specific but that the RNA itself has a structural role in the scaffold. Another potential hypothesis is that the lncRNA interacts with an RNA intermediate at the gene target site. These promoter associated RNAs, named such as they are derived from the promoter of the target gene, have been shown to be mechanistically relevant in antisense RNA (asRNA) directed transcriptional gene silencing [63] and thus have been included as a potential mechanism for lncRNA-directed epigenetic modification. The precise mechanism or combination of mechanisms for *trans*-regulation of epigenetic modification is still yet to be elucidated and may be multifactorial. For a more detailed review on the mechanism of ncRNA-directed epigenetic silencing see Beisel and Paro, 2011 [64].

4. HIV and Non-Coding RNAs

It is important to recognize the multiplicity of complex regulatory mechanisms surrounding HIV-1 transcriptional and translational control in order for us to decipher the puzzle of HIV viral latency and reactivation. In addition to chromatin remodeling complexes and the direction of the LTR, other regulatory means are present to control HIV-1 viral expression.

4.1. HIV and asRNAs

Gene expression of retroviruses usually occurs by the transcription of a single transcript, which may be differentiated through splicing events. Transcription is initiated from the 5' end in the LTR, where there are many of the necessary binding sites for transcription factors and regulators of gene expression (Figure 1). With the discovery of new antisense transcripts generated in the HTLV-1 retrovirus [65–67], the long-held belief that retroviruses transcribe their genome in a single direction has become redundant and naturally it has been speculated that a similar antisense transcript may exist

in other retroviruses such as HIV-1. The possible existence of an antisense protein (ASP) was first proposed by Miller using computational models and analysis of highly conserved regions of HIV-1 proviral DNA [68]. Since then, an asRNA coding for a putative ASP have been characterized [69], however the existence of an actual ASP protein has not yet been determined [70]. Furthermore, it was shown that the regulation this asRNA exerts over the proviral genome may not be due to the ASP, but rather the asRNA itself [71]. Therefore although the existence of the ASP is debatable, there is a growing evidence suggesting a role for the HIV expressed asRNA in controlling HIV-1 expression [72–74]. Supporting this notion a recent study by Kobayashi-Ishihara *et al.* has demonstrated that asRNAs may have a regulatory role in HIV-1 viral replication, in particular, regulation of positive sense transcription [75]. These data suggest that the aforementioned asRNA may actually be reclassified as a lncRNA due to its functions in gene regulation, however further studies will be required to elucidate the true function of this HIV expressed asRNA in regulating HIV.

Chromatin remodeling has already been described as an important mechanism for the regulation of viral latency in HIV. Chromatin remodeling in humans is facilitated, at least in part, by lncRNAs that have the ability to form RNA-protein complexes with histone modifying proteins and direct them to the target region (Reviewed in [76–78]). Although no lncRNAs have yet been discovered in HIV, their presence in a wide variety of organisms and their role in chromatin remodeling and gene expression would suggest that this is an area of potential interest when considering a possible driving mechanism in HIV latency.

4.2. HIV and miRNAs

In addition to the long asRNAs being utilized by viruses, miRNAs have also been found to be produced by viruses such as EBV and HSV-1. These miRNAs appear to be involved in the post-transcription regulation of viral mRNA transcripts and also modulating host cell gene expression, essentially with the virus hijacking host miRNA pathways for its own benefit and survival ([55], and reviewed in [79]). MiRNAs are generated from imperfect stem-loop precursors by the enzymes Drosha and Dicer. HIV-1 contains two of these such structures, TAR and RRE [80] so it seems reasonable to extrapolate that it may be able to act in the same way as other viruses, using host machinery to process its own miRNAs. A study by Benasser *et al.* hypothesized, using computational analysis that HIV was also capable of generating up to ten viable viral miRNAs (vmiRNAs) [81]. Further studies have demonstrated that the HIV TAR element is bound by and processed by host-derived Dicer to yield a vmiRNA [82–84]. This vmiRNA, processed from the HIV-1 TAR element, was found to inhibit LTR-driven gene expression, potentially through sequence homology to the TAR element and transcriptional gene silencing [82]. It has also been suggested that HIV may produce vmiRNAs as a part of its pathogenic mechanism. Findings indicate that the HIV-1 proviral genome may have the capacity to produce inhibitory vmiRNAs that interfere with host immunity [85]. These vmiRNAs have been found to theoretically have the ability to block translation of factors such as CD28, CD4 and some interleukins [85]. Supporting this model, HIV-1 has also been shown through deep sequencing

analysis, to produce viral small interfering RNAs (vsiRNAs) to further modulate both cellular and viral gene expression [86,87].

5. Final Remarks and Future Work

HIV-1 is a complex retrovirus with many regulatory mechanisms in place to control latency, reactivation and gene expression. Although these mechanisms are not yet fully understood, there has been a trend toward widening the scope of the search. Chromatin remodeling complexes and the mechanisms in which they are directed is under particularly intense study due to the increasing amounts of data implicating them as important factors in gene expression. Among the masses of data, lncRNAs seem to be particularly well represented as regulators of chromatin complexes. Further investigation into this area may see the emergence of previously unknown ncRNAs, be they miRNAs, or lncRNAs, in the epigenetic regulation of viral expression and host-virus interactions.

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Conflicts of Interest

The authors declare no conflict of interest.

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Review

Deregulation of Epigenetic Mechanisms by the Hepatitis B Virus X Protein in Hepatocarcinogenesis

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Abstract: This review focuses on the significance of deregulation of epigenetic mechanisms by the hepatitis B virus (HBV) X protein in hepatocarcinogenesis and HBV replication. Epigenetic mechanisms, DNA methylation, and specific histone modifications, e.g., trimethylation of H3 on lysine-27 or lysine-4, maintain 'cellular memory' by silencing expression of lineage-inducing factors in stem cells and conversely, of pluripotency factors in differentiated cells. The X protein has been reported to induce expression of DNA methyltransferases (DNMTs), likely promoting epigenetic changes during hepatocarcinogenesis. Furthermore, in cellular and animal models of X-mediated oncogenic transformation, protein levels of chromatin modifying proteins Suz12 and Znf198 are down-regulated. Suz12 is essential for the Polycomb Repressive Complex 2 (PRC2) mediating the repressive trimethylation of H3 on lysine-27 (H3K27me3). Znf198, stabilizes the LSD1-CoREST-HDAC complex that removes, via lysine demethylase1 (LSD1), the activating trimethylation of H3 on lysine-4 (H3K4me3). Down-regulation of Suz12 also occurs in liver tumors of woodchucks chronically infected by woodchuck hepatitis virus, an animal model recapitulating HBV-mediated hepatocarcinogenesis in humans. Significantly, subgroups of HBV-induced liver cancer re-express hepatoblast and fetal markers, and imprinted genes, suggesting hepatocyte reprogramming during oncogenic transformation. Lastly, down-regulation of Suz12 and Znf198 enhances HBV replication. Collectively, these observations suggest deregulation of epigenetic mechanisms by HBV X protein influences both the viral cycle and the host cell.

Keywords: Hepatitis B virus (HBV); HBV X protein; Hepatocellular Carcinoma (HCC); HBV replication; epigenetic regulation; DNA methylation; DNA methyl transferases (DNMTs); Polycomb Repressive complex 2 (PRC2); Suz12; suppressor of zeste 12 homolog (Drosophila); Znf198; zinc finger; MYM-type 2; LSD1-Co-REST-HDAC1

1. Introduction

Chronic hepatitis B virus (HBV) infection is a major etiologic factor in pathogenesis of hepatocellular carcinoma (HCC) [1,2]. Despite an HBV vaccine, the World Health Organization estimates 400 million people globally are chronically infected with HBV, and HCC has become the fifth most common cancer world-wide [3,4]. Liver tumors are highly heterogeneous, differing in genetic alterations, treatment, and prognosis [5]. Global transcriptome analyses of human liver tumors have identified distinct subgroups of HCCs associated with specific genetic changes and clinical prognosis [6]. In this review, of special interest is the HCC subgroup G1, identified and characterized by the transcriptome studies of Boyault *et al*. [7]. The G1 subgroup is associated with low titer HBV infection, high rate of chromosomal instability, poor prognosis, and high expression levels of AFP (alpha-fetoprotein), imprinted genes (IGFII, H19, PEG3 and PEG10), and transcription factor SOX9, a key regulator of pancreatobiliary ductal system development. Given that classification of tumors by histologic markers does not identify the cellular origin of the tumor, *i.e.*, differentiated *vs.* progenitor cell, in this review I explore mechanisms that could mediate the upregulated expression of the hepatoblast/fetal markers and imprinted genes observed in the G1 subgroup of HBV-mediated HCCs.

2. The HBV Life Cycle

Hepatitis B virus (HBV) is a small enveloped virus belonging to the hepadnavirus family [8]. It infects human hepatocytes causing acute and chronic liver infection and disease. Epidemiologic studies have established that chronic HBV infection, occurring in less than 5% of HBV infected patients, is associated with high risk of developing hepatocellular carcinoma by the fourth or fifth decade of life [1].

The HBV genome is partially double stranded and comprised of 3.2 Kb. It encodes the pre-S/S (surface antigen), the pre-C/C (core protein), the P (viral polymerase) and X open reading frames. The X protein is essential for viral transcription and replication [9,10]. Following infection of hepatocytes by the hepatitis B virus, the viral nucleocapsids are transported to the nuclear membrane where they become disassembled, releasing the viral genome into the nuclear compartment [11]. At this stage, the viral genome takes the form of relaxed, circular (RC), partially double-stranded DNA. The RC DNA is subsequently converted into covalently closed, circular DNA (cccDNA) which serves as template for transcription of the pregenomic RNA (pgRNA) and the RNA species that encode the viral proteins [12]. In turn, the pgRNA serves as the template for synthesis of the viral genome by

virus-encoded reverse transcriptase [13]. This step occurs in viral capsids in the cytoplasm [14]. Importantly, the cccDNA in the nucleus of infected cells forms chromatin-like structure by association with nucleosomes [15]. Moreover, it has been clearly demonstrated that the histone modifications associated with the viral mini-chromosome determine its transcriptional activation or repression. Specifically, chromatin immunoprecipitation assays of the cccDNA/mini-chromosome have demonstrated that lysine acetylation of H3 and H4 correlates with viral replication which depends on transcription of the pgRNA, and the level of viremia in HBV-infected patients [16–18]. These results imply that changes in the activity of chromatin modifying complexes (*i.e.*, those that mediate epigenetic histone modifications in infected hepatocytes), influence the rate of transcription from the viral mini-chromosome, and in turn the rate of HBV replication.

3. HBV and Hepatocellular Carcinoma (HCC)

Chronic infection by HBV is associated with increased risk of liver cancer development (1). Factors contributing to pathogenesis of HBV-mediated HCC include chronic liver inflammation due to continued hepatocyte death and regeneration [19]. In addition, the functions of viral proteins X [20,21], S [22] and core [23] are likely contributors to HBV-mediated hepatocarcinogenesis. In this review I focus on the role of the X protein in HBV-mediated hepatocarcinogenesis.

HBV DNA integrates into the host genome at early steps of clonal tumor expansion, the majority of tumors displaying continued expression of pX [24]. Genomic aberrations frequently occur in HBV-mediated HCC [25], but the mechanism(s) that initiate and propagate these genomic changes are not yet understood. pX is a multifunctional protein essential for the viral life cycle. Involvement of the X protein in HCC pathogenesis is supported by data from animal models revealing pX acts as a weak oncogene or a co-factor in hepatocarcinogenesis [20,21]. pX activates cellular mitogenic pathways and induces transcription of select viral and cellular genes [26,27], thereby influencing cellular proliferation mechanisms. [The reader also is referred to recent comprehensive reviews of X protein functions and HBV-mediated hepatocarcinogenesis [28,29].

Pertinent to the weakly oncogenic potential of the X protein, it was shown in a cellular model of immortalized hepatocytes that inducible expression of pX promotes DNA re-replication-induced DNA damage, propagation of damaged DNA to daughter cells, and generation of partial polyploidy (>4 N DNA) [30,31]. In this cellular model, it was determined pX prematurely activates the mitotic polo-like-kinase1 (Plk1) in the G2 phase [32]. Plk1 is a cell cycle regulated gene [33], required for checkpoint recovery after completion of DNA repair [34], allowing mitotic entry and cell cycle progression [35]. Significantly, Plk1 is over-expressed in many human cancers, including liver cancer [36], and this up-regulation correlates with poor cancer prognosis. Elevated expression of Plk1 and of a cluster of proliferation genes was also observed by microarray analyses of human HCCs, including liver tumors from chronic HBV patients [37]. Importantly, in an *in vitro* cellular model of pX-mediated hepatocyte transformation, inhibition of Plk1 suppressed transformation [31], underscoring the importance of this enzyme in HBV-induced HCC. Recent studies have linked the increased expression of Plk1 during HCC progression to down-regulation of miR-100, a microRNA that

targets Plk1 [38]. Whether miR-100 becomes down-regulated in HBV-mediated HCCs remains to be determined.

Proteomic studies have shown Plk1 phosphorylates substrates involved in a wide variety of processes [39]. A classic substrate of Plk1 is the protein claspin that participates in checkpoint activation by ATR [33]; in turn, upon completion of DNA repair, the G2/M DNA damage checkpoint is terminated by proteosomal degradation of claspin [34]. This step is initiated by phosphorylation of claspin by Plk1 at phosphodegron sites, signaling ubiquitination of the substrate and proteosomal destruction [34]. The mechanism by which Plk1 induction promotes pX-mediated oncogenic transformation remains to be determined.

4. Chromatin Modifying Proteins Suz12 and Znf198 in the X Protein Signaling Network

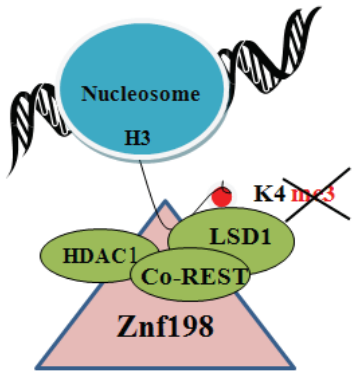
A genome-wide siRNA library screen performed in pX-expressing cells led to identification of nuclear proteins Suz12 and Znf198, both components of chromatin modifying complexes, as downstream players in the pX signaling network [40]. Specifically, siRNA-mediated knockdown of these proteins rescued X-expressing cells from DNA damage induced apoptosis, suggesting a role of these proteins in X-mediated hepatocyte transformation. Loss-of-function of Znf198 and Suz12 has been described in other human cancers [41,42]. More importantly, the ZNF198 gene located on chromosome 13q12.11 is frequently affected (~30% of cases) by loss of heterozygosity in early onset HCC, correlating with high tumor grade and HBV-related tumors [43,44]. Both Suz12 [45] and Znf198 [46] associate with promyelocytic leukemia (PML) nuclear bodies (NBs) [47]; Suz12 and Znf198 knockdown cell lines exhibit reduced numbers of PML NBs [40].

PML NBs are dynamic nuclear structures that change in composition during the cell cycle [47]; are sites of epigenetic regulation [48,49], p53 function [50], DNA repair [51]; and play a role in the innate immune response [52] and viral replication [53]. Indeed, Suz12 and Znf198 knockdown cell lines, similar to PML knockdown cell lines, exhibit reduced numbers of PML NBs, loss of p53-mediated apoptosis, and loss of DNA repair [40]. Moreover, knockdown of PML, Suz12, and Znf198 proteins by siRNA transfection enhances HBV replication [40], in a cellular model of HepAD38 cells engineered to support HBV replication in a tetracycline- inducible manner. Viruses employ various mechanisms to disrupt PML function, favoring enhanced viral replication [54] For example, an early event in Herpes Simplex Virus1 (HSV1) replication, mediated by the ICP0 protein, is dislodging of HDAC1/2 from the LSD1-CoREST-HDAC complex [55,56]. In HBV replication, PML NBs become altered in both number and morphology [57]. Furthermore, HBV replication depends on modifications of H3 and H4 associated with cccDNA, the template of pgRNA transcription [16–18]. As described above, pgRNA transcription determines the rate of HBV replication [8], and HDAC1 associated with cccDNA correlates with decline in HBV replication [18].

Interestingly, Znf198 stabilizes the LSD1-CoREST-HDAC1 chromatin modifying complex [58] that removes histone modifications associated with transcriptional activation (Figure 1). Specifically, lysine demethylase1 (LSD1) removes the activating trimethylation of histone 3 on lysine-4 (H3K4me3), and histone deacetylase 1(HDAC1) removes the activating acetylation of histones. Thus,

loss of Znf198, destabilizing the LSD1-CoREST-HDAC1 chromatin modifying complex, will maintain the transcriptionally activating histone modifications of the host chromatin as well as the viral mini-chromosome, providing yet another example of how viruses hijack cellular mechanisms to the advantage of their life cycle.

Figure 1. Diagram shows Znf198 interacting and stabilizing the LSD1-Co-REST-HDAC1 complex [58]. LSD1 removes the transcriptionally activating trimethylation of H3 on K4. HDAC1 removes the transcriptionally activating acetylation of histones.



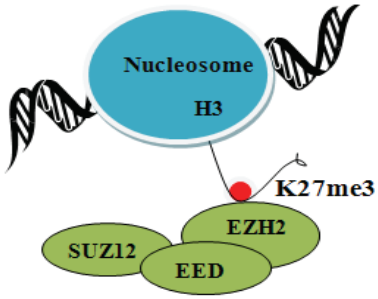
5. Suz12 Containing PRC2 Complex

Suz12 is an essential component for activity of the polycomb repressive complex PRC2, comprised of histone methylstransferase EZH2 and EED [59]. The PRC2 complex (Figure 2) mediates the trimethylation of histone 3 on lysine27 (H3K27me3), a mark of transcriptionally silent chromatin [60,61]. The PRC2 chromatin modifying complex regulates expression of many developmental and signaling genes in embryonic stem cells (ESCs). Accordingly, the PRC2 complex was proposed to have a role in the maintenance of stem cell pluripotency [62–65]. However, additional studies have refined our understanding of the role of the PRC2 complex in ESCs. Specifically, PRC2-deficient ESCs failed to maintain expression of lineage-specific genes, exhibiting impaired differentiation, lack of repression of ESC markers, and lack of activation of differentiation-specific genes [66]. These observations were interpreted to mean the PRC2 complex regulates cell fate transitions during lineage commitment [66–68].

In human embryonic fibroblasts, more than 1,000 genes are transcriptionally silenced by the PRC2 complex [62]. In untransformed hepatocytes, Suz12 chromatin immunoprecipitation assays identified a subset of genes that are repressed by the PRC2 complex, including: BAMBI, CCND2, DLK1, EpCAM, and IGFII [69]. Interestingly, Suz12 occupancy at the promoters of these genes was significantly reduced in X-transformed cells, and up-regulated expression of these genes was detected in liver tumors from animal models of X-mediated and HBV-mediated hepatocarcinogenesis [69,70]. Significantly, these genes are up-regulated in the G1 subgroup of HBV-mediated HCCs [7] and also

are expressed in hepatic cancer initiating/stem cells [71]. Intriguingly, these genes are also markers of normal hepatoblasts [70], raising a question of the mechanism allowing re-expression of hepatoblast markers during oncogenic transformation of differentiated hepatocytes. One might predict up-regulation of genes supporting re-establishment of the hepatoblast phenotype, resulting in lineage mis-specification and reversal of the differentiated phenotype.

Figure 2. Diagram shows the essential components of the PRC2 complex, namely SUZ12, EED and EZH2. EZH2 is the histone methyltransferase enzyme that trimethylates H3 on K27. This modification silences transcription.



In this dynamic process of lineage change as in hepatocyte de-differentiation, it is not understood how the PRC2 complex selects genes for repression. Although several possibilities exist including interaction of the PRC2 complex with specific transcription factors, the PRC2 complex binding to specific long, noncoding RNAs [72] as well as to short RNAs transcribed from the 5' end of genes repressed by the PRC2 complex [73], suggests a likely mechanisms for target gene selection. For example, in embryonic stem cells, the PRC2 complex regulates expression of the imprinted DLK1 gene via interaction with the long noncoding RNA Gtl2 serving as cofactor [74]. Interestingly, the DLK1 gene is expressed in hepatic progenitors and hepatoblasts [70], as well as in the G1 subgroup of HBV-mediated HCCs [7]. Moreover, poor prognosis, HBV-mediated liver tumors, referred to as the C3 subgroup in the study by Toffanin *et al* [74] also re-express EpCAM and AFP, and exhibit elevated expression of a cluster of microRNAs encoded by the imprinted DLK1-DIO3 region. Taken together, both of these studies [7,74] have identified the re-expression of hepatoblast/fetal genes in the G1/C3 subgroups of HBV-mediated HCCs, significantly, genes repressed by the PRC2 complex in differentiated hepatocytes. Furthermore, these studies suggest the down-regulation of the PRC2 complex or of its activity in the G1/C3 subgroups of HBV-mediated HCCs, raising the question of how the PRC2 complex is regulated.

6. Regulation of the PRC2 Complex

Studies by independent groups have identified several mechanisms by which the PRC2 complex is regulated. (1) Suz12 protein can be down-regulated via the action of microRNAs. Specifically miR-200 targets the 3'UTR of Suz12 [75]; (2) Ezh2, the component of the PRC2 complex containing the histone methyltransferase enzymatic activity, is inactivated by Akt-mediated phosphorylation [76]. Importantly, poor prognosis HBV-mediated HCCs, such as those of the G1 subgroup, exhibit enhanced activation of Akt [7]; (3) Loss of the protein JARID, enabling association of the PRC2 complex with target genes [77]; (4) Induction of the H3K27 demethylase (JMJD3 or KD6M) could also result in removal of the transcriptionally repressive H3K27me3 modification, likely resulting in re-expression of the targeted genes. Interestingly, recent studies have shown induction of JMJD3 in Epstein Barr virus infected cells and associated Hodgkin's lymphoma [78]; (5) Lastly, an inverse relationship was noted between protein levels of Plk1 and Suz12/Znf198 in X-induced tumorigenesis [69], suggesting Suz12 and Znf198 are down-regulated via Plk1-mediated phosphorylation, promoting their proteasomal degradation. Suz12 is phosphorylated *in vivo* at a Plk1 consensus site [79] and both Suz12 and Znf198 are *in vitro* substrates of Plk1 (unpublished data). Since Plk1 is up-regulated in HBV-mediated HCCs, and the X protein activates Plk1, these chromatin modifying proteins undergo transient down-regulation during G2 and M phases of the cell cycle. The down-regulation of Suz12 and Znf198 suggests that the respective chromatin modifying complexes are concurrently inactivated. Importantly, the LSD1-CoREST-HDAC1 complex and the PRC2 complex are tethered together via the lincRNA HOTAIR, indicating that their activities are coupled, likely acting on the same gene [80]. In ESCs, genes that contain both H3K27me3 and H3K4me3 modifications are described as "poised or bivalent" genes, awaiting activation [81]. Down-regulation of these chromatin modifying complexes induces re-activation of specific genes in the respective knockdown cell lines, during progression to X-induced transformation and in animal models of X- and HBV-mediated liver cancer development [69].

The consequence of chronic HBV infection and continued expression of X, mediating down-regulation of these proteins through each cell cycle of an HBV infected hepatocyte, for many cell generations, is not yet understood. The gene expression program of differentiated cells is maintained after cell division by propagating the identical "chromatin landscape" of the parental cell to the daughter cell via heritable histone modifications known as epigenetic marks [82]. Recent studies in *Drosophila* have shown that propagation of the heritable chromatin landscape through cell division involves chromatin-modifying proteins including the Enhancer-of-Zeste, found to associate with newly synthesized DNA [83]. It was proposed that these histone modifying enzymes re-establish the heritable histone modifications on newly assembled unmethylated histones, serving as the epigenetic mark. Whether the PRC2 complex also serves as the epigenetic mark in mammalian cells is unknown. If that turns out to be the case, the down-regulation of Suz12 and Znf198 by pX in HBV infected hepatocytes not only alters the gene expression pattern of the hepatocyte, but its down-regulation has the potential to propagate to the subsequent cell generations an altered chromatin landscape.

7. Distinct Histone Modifications and DNA Methylation Landscapes in Pluripotent *vs.* Differentiated Cells

In addition to histone modifications described above, DNA methylation is another heritable modification that is linked to gene activity and maintenance of cellular memory. For example, DNA methylation at the promoter of the pluripotency factor Oct4 in a differentiated cell maintains the Oct4 gene repressed, ensuring maintenance of the differentiated state [84]. Large scale analyses examined the link between DNA methylation and histone modifications, showing that the methylation state of H3K4 is a good predictor of promoter DNA methylation. Specifically, methylation of H3K4 disrupts promoter DNA methylation by inhibiting contact between DNA methyl transferases (DNMTs) and histones [85,86]. Global comparison of the chromatin landscape between hESCs and differentiated cells [87] using Chip-Seq approaches showed the following: (1) Differentiated cells display extensive regions with repressive histone modifications (H3K27me3 and H3K9me3) in comparison to pluripotent cells; (2) In differentiated cells there is an association of H3K27me3 with promoter hypomethylation, also suggesting a connection between histone modifications and DNA methylation. Indeed, liver differentiation *in vivo* is characterized by specific demethylation in regions enriched in H3K27me3 [88]; (3) Transcribed exons in gene regions associated with H3K36me3 exhibit DNA hypermethylation [87]. Together, these results emphasize a strong connection between histone modifications and DNA methylation in determining cellular memory. Consequently, alterations in both histone modifying enzymes as well as in DNA methyltransferases (DNMTs) have the potential to alter the heritable epigenetic landscape of the cell, thereby altering its cellular memory and identity.

8. DNA Methylation and the X Protein

Several studies have reported increased levels of DNMT1, DNMT3A, and DNMT3B and aberrant DNA methylation in HBV infected cells and HBV-mediated liver tumors [89–91]. DNMT3A and DNMT3B are *de novo* DNA methyltransferases, and DNMT1 maintains the DNA methylation pattern of the daughter strand during DNA replication, and is referred to as the maintenance methyltransferase [92]. DNMTs catalyze the addition of a methyl group to the cytosine ring of the 5'-CpG dinucleotide.

The HBV X protein was reported to induce transcription of DNMT1, DNMT3A, and DNMT3B genes [89] and to directly interact and activate the *de novo* methyltransferase DNMT3A [90]. Specific gene promoters were shown to be methylated in HBV infected cells including the IL-4, metallothionein-1F, IGFBP3, SUFU, and TIRAP [90,93]. Moreover, these DNMTs also methylate the viral DNA, resulting in a reduction in viral replication [93–95]. The significance of these DNA modifications in the pathogenesis of HCC is not understood. Recent technological advances for identification of global CpG methylation patterns have been reported [96] and will undoubtedly be applied to determine the DNA methylome of HBV-mediated HCCs. It will be interesting to explore the link between pX-mediated down-regulation of the PRC2 complex and induction of DNMTs in the cellular reprogramming of HBV infected hepatocytes.

9. Conclusion

Since HBV infection occurs in differentiated hepatocytes, requiring the transcriptional activity of hepatocyte-specific transcription factors [97], it is intriguing that subgroups of HBV-induced HCCs express hepatoblast and fetal markers.

The evidence presented in this review discussed the down-regulation of Suz12/PRC2 and Znf198/LSD1-CoREST-HDAC1 chromatin modifying complexes in X-mediated tumorigenesis [69] and the induction of the DNMTs in HBV-induced HCCs [89–91]. A third epigenetic mechanism that is likely involved in this cellular reprogramming is deregulation of microRNAs, a topic not included in this review. In the cellular context of the HBV-infected hepatocyte, it is likely that the combination of the enhanced DNMTs expression, down-regulation of the two chromatin modifying complexes and deregulated expression of microRNAs induce epigenetic reprogramming of the differentiated hepatocyte into a de-differentiated state resembling a hepatoblast/hepatic progenitor cell. This leads to re-expression of hepatoblast, fetal and imprinted genes observed in the G1 subgroup of HCCs [7] along with acquisition of enhanced proliferative potential resembling self-renewing stem cells. Based on this scenario, I propose that this combination of X-induced epigenetic processes gives rise to hepatic cancer initiating stem cells. Recent studies have established a specific molecular sequence of events mediating cellular reprogramming of somatic cells into the induced pluripotency (iPS) cells [98]. Whether epigenetic reprogramming of chronically HBV-infected hepatocytes to HCC is a random or mechanism-specific process remains to be determined.

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Conflicts of Interest

The author declares no conflict of interest.

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