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Role of Chromatin During Herpesvirus Infections

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The last two decades have witnessed exciting developments in our knowledge of how eukaryotic gene transcription is regulated. Early developments focused on the cis-regulatory elements associated with specific gene promoters and on the trans-acting factors that bind to these elements. More recent progress has revealed dynamic aspects of chromatin structure and the mechanisms whereby chromatin and its modifications influence gene expression.

DNA viruses have long served as model systems to elucidate various aspects of eukaryotic gene regulation, due to their ease of manipulation and relatively low complexity of their genomes. In some cases, these viruses have revealed mechanisms that are subsequently recognized to apply also to cellular genes. In other cases, viruses adopt mechanisms that prove to be exceptions to the more general rules. The double-stranded DNA viruses that replicate in the eukaryotic nucleus typically utilize the host cell RNA polymerase II (RNAP II) for viral gene expression. As a consequence, these viruses must reckon with the impact of chromatin on active transcription and replication. Unlike the small DNA tumor viruses, such as polyomaviruses and papillomaviruses, the relatively large genomes of herpesviruses are not assembled into nucleosomes in the virion and stay predominantly free of histones during lytic infection. In contrast, during latency, the herpesvirus genomes associate with histones and become nucleosomal, suggesting that regulation of chromatin *per se* may play a role in the switch between the two stages of infection, a long-standing puzzle in the biology of herpesviruses.

In this review we will focus on how chromatin formation on the herpes simplex type-1 (HSV-1) genome is regulated, citing evidence supporting the hypothesis that the switch between the lytic and latent stages of HSV-1 infection correlates with changes in the chromatin state of the HSV-1. Before going into the details of HSV-1, we will briefly summarize some of the recent advancements in regulation of chromatin and transcription by RNAP II as it pertains to the rest of this review.

1. Transcription in eukaryotes

Eukaryotic DNA is packaged in the form of nucleosomes, whereby approximately 147 bp of DNA is wrapped around a protein octamer that consists of two copies of each core histone (H2A, H2B, H3 and H4). Further compaction of nucleosomes is mediated by the linker histone H1 and other non-histone proteins. Although RNAP II can transcribe efficiently *in vitro* from naked DNA templates, the packaging of DNA into nucleosomes inhibits transcription. The

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past few decades have witnessed great progress in our understanding of how the inhibitory effect of chromatin on transcription can be overcome. Four principal mechanisms include the covalent modification of histone tails and globular domains, remodeling of nucleosomes, incorporation of histone variants, and removal or disruption of nucleosomes at actively transcribed genes. These four general mechanisms will be described briefly before turning to the role of chromatin and its modification during herpesvirus infections.

1. Histone modifications and transcription

Covalent or post-translational modifications of the amino-terminal tails of core histones have been extensively characterized (84), although the globular domains can also be modified (124,141,178,183,186). The most prominent covalent histone modifications include acetylation, methylation, ubiquitinylation, phosphorylation, and prolyl isomerization (84). In many cases, specific modifications on specific residues of particular histones have been correlated as either positive or negative markers of transcriptional activity (84). Genome-wide studies that employed chromatin immunoprecipitation (ChIP) assays coupled with DNA microarrays or high-throughput sequencing have shown that particular modifications are predominantly localized to distinct regions of target genes, such as the upstream regulatory regions, core promoters, or the 5' and 3' portions of the transcribed regions (144). For instance, histone H3 acetylated on lysines 9 and 14 (H3K9/K14ac) localizes to the promoter and 5' ends of actively transcribed genes. Methylation of histone H3 can be an indicator of either active or inactive transcription, depending on which lysine residue is modified. Histone H3 methylation also follows a distinct pattern of localization through the body of a gene; for example, H3K4me3 is mainly present around the transcriptional start site, whereas H3K36me3 is localized towards the middle and 3' ends of actively transcribed genes. Other H3 methylation marks, such as H3K9me3 or H3K27me3, are strictly associated with inactive transcription and are observed over broad regions of silenced genes.

In parallel with the identification of covalent histone tail modifications has come the discovery of the corresponding enzymes that catalyze these reactions. For instance, histone methyltransferases are rather specific for the target lysine or arginine residue. Histone acetylation is somewhat less specific: a given histone acetyltransferase (HAT) might modify several residues, and several different HATs might have overlapping substrate specificities. For instance, the HATs p300, CBP, and PCAF can all acetylate H3K14 (105,115,158). As a rule, the covalent marks are reversible by enzymes such as histone deacetylases (HDACs) and lysine demethylases, indicative of the highly dynamic nature of chromatin modifications and multiple potential levels of transcriptional regulation (84).

Covalent modifications of histones are thought to have two principal consequences. The first is the direct impact of modification on higher-order chromatin structure. For instance, the loss of positive charge on lysines upon acetylation is associated with relaxed chromatin structure (84). The second potential outcome is the recognition of specific histone modifications by other proteins that function as transcription factors or coactivators. Two examples of such mechanism are proteins containing bromodomains, which bind to acetylated lysines, and proteins containing chromodomains, which bind to methylated lysines (25,161). Since a number of bromo- or chromodomain-containing proteins are themselves chromatin-modifying enzymes, this recognition enables the propagation or cooperativity of histone modifications and chromatin remodeling (25,161).

2. Chromatin remodeling and transcription

The second major class of chromatin-modifying factors comprises protein complexes that utilize ATP hydrolysis to induce changes in the positions of nucleosomes on DNA and hence are called chromatin-remodeling complexes. Chromatin remodeling may result in sliding of

the nucleosomes on DNA, DNA looping on the nucleosome particle, or histone octamer transfer *in trans* (40,154).

Several families of chromatin remodeling complexes have been identified. The prototypes of these families include SWI/SNF, ISWI, INO80, and NURD/Mi-2/CHD, all of which contain an ATPase subunit and have both similar and distinct functions. For instance, the ISWI and NURD/Mi-2/CHD families are both involved in transcriptional repression, yet a separate function of the ISWI family is to induce ordered chromatin assembly. The SWI/SNF family, on the other hand, is primarily associated with active transcription. In mammals, remodelers of the SWI/SNF family are represented by two separate complexes that have hBRM and BRG1 as their ATPase subunits. In addition to their role in transcription, hBRM and BRG1 remodeling complexes in mammals are involved in processes such as cancer progression, differentiation, and development (154).

3. Incorporation of histone variants and role of histone chaperones in transcription

The third mechanism that influences the impact of chromatin on gene regulation is the incorporation of histone variants. Whereas the canonical core histones are each encoded by multiple genes that are expressed predominantly in the S phase of the cell cycle, histone variants are encoded by single-copy genes that are expressed independent of DNA replication. Histone variants are thought to exert their actions mainly by influencing the stability of nucleosomes or higher-order chromatin structure, but not by differential covalent modifications, as in most cases the sites for covalent histone modifications are conserved among the variants. Another theory postulates that exposure of different surface residues in histone variants may serve as binding sites for other proteins (70,72).

Although H1, H2A, and H3 have multiple variants, no histone variants have been identified for H2B and H4. The H2A variants in humans include H2A.X, H2A.Z, H2A-Bbd, and macroH2A, each of which has a distinct localization pattern and function (70,72). For instance, macroH2A is localized to the inactive X chromosome, where it is thought to contribute to heterochromatin formation. In contrast, H2A-Bbd is excluded from the inactive X chromosome and accumulates at actively-transcribed genes. H2A-Bbd is an exceptional histone variant in that it shares only 48% sequence identity with histone H2A and lacks a number of structural features characteristic of histone H2A family. As such, this histone variant is thought to participate in destabilization of nucleosomes, which then facilitates the recruitment of transcription factors and coactivators that facilitate active transcription (47). Although in yeast H2A.Z prevents the spread of heterochromatin, in higher eukaryotes it might also function in the formation of heterochromatin. The principal function of H2A.X is not in transcription but in DNA repair: phosphorylated H2A.X (γ -H2A.X) marks the regions of double-stranded DNA breaks and thus aids in recruiting the DNA repair machinery. Among the two major H3 variants, CENP-A is localized exclusively to the centromeres and contributes to formation of kinetochore and chromosome segregation. The other histone H3 variant, H3.3, differs from the canonical H3 by only a few amino acid substitutions but is expressed throughout the cell cycle and is present in transcriptionally active regions. A large number of histone H1 variants, which share a conserved core domain yet have more divergent N- and C-termini, have been identified in humans. Although histone H1 variants were initially thought to have redundant functions, recent findings indicate that they may also have specific roles in gene regulation (67).

The assembly of histones and histone variants into nucleosomes requires the activities of a number of proteins and protein complexes (59,156). CAF1 and HIRA are two such assembly factors that incorporate H3.1 (canonical histone H3) and H3.3 into nucleosomes in a replication-dependent and -independent manner, respectively. Another histone chaperone, Asf1a, interacts with both CAF1 and HIRA, and as such it is involved in both replication-dependent and -independent histone assembly. Assembly of other histone variants might also

be mediated by specific protein complexes, most of which are yet to be identified. For instance the SWR1 complex, which contains SWI/SNF-type remodeling activity, is involved in deposition of histone variant H2A.Z in yeast (119).

Other histone chaperones interact with RNAP II and contribute to overcoming the nucleosomal barrier to transcription. Histone chaperone NAP1 preferentially interacts with H2A-H2B dimers and mediates histone shuttling between nucleus and cytoplasm, as well as the removal of H2A-H2B dimers during transcription, which may allow further removal of histones and enable the passage of RNAP II. Two other chaperone-like factors, FACT and Spt6, remove and reassemble histones during elongation by RNAP II, enabling the passage of RNAP II through nucleosomes while maintaining the structure of chromatin and inhibiting cryptic transcripts (6,73,146).

4. Nucleosome removal during transcription

A fourth mechanism at work that might contribute to active transcription is the partial or complete depletion of nucleosomes from gene promoters or transcribed regions (61,94,187). The initial evidence that nucleosome structure is disrupted during transcription by RNAP II came from *in vitro* studies, where the absence of an H2A-H2B dimer from nucleosomes both increased the affinity for and stimulated transcription by RNAP II (48,49). In accord with these initial observations, recent *in vitro* evidence indicated that passage of RNAP II leads to the elimination of a H2A-H2B dimer from nucleosomes (78,183). In support of these *in vitro* assays are observations of increased rates of histone exchange that correlated with transcriptional activity in yeast (68) and in *Physarum* (174). Histone chaperones and chaperone-like proteins, such as FACT and Spt6 that associate with the elongating RNAP II are likely participants in this process.

Whereas H2A-H2B dimers are depleted from DNA regions traversed by RNAP II, promoters of actively transcribed or transcriptionally competent yeast genes are often relatively free of nucleosomes (32,195). Moreover, histone octamers can also be lost throughout the coding regions of highly transcribed regions in yeast (83,90,98,147,159), although another study indicated the contrary (199). High-throughput genome-wide screens have also shown that high rates of histone turnover within coding regions, but not promoters, correlate with RNAP II density (28). These findings together lead to the conclusion that active transcription by RNAP II correlates with the partial disruption or removal of nucleosomes.

2. Chromatin During Herpesvirus Infections

Herpesviruses vary greatly with respect to the cell types infected and the clinical diseases they cause, yet they share common structural features. A typical herpesvirus virion contains a linear double-stranded DNA of 120–230 kilobase-pairs packaged in an icosadeltahedral capsid. The capsid is surrounded by an amorphous protein coat, known as the tegument, and a lipid envelope in which viral glycoproteins are embedded. Common to all herpesviruses is the establishment of life-long latent infections after a phase of lytic infection. Reactivation from latency in response to any of a number of stresses results in recurrent infections. The mechanisms by which herpesviruses establish latency and reactivate remain unresolved, although various mechanisms have been proposed in recent years. In this review, we will primarily focus on the regulation of chromatin and chromatin modifications during the lytic and latent stages of a model herpesvirus, herpes simplex virus type 1 (HSV-1, also known as human herpesvirus 1 or HHV-1), as it presents a potential mechanism for the switch between the two stages of infection.

HSV-1 belongs to the Alphaherpesvirinae subfamily together with the human viruses herpes simplex virus type 2 (HSV-2 or HHV-2) and varicella zoster virus (VZV or HHV-3), and a

number of viruses that infect various animal species. HSV-1 commonly causes oral cold sores but can also cause corneal infections and encephalitis. By adulthood, most of the world population becomes seropositive for HSV-1, yet not all of those individuals present symptomatic infection.

The life cycle of HSV-1 is characterized by an initial phase of lytic infection in epithelial cells, followed by a latent phase in the neurons of the trigeminal ganglion. During the lytic phase, attachment of the virus to the host cell membrane by interaction of viral glycoproteins with cellular receptors leads to membrane fusion and the release of nucleocapsid and tegument components to the cytoplasm. The viral capsid is then transported to the nuclear pores, through which the viral genome is released into the nucleus. The major viral transcriptional activator protein, VP16, which is one of the tegument proteins, is also transported to the nucleus by mechanisms yet undefined. VP16 forms a complex with two cellular proteins, Oct-1 and HCF-1, and binds to specific *cis*-acting sequences in the promoters of immediate early (IE) genes to stimulate their transcription (189). Expression of delayed early (DE) and late (L) genes is, in turn, dependent on some of the IE proteins, such as ICP4 (140,186).

Following the release of infectious virions from epithelial cells at the primary site of infection, some HSV-1 virions infect the surrounding sensory neurons. The viral nucleocapsid is transported via retrograde axonal transport to the cell bodies of the neurons in the trigeminal ganglion, where HSV-1 establishes latency. During the latent phase of infection, the viral genome is maintained as a circular episome and viral gene expression is repressed, with the exception of the latency-associated transcript gene (LAT), which is the only gene continuously transcribed during latency. Stress stimuli, such as UV exposure or thermal injury, lead to reactivation of the virus by an unknown mechanism. HSV-1 then travels through the sensory neurons by anterograde transport and causes recurrent infections in the epithelial cells, usually at the same location as the primary infection.

Several related aspects regarding the role of chromatin in the context of HSV-1 infection have drawn significant attention in recent years. One question is how the viral genome remains substantially non-nucleosomal during lytic infection. Another issue is the mechanism of the transition from lytic infection, in which the viral genomes are predominantly histone-free, to latency, in which viral genomes are nucleosomal. A third question seeks the molecular mechanism by which the viral genomes are released from the inhibitory effects of chromatin during reactivation from latency. We and others have proposed that the regulation of chromatin on the viral genome itself may be a determining factor in the switch between lytic and latent infections. In this section, we will focus on some of the recent developments on this subject. For further insight, see the excellent recent review by Knipe and Cliffe (80).

1. Chromatin on the HSV-1 genome during lytic infection

Unlike the small DNA tumor viruses of the polyomavirus and papillomavirus families, the genome of HSV-1 is not packaged with histones in the virion particle (18,133,136); instead, the polyamine spermine provides the counterions for the phosphates of viral DNA (45). A number of years ago, nuclease-digestion studies indicated that the viral DNA remains predominantly free of nucleosomes throughout the lytic infection (100,101,121). Moreover, histones are excluded from viral replication compartments of infected cell nuclei (120,163), and GFP-tagged linker histone variants in infected cells have a higher mobility assessed by fluorescence recovery after photobleaching (FRAP) assays (20). More recently, ChIP assays employed by several groups have indicated that histones (typically represented by histone H3) are present on the viral genome at much lower levels than on cellular genes; that is, the fraction of input DNA that is immunoprecipitated by anti-histone antibodies is much lower for viral DNA than for cellular DNA present in the same sample (63,65). The low levels of histones on the viral genome can be interpreted in several ways. The first possibility is that most viral

genomes carry a few randomly-distributed histones throughout the viral genome. Alternatively, chromatin might form on a small fraction of viral genomes (perhaps as an innate defense mechanism against foreign DNA), whereas the rest of transcriptionally active viral genomes remain free of histones. A third possibility is that histone deposition on a small fraction of viral genomes might be a requirement for engaging those genomes in the transcriptional activation mechanisms typically employed for host genes. Observations that histone modifications associated with active transcription, such as H3K4me3 and H3K9/K14ac, are found on the viral genome during lytic infection (63,65,76) would be consistent with the first or third models.

Studies probing the possible mechanisms of histone depletion from and histone modifications on the viral genome during lytic infection have focused on recruitment or displacement of chromatin-modifying coactivators by viral or cellular regulatory proteins. The following sections will summarize some of these recent findings.

Role for VP16—VP16 is the main transcriptional activator of IE genes and has served as a model transcriptional activator for decades, often in artificial experimental settings in which the VP16 transcriptional activation domain (VP16 AD) is fused to a heterologous DNA-binding domain such as that from the yeast Gal4 protein (151). In both *in vitro* experiments and in transformed yeast or transfected mammalian cells, the VP16 AD can interact with basal transcription factors such as TFIID, TFIIA, TFIIB, and TFIIH (7,62). The VP16 AD can also interact with a number of transcriptional coactivators that potentiate transcription and can recruit these coactivators to promoters of target genes. Some of these coactivators include p300/CBP HATs (8,57,178,184), PCAF and GCN5 HATs (57,178,180,184), and SWI/SNF remodeling complexes (53,125,126,196). Interactions of VP16 AD with coactivators may induce decondensation of a highly compact 90 Mbp heterochromatic amplified chromosome region, independent of transcriptional activity (12). A similar study also suggested that chromatin decondensation mediated by VP16 AD is not localized but rather propagates over larger regions (>100 kbp) (177). Recruitment of SWI/SNF by VP16 AD also leads to eviction of histone octamer from reconstituted mononucleosomes and nucleosome arrays *in vitro* (53). These findings all point to an attractive model in which VP16 recruits chromatin-modifying coactivators in order to regulate chromatin formation on the HSV-1 genome during different stages of infection.

We have tested certain aspects of this model during HSV-1 lytic infection using a mutant virus strain (designated RP5) that lacks sequences encoding the VP16 AD. During lytic infection by RP5, expression of IE genes was greatly impaired (168,192), indicating the crucial role of the VP16 AD in initiating the viral gene expression cascade. Moreover, RP5 could not effectively establish latent infections in the central or peripheral nervous system of immunocompetent mice (168). ChIP studies using cells infected with RP5 and or its wild-type parent strain, KOS, indicated that the HATs p300 and CBP and the chromatin remodeling enzymes hBRM and Brg-1 are recruited to HSV-1 IE gene promoters in a manner mostly dependent on the presence of the activation domain of VP16 (63). Interestingly, p300 and CBP are also components of the ND10 structures, which assemble on the incoming viral genomes and become replication compartments at later stages of infection (97,111,118). Although ND10 structures are thought to be inhibitory for viral gene expression, the presence of p300 and CBP in these structures suggests that some components of ND10 may be beneficial for viral gene expression. ChIP assays also indicated that histone H3 levels throughout the RP5 genome were higher than on wild-type viral genomes (63).

These observations are consistent with a model in which histone deposition on the IE genes is reversed by the activities of chromatin-modifying coactivators recruited by VP16. An extension of this model predicts that the functions of these coactivators might be essential (or at least important) for effective activation of IE gene expression. This prediction was tested by

experiments in which the expression of particular coactivators was disrupted by siRNAs prior to viral infection. Contrary to the predictions, such siRNAs had little or no effect on viral IE gene expression, at either high or low multiplicities of infection (95). Even when combinations of siRNAs were used to circumvent potential redundancy among or between various classes of coactivators, viral gene expression was not inhibited. Moreover, cell lines in which various coactivators are absent or defective were fully capable of supporting viral gene expression (95). These results indicate that the coactivators tested are not essential for VP16-mediated activation of IE gene expression during lytic infection. The possibility that coactivators are required during reactivation from latency, when the viral genome transitions from a nucleosomal to a nucleosome-free state, is addressed more in detail in subsequent sections of this review.

Role for HCF-1 and histone methylation—The cellular protein HCF-1 has been known for some time as a component of the stable VP16-induced complex (VIC) on IE promoters during lytic infection (44,87,88,189). Recent evidence suggests that HCF-1 may also influence histone modifications on HSV-1 DNA. HCF-1 can interact both with the Sin3 histone deacetylase complex and with the Set1/Ash2 histone H3K4 methyltransferase complex (190), which are associated with transcriptional repression and activation, respectively. Although it seems contradictory that HCF-1 interacts with these complexes with different transcriptional outcomes, VP16 selectively binds to HCF-1 that is associated with Set1/Ash2, but not Sin3 (190). Consistent with this observation, promoters of several temporal classes of HSV-1 genes were found to associate with H3K4me3 in lytically infected cells (65). Although disruption of Set1 expression by RNAi resulted in a decrease in H3K4me3 levels on viral genes, the impact on IE gene expression was rather a modest defect and evident only at later times in infection (65). A role for HCF-1 and Set1 was also suggested by Narayanan et al. (124) for VZV IE gene expression. In this study, HCF-1 was shown to be required for the recruitment of Set1 to the VZV IE62 promoter in transfection-based assays and during lytic infection. Further studies are needed to clarify the importance of Set1 and histone methylation for viral IE gene expression.

Role for ICP0 and ND10—ICP0 is a multi-functional IE protein that may contribute to regulation of chromatin on HSV-1 DNA. Although ICP0 does not directly bind to DNA, it stimulates transcription from all kinetic classes of viral promoters (33,42,131,132,142). However, absence of ICP0 results in decreased viral gene expression only at low, but not at high, multiplicities of infection (11,15,71,150,165). In addition, the requirement for ICP0 is dependent on the cell type; for example, ICP0 is not required for productive infection in U2OS osteosarcoma cells even at low MOIs (193).

One of the ways that ICP0 may activate transcription is by stimulating the degradation of the host PML protein, leading to disruption of ND10 structures (35). ND10 structures form around the incoming viral DNA and are implicated in transcriptional repression of viral genomes (38,127). Disrupting the expression of ND10 components such as PML or Sp100 partially complemented a viral ICP0 null mutation (37,38). In contrast, overexpression of PML or blocking the ICP0-mediated disruption of ND10 structures had no inhibitory effect on viral gene expression (111). Transcriptional coactivators such as p300 and CBP, which are associated with active transcription, also colocalize with ND10 structures (97,111,118). Therefore, disruption of ND10 structures by ICP0 may not only relieve a general repression mechanism, but also may allow the relocalization of factors that may positively regulate viral transcription. Interestingly, the ICP0 protein of bovine herpesvirus 1 can associate with p300 HAT (198), but it is not clear if p300 is a partner for HSV-1 ICP0 in mediating the changes in chromatin structure in the context of lytic infection.

ICP0 may also prevent heterochromatin formation more directly by inhibiting the activity of histone deacetylases (HDACs). ICP0 interacts with several mammalian HDACs (110) and

forms a complex with the REST/CoREST/HDAC repressor complex, leading to the dissociation of HDAC1 from the complex (51,52). Although HDAC inhibitors, trichostatin A and sodium butyrate, increased viral gene expression during infection by ICP0 mutant viruses in some systems (138,139), conflicting results were obtained in the relatively non-permissive human fibroblasts, where trichostatin A had no effect on the replication of ICP0 mutant HSV-1 (37). Interestingly, a recent study indicated that the absence of ICP0 correlated with an increase in histone H3 levels and a decrease in the fraction of H3K9/K18ac on the viral genome during lytic infection, perhaps as a result of the increase in histone H3 occupancy on the viral genome rather than a decrease in histone acetylation *per se* (17). It should also be noted that unlike HDAC inhibitors which induce global changes in histone acetylation, ICP0 does not increase the acetylation of histone H4 (110). As such, whether HDACs contribute directly to the silencing of viral genomes and whether an important function of ICP0 is to block HDACs to allow viral gene expression remains an open question.

ICP0 also promotes the degradation of two histone H3 variants, the CENP-A and CENP-C kinetochore proteins, thereby inducing mitotic arrest or abnormal cytokinesis (34,109). Whether this function of ICP0 is important for the outcome of viral infection is not yet known. Given that the viral genomes are nucleosomal during latent infection and that ICP0 may play a crucial role during reactivation, one attractive hypothesis is that CENP-A and CENP-C associate with viral genomes during latent infection and that ICP0 has a requisite role in removing these proteins from the viral DNA during reactivation.

Other viral and cellular proteins—The protein kinase encoded by the viral *Us3* gene may also influence chromatin-related events, based on evidence that the Us3 kinase blocks the activity of HDAC1/2 (presumably by phosphorylation) and that the Us3 kinase can enhance expression of a reporter gene transduced into U2OS cells (138,139). However, the evidence connecting the effects of Us3 on HDACs with the effect on gene expression is at present only circumstantial.

One of the DE proteins, the single-stranded DNA binding protein ICP8, coprecipitates hBRM and Brg-1 remodeling enzymes (172). The functional consequence of this interaction during lytic infection is not yet well-defined. This association is a useful reminder that the chromatin remodeling activity of hBRM and Brg-1 may contribute to both transcription and replication at different stages of infection by depleting the histones from the viral genome.

Another mechanism that might conceivably prevent histone deposition on the viral genome during lytic infection is the formation of viral “chromatin” comprising viral proteins. The possibility that proteins other than cellular histones might associate with the viral genome is not unprecedented. For instance, during spermatogenesis conventional histones are replaced by protamines, which are rich in arginine and provide very high levels of compaction (77). In addition, the core protein VII of adenoviruses, which also replicate in the nucleus, associates with the viral genome throughout infection (191). At later stages of HSV infection both ICP4 and ICP8 accumulate in viral replication compartments (81). At present, no quantitative data exist to show whether these proteins coat the viral genomes during lytic infection to an extent that might prevent histone deposition. Moreover, given that both ICP4 and ICP8 are synthesized *de novo* in infection, this hypothetical mechanism would not keep the viral genomes free of histones at earlier stages of infection.

The preceding paragraphs discuss several viral proteins that modulate the cellular chromatin and transcription machinery. In addition, however, the nuclear architecture of the infected cell may influence heterochromatin formation on the viral genome. A recent report indicated that absence of lamin A, a major structural component of the nuclear lamina, resulted in defects in viral gene expression and replication as well as a significant increase in heterochromatin

formation on the viral genome (162). This provides an attractive model in which the localization of incoming viral genomes to specific regions in the nucleus may inherently prevent heterochromatin formation on the viral genome and provide an easy access to transcription machinery of the host. The details of such a mechanism, including any involvement by the nuclear pore complexes through which viral DNA enters the nucleus, remain fertile grounds for future investigation.

2. Chromatin during HSV-1 latency

The release of HSV-1 from epithelial cells at the primary site of infection can lead to subsequent infection of surrounding sensory neurons, with two potential outcomes. Infection of some sensory neurons by HSV-1 may result in lytic infection (41,85,181) leading to cell death and clearance of these neurons from the trigeminal ganglia. However, in another fraction of sensory neurons, latent viral infections are established in which lytic gene expression is suppressed and the latency-associated transcript (LAT) becomes the only viral gene that is continuously expressed (164). Splicing of the primary 8.3-kb LAT leads to the accumulation of two stable introns in the nucleus (39,182). Although some studies suggest that LAT encodes one or more polypeptides (30,175), the currently prevailing model asserts that LAT is not translated (31). One of the functions of LAT is to reduce the expression of lytic genes during both acute (41) and latent infections (16) in sensory neurons as well as in cultured neuronal cells (114). An exciting recent development provides insight into how LAT may mediate the suppression of lytic genes. Several microRNAs (miRNAs) were found to be encoded within the LAT primary transcript of both HSV-1 and HSV-2 (169,170,179). These miRNAs can down-regulate IE gene expression in transfection-based assays (170,179). Future work will address whether point mutations in LAT that block the down-regulation of IE gene expression indeed affect establishment of or reactivation from latency. LAT can also block apoptosis in rabbit ganglia or when expressed ectopically in cultured cells (135), although the mechanism is not yet fully defined. Absence of LAT correlates with increased productive infection and cell death in neuronal cells, which might be explained by the effects of LAT on lytic gene expression and apoptosis (176). For more insight on the functions of LAT, we refer the readers to recent reviews (9,80).

The promoter region of LAT shows neuronal specificity (5,200) and contains a TATA-box as well as regulatory elements about 700 bp upstream of the transcriptional start site (29). In addition, an enhancer that is responsible for long-term LAT expression maps downstream of the transcriptional start site (107,108). Although the transcription factors that bind to the LAT promoter have not yet been completely defined, potential regulators include ATF/CREB (75), STAT1 (86) and EGR (171). Recently, insulator-like elements that are bound by CCCTC-binding factor (CTCF) were identified upstream of the LAT promoter and in the LAT intron (3); these elements may contribute to regulation of chromatin on the viral genome during latency, as explained in more detail below.

Histone modifications during latency—In contrast to lytic infection, during latency the viral genome is assembled into nucleosomes (27) and is maintained as a circular episome (117,148,149). The promoter and the enhancer of the LAT gene associate with higher levels of H3K9/K14ac relative to the transcriptionally inactive ICP0 gene in mouse models of latency (92,93). Similarly, another active transcription mark, H3K4me2, was enriched on the LAT enhancer when compared with IE gene promoters in latently infected rabbit neurons (46). Conversely, during the establishment of latency, viral lytic genes progressively associate with H3K9me, indicative of heterochromatin formation on the viral genome (185). Prevention of heterochromatin spreading into the LAT region is thought to be mediated in part by CTCF and the insulator-like elements upstream of LAT promoter and in the LAT intron (3). In the course of reactivation from latency by explantation of infected mouse dorsal root ganglia, concomitant

with the decrease in LAT RNA abundance, H3K9/K14ac association decreases on the LAT enhancer but increases on the now transcriptionally active ICP0 promoter (2). Consistent with these findings is the observation that inhibition of HDAC activity by intraperitoneal sodium butyrate injection also results in acetylation of histones on the lytic genes and reactivation from latency in ocularly-infected mice (128).

Several groups have attempted to recapitulate *in vivo* latent infections by employing cell culture-based quiescent infections established either by infection of fibroblasts by replication-defective HSV-1 (58) or by differentiating rat pheochromocytoma cells (PC12) into neurons and infecting with wild-type HSV-1 in the presence of acyclovir (22). Using the former model, the HSV-1 genome was found to associate with heterochromatin protein HP1, but not other heterochromatin marks such as H3K9me (36). On the other hand, another study using a similar model showed that quiescent viral genomes associated with high levels of H3K9me3, and upon reactivation from quiescence, increasing levels of acetylated histone H3 were present on lytic promoters (19). In the second model of quiescence, HDAC inhibitors stimulated the production of infectious virions, suggesting a role for histone acetylation in reactivation of viral gene expression (23). In contrast, trichostatin A did not increase de-repression of quiescent HSV-1 genomes in human fibroblasts (130,141,173). These observations collectively indicate that in most systems histone modifications and the transcriptional activity of the viral genome correlate with each other. What remains uncertain is whether histone modifications cause changes in viral gene expression or if, vice versa, changes in gene expression result in altered histone patterns. In either case, the mechanistic details remain to be uncovered.

Factors that mediate the changes in chromatin on the viral genome during latency and reactivation from latency—Abundant evidence has established that, during HSV-1 latency, lytic gene expression is repressed and the viral genome (with the exception of the LAT gene) associates with heterochromatin. However, the mechanisms that mediate these changes in the chromatin structure of the viral genome during establishment of and reactivation from latency remain poorly defined. The components of the VP16-induced complex (VIC) and LAT are leading candidates as potential mediators of these processes.

Given that IE gene transcription is repressed in latently infected neurons, various studies have addressed whether latency is a result of inhibiting VIC formation on IE gene promoters. Repression of IE gene expression during latency cannot be solely attributed to the absence of VP16, as ectopic expression of VP16 did not prevent the establishment of latency in mice infected from the ocular route (160). This same study found that ectopic expression of VP16 was not sufficient to induce reactivation from latency as indicated by the absence of infectious viruses in tears and in the explanted trigeminal ganglia on infected mice (160). In contrast, another group reported that, when expressed from adenoviral vectors, VP16, ICP0, and ICP4 can each induce reactivation from latency in explanted trigeminal ganglia (56). Given that VP16 is phosphorylated on multiple serines (134), differential phosphorylation of VP16 in neuronal cells may lead to repression of IE gene expression during latency, although no evidence currently supports this possibility.

Other studies have indicated that the inhibition of IE gene expression may be due to the modulation of VIC components other than VP16, namely HCF-1 and Oct-1. HCF-1 is localized to the cytoplasm in sensory neurons *in vivo*, but is transported to the nucleus under conditions that induce reactivation of latent HSV-1 (89). Interestingly, a recent study indicated that HCF-1 localizes to Golgi apparatus in unstimulated sensory neurons, and disruption of Golgi by brefeldin A treatment leads to accumulation of HCF-1 in the nucleus, indicating that regulation of HCF-1 localization may be an important factor between the transition from latent to lytic infection (82). Given that HCF-1 was shown to be responsible for nuclear import of VP16 (96), it will be interesting to address whether VP16 is sequestered in the cytoplasm upon

infection of neuronal cells and whether VP16 is translocated to the nucleus upon reactivation from latent infection. Another mechanism that may explain the repression of IE gene transcription is the low level of Oct-1 expression in ganglionic sensory neurons (55,60). Although one hypothesis suggests that competition of Oct-2 with Oct-1 for binding to IE promoters might repress IE gene expression (74,102), very low levels of Oct-2 expression in sensory neurons argue against this possibility (55,60).

As explained in detail above, during latency, the viral genome takes a form resembling heterochromatin with the exception of the actively transcribed LAT gene. Interestingly, absence of LAT expression correlates with an increase in euchromatin and decrease in heterochromatin marks on the viral genome (185), suggesting that LAT expression may be required for the heterochromatin formation on the viral genome. In addition, absence of LAT resulted in an increase in lytic gene expression during latency (16,41,114). Therefore, it is important to distinguish whether LAT represses lytic gene expression by directly inducing the heterochromatin formation or indirectly by inhibiting lytic gene transcription, which may also lead to heterochromatinization of the viral genome. Since LAT accumulates in the nucleus at high levels without being localized to distinct foci that contain the viral genomes, it seems unlikely that LAT is directly involved in heterochromatin formation on the HSV-1 genome. The recent identification of LAT-encoded miRNAs that target IE genes (122,179) may explain how LAT induces the repression of lytic gene expression and formation of heterochromatin on the viral genome. Although LAT may participate in regulation of chromatin on the latent viral genome, it is important to note that not all latently infected neurons express LAT (116, 157) and that absence of LAT does not preclude establishment of latency (69,99). Therefore, LAT itself cannot not be the only factor that regulates viral chromatin during latent infection.

3. Chromatin during the infection cycle of other herpesviruses

The preceding sections detail the current state of understanding of the regulation of chromatin during both lytic and latent stages of HSV-1 infection. Other herpesviruses are also subject to silencing by heterochromatin formation on the viral genome during latent infections and overcome this chromatin barrier during reactivation from latency or lytic infections. The following paragraphs will summarize some of these mechanisms in human cytomegalovirus (HCMV or HHV-5), Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8), and Epstein-Barr virus (EBV or HHV-4) infections.

Cell culture (66) and *ex vivo* (145) model systems of HCMV infection, as well as *in vivo* murine CMV (MCMV) infections (106), have all indicated a mode of chromatin regulation similar to that of HSV-1 in many respects. For instance, upon establishment of latency, the major IE promoters of MCMV and HCMV associate with HP1 (106,123,145), HDACs (106,123), and histones that carry inactive transcription marks (66,106,145). In contrast, during productive infection and reactivation from latency, the major IE promoter is associated with acetylated histones (66,106,123,145). Other findings also support the idea that histone acetylation might play an important role in HCMV infections. First of all, HDAC inhibitors increase the permissiveness of otherwise nonpermissive cells for viral infection (123). Second, during lytic infection, virion protein pp71 induces the degradation of Daxx, a component of ND10 domains that repress transcription through HDACs (152,153). Third, the major IE proteins IE72 and IE86 interact with and block the activity of HDACs (129). Finally, the IE86 protein interacts with p300/CBP (64) and PCAF HATs (10) in order to modulate the cell cycle and potentiate the transcription of viral genes, respectively. These findings are consistent with a common theme observed in HSV-1 infections: during lytic infection, various viral factors block the formation of heterochromatin on the viral genome and recruit transcriptional coactivators that covalently modify the histones and induce active transcription.

The Rta/ORF50 protein of KSHV is the main viral transcriptional activator protein and triggers the latent-to-lytic infection switch in KSHV-infected cells. Like VP16 of HSV-1, Rta/ORF50 interacts with CBP, which augments the transcriptional activity of Rta in a heterologous expression system (54). Although our knowledge about chromatin and histone modifications on the KSHV genome during lytic and latent infections is limited, a few studies indicated that histone acetylation may play a role. For instance, reactivation of KSHV from latency can be mediated by sodium butyrate, an HDAC inhibitor, leading to dissociation of latency-associated nuclear antigen (LANA) from the ORF50 promoter (112,113). Concomitant with the dissociation of LANA, the ORF50 promoter associates with acetylated histones and the Brg-1 chromatin remodeling complex (112,113). LANA mediates transcriptional repression and heterochromatin formation on the viral genome likely by interacting with the mSin3A co-repressor complex (91), HP1 (104), and SUV39H1 histone methyltransferase (155). Although LANA and LAT are not genetically homologous, the repressor function of LANA resembles that of LAT, which induces heterochromatin formation on the HSV-1 genome by a currently elusive mechanism (185). A distinct feature of LANA is its interaction with histone H2A-H2B dimers, which mediates the maintenance of KSHV episomes during latency (4). An intriguing possibility is that this interaction between LANA and histone H2A-H2B may also contribute to transcriptional regulation by LANA. Interestingly, LANA also interacts with CBP; however, this interaction results in the inhibition of the HAT activity of CBP (103). The model emerging from these findings is that KSHV chromatin is regulated dynamically during lytic and latent stages of infection in a way similar to that of HSV-1. Yet while many studies have focused on histone acetylation and deacetylation as the major switch between lytic and latent KSHV infections, other covalently modified histones that correlate with active transcription, such as H3K4me3, might also associate with the viral genome. Future work should more completely define the KSHV chromatin and identify the factors that mediate these changes.

A number of viral antigens that are expressed during EBV latency interact with some of the same transcriptional coactivators that VP16 associates with. For instance, EBV nuclear protein 2 (EBNA2), an essential protein for latency and B-cell immortalization, associates with the p300, CBP, and PCAF HATs (184), as well as with components of the Brg-1 chromatin remodeling complex (188), which all contribute to EBNA2's transactivation potential. Interestingly, EBNA3C, another critical component for EBV-mediated B-lymphocyte immortalization, can act as both an activator and a repressor of transcription depending on its interaction partners at a given promoter. For instance, EBNA3C interacts with both transcriptional coactivators such as p300 (166) and co-repressors including HDACs (79,143) and mSin3A (79). However, it is currently not known whether recruitment of these coactivators by EBNA2 and EBNA3C results in covalent modification of histones on target promoters.

Other EBV proteins, such as BRLF1 (Rta) and BZLF1 (Zta), which induce the switch from latent to lytic infection, also interact with CBP for enhanced transcription (1,167,197). Recruitment of CBP by Zta to IE promoters results in increased histone acetylation (26), suggesting an active role for histone modifications in mediating reactivation from latency. Others have observed similar correlations between the transcriptional status of EBV genes and histone modifications, in particular histone acetylation, during different stages of infection. For instance, during latency, the promoter of latent membrane protein 2A (LMP2A) is enriched in acetylated histone H3 and H4 and in H3K4me2, the levels of which correlate with the amount of LMP2A transcript (43). In contrast, the promoter of the transcriptionally inactive BZLF1 gene is silenced by recruitment of class II HDACs during latency (50). The transcriptional activity of the LMP1 and EBNA2 promoters during latency also correlates with presence of active or inactive histone marks (14,24). The hypothesis that histone acetylation contributes to the switch from latent to the lytic cycle is supported by the observation that the HDAC inhibitor TSA resulted in an increase in the levels of acetylated histone H4 on the Rta promoter and induced expression of the viral lytic proteins Rta and Zta (13). However, a more detailed study

indicated that although HDAC inhibitors can increase the levels of histone acetylation on viral lytic gene promoters, they were not sufficient to trigger reactivation from latency (21). Therefore, regulation of the switch from latent to lytic infection may not be simply explained by histone modifications, in spite of the striking correlation between transcriptional activity and the state of chromatin.

These findings clearly indicate that in all herpesviruses, during lytic infection, actively transcribed viral genes are either devoid of histones or associate with histones that carry active transcription marks. In contrast, during latency, most viral genes are not transcribed and are packaged in a form resembling heterochromatin. Strikingly, most of the changes during the switch between latent and lytic infections by various herpesviruses are associated by the recruitment of similar host factors, such as p300 and CBP HATs. In return, the host cell tries to block herpesvirus infections by silencing the viral genome mainly by recruitment of HDACs and HP1 to the viral DNA. Whether other mechanisms are involved in this tug of war will be the subject of future research.

3. PERSPECTIVES

The prevailing evidence at this time clearly establishes that various herpesviruses target similar components of the host's transcriptional machinery, despite differences in the composition of their genomes. Another emerging theme is the correlation between the transcriptional status of the viral genomes and the histone marks that associate with those genomes. However, these correlations should not necessarily be interpreted as representing causal relationships. In other words, histone modifications may not be the *cause* of the switch from latent to lytic herpes infection, but rather a *result* of transcriptional activity. Moreover, given that histone modifications are mediated by enzymes that are recruited by specific DNA binding proteins such as transcriptional activators, it is of crucial importance to identify the cellular or viral factors that bring about these changes in the state of viral chromatin, rather than relying on histone modifications as being the sole determining factors.

Despite the often implicit or tacit assumptions, viral gene expression during lytic infection may in fact not be regulated by mechanisms similar to those that govern cellular genes, perhaps highlighted by the fact that histones are not deposited at high levels on the viral genome to begin with. In contrast, during reactivation from latency, the viral genomes are heavily nucleosomal and as such resemble cellular genes. Therefore, reactivation from latency is more likely to be mediated by mechanisms similar to those that activate cellular genes.

A potential limitation in the analysis of viral chromatin is the ChIP technique and how the data obtained in these assays should be interpreted. For instance, during lytic infection one would expect that not all viral genomes will enter the host cell nuclei at the same time and not all of them will be activated transcriptionally. Yet, what precipitates in the IP reaction will be a population of these heterogeneous viral genomes, which although not testable, are assumed to immunoprecipitate at similar efficiencies. The problem of heterogeneity becomes even more problematic during reactivation from latency, where only a small fraction of viral genomes may reactivate and as such it may be difficult to assay the changes in the chromatin structure of this small fraction of reactivating viral genomes. Therefore, care should be taken while interpreting the results obtained from ChIP assays of infected cells.

Another concept that warrants further investigation is the mechanism(s) by which histones are depleted from the viral genome during lytic infection. Although some studies indicated that active transcription marks are present on the viral genome during lytic infection, the density of histones on viral DNA seems far lower than on cellular genes. Likely candidates in this process include the histone chaperones and assembly factors. For example, the histone chaperone HIRA is localized to PML bodies in senescent cells (194). A recent study has

indicated that HIRA might be involved in the deposition of H3.3 on the HSV-1 genome, and disruption of HIRA expression impaired viral gene expression and replication modestly (137). According to this study, histone deposition by HIRA might be necessary for optimal viral gene expression during lytic infection. Considering that histones are under-represented on the HSV-1 genome, future studies are necessary to address whether HIRA or other histone chaperones are important for viral gene expression or whether they are direct targets of HSV-1 proteins during lytic infection.

Although histone deposition by chaperones and removal by chromatin-remodeling enzymes is one potential mechanism to account for the low density of histones on viral DNA, an alternative is that histones may not be deposited at all on a large fraction of the viral genomes, and thus histone modifications may not matter for viral gene expression. In line with this idea, disrupting the expression of various transcriptional coactivators that are recruited by VP16 had no substantial effect on viral IE gene expression (95). To date, little is known about how histone deposition on viral genomes is prevented during lytic infection.

Another potential mechanism of histone depletion from the HSV-1 genome during lytic infection is transcription by RNAP II itself. The rate of transcription by RNAP II correlates with depletion of histones (61,94). Consistent with this model are recent observations that inhibition of RNAP II transcription leads to a gradual increase in histone occupancy on the HSV-1 genome (SK, unpublished observations). This would also be consistent with the notion that histone changes on viral DNA might be a consequence, rather than a cause, of changes in viral gene expression.

Although we have a better picture of the regulation of chromatin on the HSV-1 genome during lytic and latent stages of infection, we are far from understanding how the changes on HSV-1 chromatin are mediated and whether they matter for different stages of infection. Therefore, future studies are necessary to explore whether alternative mechanisms explained above operate during herpes infections.

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