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dissertation entitled

CHROMATIN AND COACTIVATORS IN HERPES SIMPLEX VIRUS TYPE-1 GENE REGULATION

presented by

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has been accepted towards fulfillment
of the requirements for the

Doctoral degree in Cell and Molecular Biology

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April 10, 2009
Date

CHROMATIN AND COACTIVATORS IN HERPES SIMPLEX VIRUS TYPE-1 GENE REGULATION

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Cell and Molecular Biology

2009

ABSTRACT

CHROMATIN AND COACTIVATORS IN HERPES SIMPLEX VIRUS TYPE-1 GENE REGULATION

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The virion protein 16 (VP16) of HSV-1 serves as a prominent model for studying transcriptional activation in eukaryotes. During lytic infection, HSV-1 immediate early (IE) gene expression is stimulated by the virion-borne transactivator protein VP16. In heterologous expression systems, the VP16 activation domain (AD) can recruit various coactivators such as the p300/CBP histone acetyltransferases (HATs) and the Brm and Brg-1 chromatin remodeling complexes. Given prior findings that the HSV-1 genome is mainly non-nucleosomal during lytic infection, we hypothesized that such chromatin-modifying coactivators modify and remodel nucleosomes on the viral genome enabling IE gene expression. We and others have shown that during lytic infection the actively transcribed viral gene promoters and ORFs associate with acetylated and methylated histone H3. Moreover, we have shown that the p300, CBP, Brm and Brg-1 were recruited to IE gene promoters in a manner largely dependent on the VP16 AD. Therefore, we hypothesized that the coactivators that are recruited by VP16 directly contribute to IE gene expression.

This dissertation describes my efforts to elucidate whether the transcriptional coactivators that are recruited by VP16 are important for viral gene expression and to understand the mechanism behind histone depletion from the HSV-1 genome. Disruption of the expression of p300, CBP, PCAF and GCN5 HATs by RNA interference did not reduce IE gene expression during lytic infection. These results were supported by our findings that IE gene expression was not impaired in mutant cell lines that did not express

functional coactivators. These results suggested that transcriptional coactivators are not important for IE gene expression.

Given that coactivators are not required for IE gene expression, and that the viral genome is depleted of histones during lytic infection, we have analyzed possible mechanisms of histone depletion from the viral genome during various stages of lytic infection. We specifically asked whether there is a role for VP16 AD, transcription *per se* or IE proteins in this process. To address this question, we employed chromatin immunoprecipitation (ChIP) assays to detect the presence of all four core histones on different regions of the viral genome during different stages of lytic infection. These studies have indicated that VP16 and RNA Polymerase II (RNAP II) contribute to histone depletion from IE promoters and coding regions, and that IE proteins are also involved in preventing histone deposition at later stages of infection from other regions of the viral genome.

Overall, we conclude that the HSV-1 genome stays free of nucleosomes by the action of VP16, RNAP II and IE proteins during lytic infection. Given that transcriptional coactivators are not required for viral gene expression during lytic infection, we are proposing that histones may be prevented from being deposited on the viral genome, rather than being deposited and then removed from the viral genome. Our ChIP results also support this model. As such, the low amount of histones present on the viral genome during lytic infection may not matter for the outcome of infection. Future research will focus on the detailed mechanism that keeps the histones off the viral genome, and the mechanism of how VP16 mediates transcription from nucleosomal templates *in vivo*. It will also be important to establish model systems where the necessity for coactivators in reactivation from latency can be tested effectively.

This dissertation is dedicated to my family.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Steven J. Triezenberg, for teaching me how to become an independent scientist. I appreciate his dedication and enthusiasm for science and his unending patience in mentoring. I will fondly remember my time as a graduate student in his lab.

I would also like to thank my thesis committee members, Andrea Amalfitano, Bill Henry, Michele Fluck and Richard Schwartz for their support and assistance over the last five years at MSU.

I would also like to thank all of the past and present members of the Triezenberg laboratory. In particular, I would like to thank Francisco Herrera, Dean Shooltz and Kanchan Pavangadkar for their advice and help in the early days of my graduate training. I am grateful to James Doroghazi, Martha Roemer, Sarah DeVos and Jennifer Klomp for their direct contributions to this dissertation. I would like to thank Glen Alberts and Xu Lu for their advice and guidance in the lab.

I am grateful to my family overseas for supporting and encouraging me throughout my graduate career. I value their understanding of the difficult times I had to go through over the last five years. I would also like to thank to my friends of all nationalities at MSU and VARI for making graduate school a colorful and memorable experience.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter II:

Chapter III:

Chapter IV:

LIST OF ABBREVIATIONS

ChIP	Chromatin immunoprecipitation
DE	Delayed early
HAT	Histone acetyltransferase
HSV-1	Herpes simplex virus type-1
HSV-2	Herpes simplex virus type-2
IE	Immediate early
L	Late
RNAP II	RNA polymerase II
Seq-ChIP	Sequential chromatin immunoprecipitation
VP16	Virion protein 16 from herpes simplex virus
VP16 AD	Activation domain of VP16

CHAPTER ONE

Kutluay, Sebla, B., Triezenberg, Steven, J. (2009). Role of Chromatin During Herpesvirus Infections. BBA General Subjects.

Chapter 1

LITERATURE REVIEW

1. INTRODUCTION

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 subsequently are recognized to also apply 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, the exact mechanism of which is 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.

1.1. 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 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.

1.1.1. 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, 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 trancriptional 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).

1.1.2. 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).

1.1.3. 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-temini, 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).

1.1.4. 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.

1.2. 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).

1.2.1. 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 modest 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 that 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.

1.2.2. 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.

1.2.3. Chromatin during the infection 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.

2. 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 heterogenous 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.

3. ACKNOWLEDGEMENTS

We apologize to colleagues whose original work was not cited because of space limitations. This work was supported in part by NIH grant AI-064634 to SJT, a predoctoral fellowship from the Greater Midwest Affiliate of the American Heart Association to SBK, and the Van Andel Research Institute. Thanks to Dr. Xu Lu and David Nadziejka for their comments on the manuscript.

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CHAPTER TWO

Kutluay, Sebla, B., DeVos, Sarah, L., Klomp, Jennifer, E., Triezenberg, Steven, J. (2009). Transcriptional Coactivators are not Required for Herpes Simplex Virus Type 1 Immediate Early Gene Expression in vitro. Journal of Virology. 83(8): 3436-49.

Chapter 2

ROLE OF TRANSCRIPTIONAL COACTIVATORS IN HSV-1 INFECTION

1. ABSTRACT

Virion protein 16 (VP16) of herpes simplex virus type 1 (HSV-1) is a potent transcriptional activator of viral immediate early (IE) genes. The VP16 activation domain can recruit various transcriptional coactivators to target gene promoters. However, the role of transcriptional coactivators in HSV-1 IE gene expression during lytic infection had not been fully defined. We have shown previously that transcriptional coactivators such as the p300 and CBP histone acetyltransferases and the BRM and Brg-1 chromatin remodeling complexes are recruited to viral IE gene promoters in a manner mostly dependent on the presence of the activation domain of VP16. In this study, we tested the hypothesis that these transcriptional coactivators are required for viral IE gene expression during infection of cultured cells. Disrupted expression of the histone acetyltransferases p300, CBP, PCAF, or GCN5 or the BRM and Brg-1 chromatin remodeling complexes did not diminish IE gene expression. Furthermore, IE gene expression was not impaired in cell lines that lack functional p300, or BRM and Brg-1. We also tested whether these coactivators are required for VP16-dependent induction of IE gene expression from transcriptionally inactive viral genomes associated with high levels of histones in cultured cells. We found that disruption of coactivators also did not affect IE gene expression in this context. Thus we conclude that the transcriptional coactivators that can be recruited by VP16 do not contribute significantly to IE gene

expression during lytic infection or induction of IE gene expression from nucleosomal templates *in vitro*.

2. INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a large double-stranded DNA virus that establishes life-long latency in sensory neurons after an initial phase of lytic infection in epithelial cells. Viral gene expression during lytic infection is initiated by VP16, a tegument-associated transcriptional activator protein that stimulates the transcription of viral immediate early (IE) genes (6). VP16 is recruited to viral IE gene promoters through *cis*-regulatory elements with a consensus sequence of 5' TAATGARAT, in association with two host cell proteins, Oct-1 and HCF-1 (74). VP16, by its activation domain (AD), interacts with various general transcription factors and recruits the host RNA polymerase II (RNAP II) (12, 24, 29, 43, 75).

The packaging of eukaryotic DNA in the form of chromatin presents a significant impediment to the transcriptional machinery (42). This barrier can be overcome by activator-dependent recruitment of coactivator protein complexes with either of two types of enzymatic activities. Some coactivators covalently modify histones by acetylation, methylation, phosphorylation, ubiquitinylation, sumoylation, ADP-ribosylation or proline isomerization (30, 42). Some covalent histone modifications, such as acetylation of lysine 9 and lysine 14 of histone H3 (H3K9/K14ac) or trimethylation of lysine 4 of histone H3 (H3K4me3), are marks of active transcription. In contrast, methylation of other lysine residues on histones is typically indicative of inactive transcription and heterochromatin formation (30). The second class of coactivators hydrolyzes ATP in the process of remodeling the position of nucleosomes along DNA or in removing nucleosomes from DNA (11, 59).

The interaction of transcriptional activators with coactivators has often been

explored using a chimeric protein, Gal4-VP16 (58), comprising the DNA-binding domain of the *Saccharomyces cerevisiae* Gal4 protein and the activation domain (AD) of HSV-1 VP16. The VP16 AD can physically interact with and recruit transcriptional coactivators such as the histone acetyltransferases (HATs) p300 (KAT3B) and CBP (KAT3A) (3, 17, 25, 34, 67, 70), PCAF (KAT2B) (70) and GCN5 (KAT2A) (23, 38, 63, 67, 68), or the ATP-dependent chromatin remodeling enzymes BRM and Brg-1 (16, 46, 49, 50) to potentiate transcription from nucleosomal templates. However, the role of coactivators in the context of HSV-1 infection is not yet well-defined, in part because of prior evidence that the HSV-1 genome is predominantly non-nucleosomal during lytic infection (40, 41, 47).

We and others have recently shown that histones, most often represented experimentally by histone H3, are present on the HSV-1 genome during lytic infection, but at lower levels than cellular genes (20, 22, 28, 35, 52). Furthermore, active transcription marks such as H3K9/K14ac and H3K4me3 have been associated with viral genes during lytic infection (20, 22, 28, 35). We have also shown that, at early times during lytic infection, the p300 and CBP HATs and the BRM and Brg-1 chromatin remodeling enzymes are recruited to viral IE gene promoters in a manner mostly dependent on the presence of VP16 AD (20). Similarly, the Set1 histone methyltransferase, which is recruited by HCF-1, was shown to contribute to optimal HSV-1 gene expression (22). These results suggest that, during lytic infection, nucleosomes might be deposited on the viral genome, and yet recruitment of transcriptional coactivators could result in modification and removal of the histones from the viral genome similar to actively transcribed genes in the host cell genome (4, 19, 33,

56).

Based on this model, we have hypothesized that the transcriptional coactivators that are recruited by VP16 are required for IE gene expression during lytic infection. From this hypothesis, we predicted that disrupting the expression of a coactivator will diminish IE gene expression by allowing formation of an inactive chromatin structure on the viral genome. Here we show that, contrary to our hypothesis, disrupting the expression of various coactivators by RNA interference (RNAi) did not decrease IE gene expression in HSV-infected cells under most conditions tested. In parallel with these findings, IE gene expression was not impaired in SiHa cells, which do not express functional p300, or in SW13 and C33-A cells neither of which express the BRM and Brg-1 remodeling enzymes. Moreover, restoration of BRM and Brg-1 activity to SW13 or C33-A cells had no substantial effect on IE gene expression, indicating that neither BRM nor Brg-1 remodeling enzymes are essential for IE gene expression.

If not important for lytic infection, we then hypothesized that coactivators may be required during reactivation from latency, during which the viral genomes are nucleosomal. We have not yet tested the requirement of coactivators during reactivation from latency *in vivo*; instead, we used *in vitro* conditions in which viral genomes are heavily occupied with nucleosomes in cultured cells. To this end, we employed a mutant virus strain (RP5) that lacks sequences encoding the activation domain of VP16. During RP5 lytic infection, IE gene expression is reduced dramatically (66, 76) and histones associate with the RP5 genomes at higher levels than the wild-type genomes (20). Moreover, p300 and CBP HATs or BRM and Brg-1 remodeling enzymes are not recruited efficiently to RP5 IE promoters (20). Although IE gene expression from the

RP5 genome was induced significantly upon superinfection by HSV-2, none of the transcriptional coactivators were required for this induction. We conclude that the coactivators that are recruited by VP16 are not essential for IE gene expression during lytic infection *in vitro*, regardless of the nucleosomal status of the viral genome.

3. METHODS

Cell lines and viruses: HeLa (ATCC# CCL-2), SW-13 (ATCC# CCL-105), Vero (ATCC# CCL-81) and telomerase-transformed human foreskin fibroblasts (HFFs) provided by Wade Bresnahan, were grown in Dulbecco's modified Eagle medium (Invitrogen) containing 110 mg/l sodium pyruvate and 10 % fetal bovine serum (Invitrogen). SiHa (ATCC# HTB-35) and C33-A (ATCC# HTB-31) cells were grown in minimum essential medium containing Earle's salts supplemented with non-essential amino acids, 1 mM sodium pyruvate and 10 % fetal bovine serum. The RP5 strain of HSV-1, which lacks sequences encoding the activation domain of VP16, and the RP5R (RP5 rescue) strain have been previously described (66). HSV-1 strains KOS, RP5R and RP5 and HSV-2 strain G were prepared and titers were determined using Vero cells. In some experiments, cycloheximide (100 μg/ml) was added to the medium for 30 minutes prior to and during infection to inhibit protein translation.

Plasmids and transfections: A p300 expression plasmid, pCI-FLAG-p300, was provided by Yoshihiro Nakatani (5). The pCG-BRM, pBJ-Brg-1, dnBRM and dnBrg-1 expression plasmids were obtained from Bernard Weissman and David Reisman (1, 64). SiHa cells were transfected using jetPEI (Polyplus) transfection reagent according to the manufacturer's instructions. SW13 and C33-A cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

siRNAs and transfections: For each target coactivator, two siRNA duplexes were purchased from Qiagen with the exception of CBP_1 (Dharmacon). The catalogue

numbers and sequences of siRNAs are given in Table 1. For siRNA transfections, $1.5x10^5$ HHFs were plated per well in 6-well cell culture plates one day prior to transfection. siRNA duplexes were transfected at 10 nM (for single and double transfections) or 20 nM (for quadruple transfections) total concentration using Silentfect transfection reagent (Bio-Rad) according to manufacturer's instructions, with the exception that the siRNA duplexes and the transfection reagent were diluted in OPTIMEM reduced-serum medium (Invitrogen).

Gene expression assays and Q-RT-PCRs: Total cellular RNA was isolated using Trizol reagent (Invitrogen). Total RNA was reverse-transcribed using random primers in a commercial reverse transcription system (Promega). The cDNA was used as template in quantitative real time PCR (Q-RT-PCR) assays using SYBR Green Master Mix (Roche) and ABI 7500 Real-Time PCR System (Applied Biosystems). Gene expression was first normalized against 18S rRNA and then to appropriate controls by the $2^{-\Delta\Delta Ct}$ method. For chromatin immunoprecipitation assays data was analyzed using the standard curve method explained in more detail in the following section. Primer pairs used in this study are indicated in Table 2. Other primer pairs have been previously described (20, 54). For statistical analysis of gene expression, four or more biological replicates of a given experiment were analyzed by Student's *t*-test.

Immunoblotting: Total cell lysates were prepared by RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1mM EDTA, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (10 mM PMSF, 5 μg/ml aprotinin and leupeptin).

25-50 μg of the lysates were run on 6, 8 or 10% SDS-PAGE and transferred to PVDF membranes. Blots were blocked in 5 % nonfat dry milk-TTBS (100 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) overnight at 4 °C under constant agitation. The blots were then incubated for two hours with the primary antibodies diluted in TTBS supplemented with 1 % BSA (Invitrogen). Antibodies specific for p300 (SC-584), CBP (SC-369), BRM (SC-6450), Brg-1 (SC-10768), PCAF (SC-13124) and GCN5 (SC-6303) were obtained from Santa Cruz Biotechnology. Antibodies specific for CD44 (156-3C11) and GAPDH (ab9484) were purchased from Cell Signaling Technology and Abcam, respectively. Blots were then incubated with the proper HRP-conjugated secondary antibodies and visualized using a chemiluminescence detection system (Pierce) and Chemi-Doc Imaging System with Quantity One software (Bio-Rad).

Chromatin immunoprecipitation: Chromatin immunoprecipitation was performed as explained before with minor modifications (20). Briefly, confluent plates of HFF cells were infected with RP5 or RP5R strains of HSV-1 at an MOI of 0.025 pfu/cell and 5 pfu/cell, which corresponded to about 8-10 viral genomes per cell for each infection. At 6 hpi infections were stopped by addition of formaldehyde to cell culture plates at a final concentration of 1 %. Chromatin was isolated and sonicated using Branson Digital Sonifier-450 to obtain 200-1000 bp DNA fragments. Protein-DNA complexes were immunoprecipitated using 5 μg of antibodies against histone H3 (Abcam, ab1791). Protein-DNA complexes were collected by Protein G-agarose beads (Invitrogen). After several washes, the protein-DNA complexes were eluted and reverse-crosslinked overnight at 65°C, in the presence of 200 mM NaCl and 10 μg RNaseA. Samples were

then precipitated with ethanol, digested with proteinase K (Roche) at 42 °C for 2 hours and purified with Qiagen spin columns using the gel extraction protocol. The presence of viral and cellular DNA fragments in the immunoprecipitated material was analyzed by quantitative real-time PCR using SYBR Green Master Mix (Roche) and ABI 7500 Real-Time PCR system (Applied Biosystems). A standard curve using serial 3-fold dilutions of input samples (1, 0.3, 0.1, 0.04 %) was produced to quantitate the signals from immunoprecipitation samples. Background signals, obtained from immunoprecipitation reactions performed in the absence of antibodies (no antibody control), were subtracted from signals obtained from immunoprecipitation samples [referred to as "% input (IP-noab)]. When necessary, data was further normalized against the cellular control U3 snRNA promoter, by dividing the "% input (IP-noab)" value for the viral DNA by that of the cellular DNA, to account for the differences in immunoprecipitation efficiencies.

4. RESULTS

RNAi of p300 and CBP HATs does not diminish HSV-1 IE gene expression:

The related HAT enzymes p300 and CBP can potentiate VP16 AD-dependent transcriptional activation from reconstituted nucleosome arrays *in vitro* or from reporter plasmids *in vivo* (3, 17, 67, 70). We have previously shown that both p300 and CBP are recruited to IE gene promoters in a manner mostly dependent on the presence of VP16 AD (20). Others have also indicated that p300 and CBP are recruited to PML bodies that become viral replication compartments later in infection (37, 44, 45). However, a direct role for p300/CBP in viral gene expression has not yet been established. The present study was undertaken to address this gap in our understanding.

We have previously shown that disrupting the expression of p300 by plasmid-based RNAi in HeLa cells did not affect IE gene expression (35). However, this finding was complicated by several considerations. First, p300 and CBP in some contexts have been shown to be redundant (26, 69) and therefore knocking down p300 itself may not have been sufficient to affect IE gene expression. Second, RNAi is not 100 % efficient, and thus the residual levels of p300 might have been sufficient for IE gene expression. Third, analysis of coactivators in HeLa cells might be inherently flawed due to the presence in HeLa cells of the papillomaviral proteins E6 and E7, which affect the activities of p300 and CBP (55, 79).

To overcome these potential problems, we disrupted the expression of p300 and CBP, both separately and in combination, by multiple siRNA duplexes in telomerase-transformed human foreskin fibroblasts (HFFs). Steady-state levels of p300 and CBP protein (Fig. 2.1A) and mRNA (Fig. 2.1B) were significantly and specifically reduced by

siRNA duplexes designed to target these two related proteins. One siRNA duplex targeting p300 (p300_1) reduced p300 protein expression more than p300 mRNA expression, which likely reflects a block in mRNA translation rather than mRNA degradation.

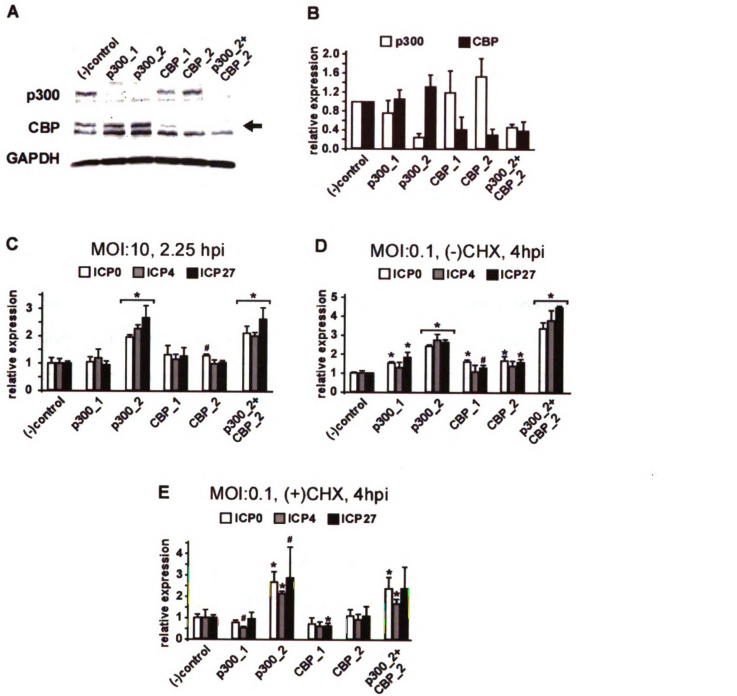
We then tested whether IE gene expression was reduced by disruption of p300 and CBP in HFFs. To address this, HFFs were transfected with siRNA duplexes as described above and were then infected with wild-type HSV-1 at a multiplicity of infection (MOI) of 10 plaque-forming units per cell (pfu/cell). At 2.25 hours post infection (hpi), when IE gene expression is robust, total RNA was isolated and further processed for analysis of IE gene expression. To our surprise, disruption of neither p300 nor CBP resulted in reduced expression of the ICP0, ICP4 or ICP27 mRNAs (Fig. 2.1C). Moreover, even simultaneous knockdown of p300 and CBP had no deleterious effect on viral IE gene expression. One of the p300-specific siRNA duplexes (p300_2), either alone or in combination with the CBP_2 duplex resulted in a statistically significant increase in IE gene expression, quite the contrary of the expected outcome. We suspect that this increase represents an off-target effect of that specific siRNA, as the other duplex that also targets p300 (p300_1) did not show a similar effect.

The initial assays shown in Fig. 2.1C were conducted using relatively high MOI (10 pfu/cell). We considered whether a requirement for p300 and CBP might be more evident during low multiplicity infections, in which the viral genome might be more prone to transcriptional repression by deposition of host histones. HFF cells transfected with the various siRNA duplexes were infected at low MOI (0.1 pfu/cell) and RNA harvested at 4 hpi was analyzed for IE gene expression using quantitative RT-PCR (Fig.

2.1D). The results (Fig. 2.1D) are comparable to the high MOI infections; siRNAs targeting p300 or CBP (or both together) have no deleterious effect on viral IE gene expression. In fact, ICP0 and ICP27 but not ICP4 expression showed a modest but statistically significant increase when p300 and CBP are knocked down either separately or together. These results suggest that p300 and CBP are not required for IE gene expression at low MOI, and if anything, they may act to repress IE gene transcription.

The IE gene products ICP4 and ICP0 themselves have activities that regulate IE gene transcription; for example, ICP0 might bypass the requirement for coactivators by disrupting the REST/CoREST/HDAC repressor complex (13, 14). To prevent any feedback on IE gene expression by the IE proteins themselves, parallel experiments were conducted in the presence of cycloheximide, a translation inhibitor. The results (Fig. 2.1E) again show that siRNAs targeting p300 or CBP have no deleterious effect on viral IE gene expression. Disruption of p300 by the p300_2 duplex, either alone or in combination with CBP_2, caused a statistically significant increase in expression of all IE genes during low multiplicity infections (Fig. 2.1D, E), an observation that contradicts our original hypothesis. This increase is likely due to an off-target effect of the p300_2 siRNA duplex, since the other p300 siRNA did not have a similar effect. We conclude that the HATs p300 and CBP are neither required nor redundant for activation of IE gene expression by VP16.

Figure 2.1: Disruption of p300 and CBP expression by RNAi does not decrease HSV-1 IE gene expression. Human foreskin fibroblasts (HFFs) were transfected with siRNA duplexes targeting p300, CBP or a negative control non-targeting siRNA duplex. **(A)** Immunoblot showing p300, CBP and GAPDH protein levels 48 hours after siRNA transfection. Arrow indicates the CBP-specific band. **(B)** Q-RT-PCR analysis of p300 and CBP expression in siRNA-transfected and KOS-infected HFFs. Values for each target are represented relative to the negative control siRNA signal. Histograms represent the average of 6 independent experiments and error bars represent the standard deviation. **(C)** siRNA-transfected HFFs were infected with HSV-1 KOS strain at an MOI of 10 pfu/cell. Expression of viral IE genes (ICP0, ICP4, ICP27) at 2.25 hours post infection was analyzed by Q-RT-PCR. **(D)** siRNA-transfected HFFs were infected with HSV-1 at an MOI of 0.1 pfu/cell. IE gene expression at 4 hpi was analyzed by Q-RT-PCR. **(E)** siRNA-transfected HFFs were pretreated with 100 μg/ml cycloheximide for 30 minutes and then infected with HSV-1 at an MOI of 0.1 pfu/cell in the presence of 100 μg/ml cycloheximide for 4 hours. IE gene expression was analyzed by Q-RT-PCR. Data in panels C, D and E represent the average of two independent experiments, each done with biological duplicates. Error bars represent the standard deviation based on these four samples. Mean values that differ significantly from those obtained from cells transfected with negative control siRNA are indicated by (*) for $p<0.01$ or by (#) for $0.01<p<0.05$ as determined by Student's *t* test.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p300_1
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CBP_1
CBP_2
p300_2+ CBP_2
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MOI:0.1, (-)CHX, 4hpi
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p300_2+ CBP_2

IE gene expression is not impaired in SiHa cells but is augmented by expression of wild-type p300:

Although p300 was knocked down efficiently by siRNAs in HFFs, we were concerned that the residual expression of p300 might still be sufficient to enable IE gene expression. To address this, we analyzed IE gene expression in SiHa cervical carcinoma cells, which express a mutated form of p300 that lacks the bromodomain (53). Since both SiHa and HeLa cells are derived from cervical carcinomas and are transformed by human papillomaviruses, we reasoned that comparing IE gene expression in these two cell lines would be a legitimate approach to test whether p300 is required for transcription of IE genes. SiHa and HeLa cells were infected with KOS at 1 pfu/cell and IE gene expression at 2 hours post-infection was analyzed by Q-RT-PCR. Contrary to our hypothesis, IE gene expression in SiHa cells was not significantly different than HeLa cells (Fig. 2.2A). We also tested whether supplementing SiHa cells with fully functional p300 might further enhance viral IE gene expression. To that end, SiHa cells were transfected with a wild-type p300 expression plasmid or an empty plasmid. Overexpression of wild-type p300 in SiHa cells (Fig. 2.2B) resulted in increases in ICP0, ICP4 and ICP27 expression that were modest but statistically significant (Fig. 2.2C), suggesting that although p300 is not required, it may potentiate the transcription of viral IE genes in SiHa cells.

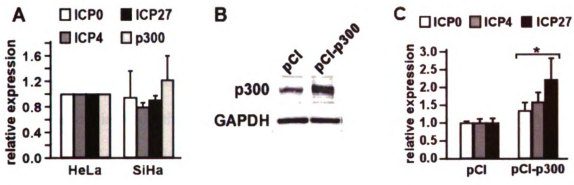


Figure 2.2: HSV-1 IE gene expression is not impaired in SiHa cells but is augmented in the presence of wild-type p300. **(A)** HeLa and SiHa cells were infected with HSV-1 strain KOS at an MOI of 1 pfu/cell. IE gene (ICP0, ICP4, ICP27) and p300 mRNA levels at 2 hpi were analyzed by Q-RT-PCR. Values for each gene tested in SiHa cells are represented relative to HeLa cells. Error bars show the range between the averages of two independent experiments. **(B)** SiHa cells were transfected with 2.5 μg of pCI (empty) or pCI-p300 plasmids. p300 and GAPDH expression was analyzed 24 hours after transfection by immunoblotting. **(C)** SiHa cells were transfected as in (B) and were infected with HSV-1 KOS strain at an MOI of 5 pfu/cell. IE gene expression (ICP0, ICP4, ICP27) at 2 hpi was analyzed by Q-RT-PCR. Values for each viral gene tested in pCI-p300 transfected cells are represented relative to cells transfected with empty plasmid (pCI). Error bars indicate standard deviations (n = 4). Mean values that vary significantly ($p < 0.01$, Student's *t* test) from those obtained from cells transfected with vector plasmid are indicated (*).

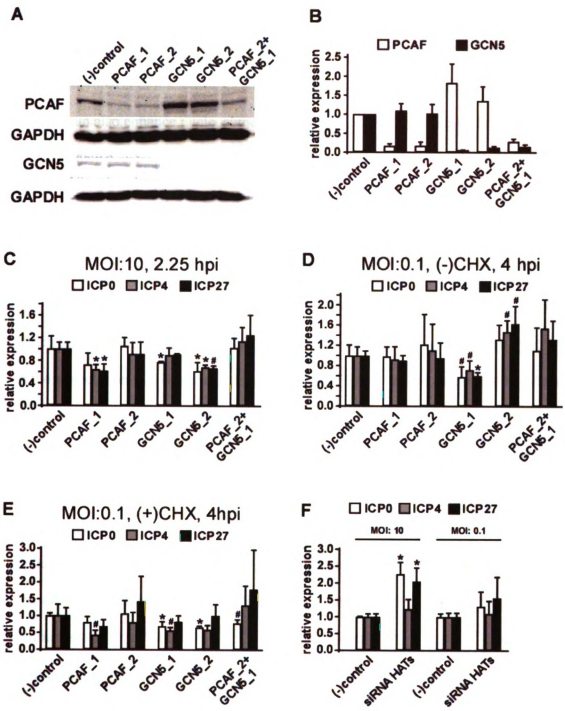
Disruption of PCAF and GCN5 HATs does not affect HSV-1 IE gene expression:

In addition to p300 and CBP, other HATs such as PCAF and GCN5 are known to interact with the activation domain of VP16 (68, 70). We therefore considered whether PCAF and GCN5 might be preferentially required for IE gene expression. To address this, IE gene expression was analyzed in HFFs in which the expression of PCAF and GCN5 was diminished either separately or together by siRNA duplexes. The siRNAs targeting PCAF and GCN5 in HFFs were both effective and specific for their respective targets, as indicated by immunoblotting (Fig. 2.3A) and Q-RT-PCR (Fig. 2.3B). We then infected these cells with HSV-1 at high MOI (10 pfu/cell) and analyzed IE gene expression at 2.25 hpi. As we observed for p300 and CBP, IE gene expression was not affected significantly under most circumstances when PCAF and GCN5 (separately or in combination) were knocked down (Fig. 2.3C). Transfection of two siRNAs (PCAF_1 and GCN5_2) caused a slight but statistically significant decrease in IE gene expression. However, since comparable effects were not exhibited by the PCAF_2 and GCN5_1 duplexes, which block target protein expression to similar levels as do the PCAF_1 and GCN5_2 duplexes, we cannot attribute these modest changes in IE gene expression to the effects on disruption of PCAF or GCN5. Parallel experiments were performed at low MOI (0.1 pfu/cell), to see whether a requirement for PCAF and GCN5 might be more apparent under conditions of a lighter viral genome load. In most cases, IE gene expression did not change significantly when PCAF and GCN5 expression was reduced (Fig. 2.3D). Although the GCN5_1 duplex reduced IE gene expression, GCN5_2 duplex caused the opposite effect. Therefore, although these siRNAs alter IE gene expression,

we do not think that these modest effects on IE gene expression are biologically meaningful. To rule out the possibility that IE proteins themselves were masking a requirement for coactivators in the activity of VP16 on IE gene expression, we repeated the low MOI experiments in the presence of cycloheximide to block IE protein expression. Although the decrease in ICP0 and ICP4 expression when GCN5 was knocked down (with either of two siRNA duplexes) was statistically significant, in general the expression of IE genes was not impaired when PCAF and GCN5 were knocked down either separately or in combination (Fig. 2.3E). Collectively, these results suggest that although VP16 can recruit PCAF and GCN5 in artificial contexts, PCAF and GCN5 are neither required substantially nor redundant for IE gene transcription during lytic infection.

To address whether different classes of HATs are redundant for IE gene expression, we then simultaneously disrupted the expression of p300, CBP, PCAF and GCN5 HATs in HFFs, and infected these cells at high (10 pfu/cell) or low (0.1 pfu/cell) MOI with HSV-1. As indicated in Figure 2.3F, regardless of the MOI, disruption of all four HATs did not reduce viral IE gene expression; if anything, we observed a slight but significant increase in ICP0 and ICP27 expression. These results suggest that p300, CBP, PCAF and GCN5 HATs are not redundant for IE gene expression during lytic infection *in vitro*.

Figure 2.3: RNAi of PCAF and GCN5 does not decrease HSV-1 IE gene expression. HFFs were transfected with siRNA duplexes targeting PCAF, GCN5 or a negative control non-targeting siRNA duplex. **(A)** Western blots showing levels of PCAF, GCN5 and GAPDH proteins 48 hours after siRNA transfection. **(B)** Q-RT-PCR analysis of PCAF and GCN5 expression in siRNA-transfected and KOS-infected HFFs. Data represent the average of six independent experiments, and error bars represent the standard deviation. **(C)** siRNA-transfected HFFs were infected with HSV-1 at an MOI of 10 pfu/cell. Total RNA was isolated at 2.25 hpi and IE gene mRNA levels (ICP0, ICP4, ICP27) were analyzed by Q-RT-PCR. **(D)** siRNA-transfected HFFs were infected with HSV-1 at an MOI of 0.1 pfu/cell. IE gene expression at 4 hpi was analyzed by Q-RT-PCR. **(E)** siRNA-transfected HFFs were pretreated with 100 μg/ml cycloheximide for 30 minutes and then infected with HSV-1 at an MOI of 0.1 pfu/cell in the presence of cycloheximide. IE gene expression at 4 hpi was analyzed by Q-RT-PCR. Panels C-E present the average of four replicates, and error bars represent standard deviations. **(F)** HFFs were transfected with siRNA duplexes targeting p300, CBP, PCAF, GCN5 or a negative control non-targeting siRNA duplex and infected with HSV-1 at an MOI of 10 or 0.1 pfu/cell. IE gene expression at 2.25 and 4 hpi for high and low MOI, respectively, were analyzed by Q-RT-PCR. Data shown are from a representative experiment done with biological triplicates; error bars indicate the standard deviation. Experimental samples whose mean values differ significantly from those obtained from cells transfected with negative control siRNA are indicated by (*) for $p<0.01$ or by (#) for $0.01<p<0.05$ as determined by Student's *t* test.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PCAF_2
GCN5_1
GCN5_2
PCAF_2+ GCN5_1
PCAF
GAPDH
GCN5
GAPDH
B
PCAF
GCN5
relative expression
2.5
2.0
1.5
1.0
0.5
0.0
(-)control
PCAF_1
PCAF_2
GCN5_1
GCN5_2
PCAF_2+ GCN5_1
C
MOI:10, 2.25 hpi
ICP0
ICP4
ICP27
relative expression
1.6
1.2
0.8
0.4
0.0
D
MOI:0.1, (-)CHX, 4 hpi
ICP0
ICP4
ICP27
relative expression
2.0
1.6
1.2
0.8
0.4
0.0
E
MOI:0.1, (+)CHX, 4hpi
ICP0
ICP4
ICP27
relative expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
F
ICP0
ICP4
ICP27
MOI: 10
MOI: 0.1
relative expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
(-)control
siRNA HATs
(-)control
siRNA HATs

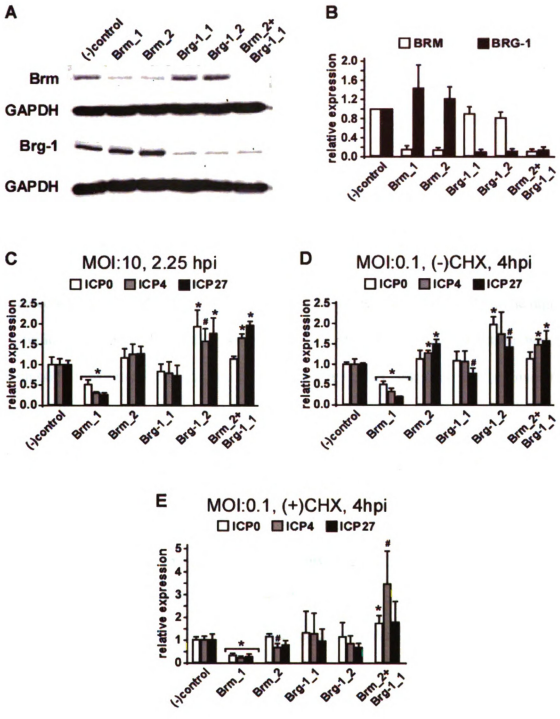
RNAi of BRM and Brg-1 chromatin remodeling complexes does not affect IE gene expression:

Several lines of evidence indicate that ATP-dependent chromatin remodeling complexes can be recruited by the activation domain of VP16 to nucleosomal templates leading to disruption of nucleosomes and transcriptional activation (46, 49, 50, 72, 78). VP16 AD can also enhance nucleosome eviction by the SWI/SNF remodeling complex from mononucleosomal templates *in vitro* (16). Therefore, we hypothesized that BRM and Brg-1 chromatin remodeling complexes, the mammalian homologues of the yeast SWI/SNF complex (72), might remove the nucleosomes from the viral genome and enable active transcription.

To address this, as in previous sections, we analyzed IE gene expression in HFFs in which the expression of BRM and Brg-1 remodeling enzymes was disrupted by RNAi, with the expectation that IE gene expression would diminish in the absence of BRM and Brg-1. Immunoblotting (Fig. 2.4A) and Q-RT-PCR (Fig. 2.4B) results indicate that BRM and Brg-1 were knocked down very efficiently and specifically by both siRNA duplexes against each target. However, disruption of BRM and Brg-1 expression by most siRNA duplexes (with the exception of Brm_1) did not reduce IE gene expression at high MOI (10 pfu/cell) during lytic infection (Fig. 2.4C). To the contrary, IE gene expression was increased in the presence of some siRNAs, most notably Brg-1_2. In parallel experiments performed at low MOI (0.1 pfu/cell), transcription of some IE genes during lytic infection was affected by one but not by both of the siRNA duplexes targeting either BRM or Brg-1 (Fig. 2.4D). Therefore, we conclude that reduced levels of the BRM or Brg-1 remodeling enzymes do not affect IE gene transcription substantially. Moreover,

similar results were also obtained when the viral infection was performed in the presence of cycloheximide to prevent the interference of IE proteins in IE gene transcription (Fig. 2.4E). Interestingly, under all conditions tested, expression of most IE genes increased significantly when Brm and Brg-1 were knocked down together (Fig. 2.4C, D, E), suggesting that Brm and Brg-1 might be acting redundantly in a manner contrary to our original hypothesis, i.e., to inhibit rather than to support IE gene expression. Although primarily associated with transcriptional activation, Brm and Brg-1 have been shown to potentiate transcriptional repression by the Rb tumor suppressor protein (15).

Figure 2.4: Disruption of BRM and Brg-1 expression does not decrease HSV-1 IE gene expression. HFFs were transfected with the indicated siRNA duplexes targeting BRM, Brg-1 or a negative control non-targeting siRNA duplex. (A) Western blot showing BRM, Brg-1 and GAPDH expression in HFFs 48 hours after siRNA transfection. (B) Q-RT-PCR analysis of BRM and Brg-1 in HFFs after siRNA transfection and KOS infection. Data represent the average of six independent experiments. Error bars represent the standard deviation. (C) After siRNA transfection, HFFs were infected with HSV-1 at an MOI of 10 pfu/cell. IE gene expression at 2.25 hpi was analyzed by Q-RT-PCR. (D) siRNA-transfected HFFs were infected with HSV-1 at an MOI of 0.1 pfu/cell. IE gene expression at 4 hpi was analyzed by Q-RT-PCR. (E) After siRNA transfection, HFFs were pretreated with 100 μg/ml cycloheximide for 30 minutes and then infected with HSV-1 at an MOI of 0.1 pfu/cell in the presence of 100 μg/ml cycloheximide. Total RNA was isolated at 4 hpi and IE gene expression was analyzed by Q-RT-PCR. C to E present the mean of four biological replicates; error bars represent standard deviations. Mean values that differ significantly from those obtained from cells transfected with negative control siRNA are indicated by (*) for $p<0.01$ or by (#) for $0.01<p<0.05$ as determined by Student's *t* test.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(-)control
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Brm_2
Brg-1_1
Brg-1_2
Brm_2+ Brg-1_1
Brm
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BRM
BRG-1
relative expression
2.0
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0.4
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C
MOI:10, 2.25 hpi
ICP0
ICP4
ICP27
relative expression
2.5
2.0
1.5
1.0
0.5
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D
MOI:0.1, (-)CHX, 4hpi
E
MOI:0.1, (+)CHX, 4hpi
5
4
3
2
1
0

IE gene expression in cell lines with defective BRM and Brg-1 expression:

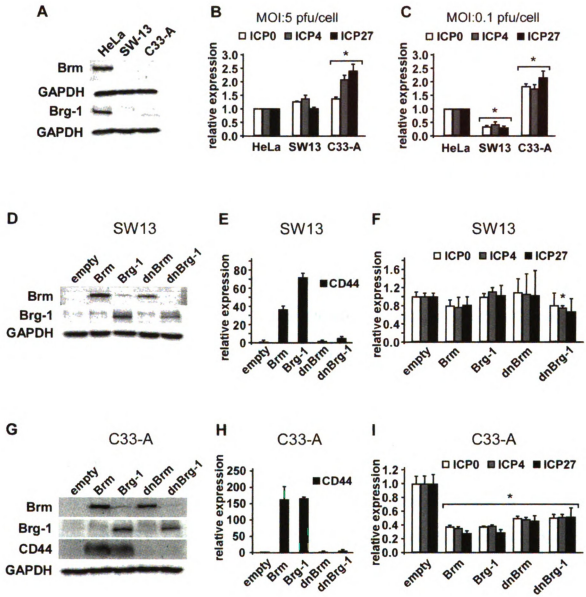
As a complementary strategy to RNAi, we also analyzed IE gene expression in cell lines that do not express BRM and Brg-1 enzymes (57, 73). The immunoblot shown in Figure 2.5A confirms that the levels of BRM and Brg-1 proteins are dramatically reduced in SW13 (adrenal carcinoma) and C33-A (cervical carcinoma) cells as compared with HeLa cells.

We then asked whether IE gene expression was impaired in SW13 and C33-A cells. Parallel cultures of HeLa, SW13 and C33-A cells were infected with HSV-1 at high (5 pfu/cell) or low (0.1 pfu/cell) MOIs. Q-RT-PCR assays of viral IE gene expression revealed no defect in SW13 cells and about a 2-fold increase in C33-A cells in the high multiplicity infections (Fig. 2.5B). At low MOI (0.1 pfu/cell), IE gene expression was about 60 % lower in SW13 cells, but 2-fold higher in C33-A cells (Fig. 2.5C). Given that both cell lines are defective for BRM and Brg-1, we cannot attribute the deficit in SW13 cells at low MOI to a requirement for these coactivators.

To further test for potential contributions by BRM and Brg-1, we transfected SW13 or C33-A cells with plasmids expressing either wild-type BRM or Brg-1 or dominant-negative forms that lack ATPase activity (Fig. 2.5D, G). The endogenous gene encoding the cell surface marker CD44 served as a positive control, since CD44 is known to be regulated by BRM and Brg-1 in these cells (64, 65). As expected, CD44 mRNA expression was induced in both SW13 and C33-A cells upon expression of BRM and Brg-1 but not the dominant negative BRM and Brg-1 (Fig. 2.5E, H), indicating that the BRM and Brg-1 proteins ectopically expressed in these cell lines are functional. When these cells were subsequently infected with HSV-1, IE gene expression in SW13 cells

that express wild-type BRM and Brg-1 was not significantly different ($p > 0.05$, by Student's *t*-test) than cells that were transfected with an empty plasmid or with plasmids encoding the dominant negative forms of BRM or Brg-1 (Fig. 2.5F). Curiously, IE gene expression in C33-A cells expressing wild-type BRM or Brg-1 was approximately 60% lower than in parallel cells transfected with empty vector (Fig. 2.5I). However, this reduction seems independent of the catalytic activity of BRM and Brg-1, since expression of the dominant-negative forms of BRM and Brg-1 also reduced IE gene expression to similar levels (Fig. 2.5I). This suggests that BRM and Brg-1 do not repress IE gene expression, as suggested above by RNAi assays (Fig. 2.4). In other words, if BRM and Brg-1 were inhibitory for IE gene expression, restoring BRM and Brg-1 expression in both SW13 and C33-A cells would decrease IE gene expression. These results together with the RNAi assays described above lead us to conclude that the chromatin remodeling enzymes BRM and Brg-1 are neither required nor redundant for IE gene expression during lytic infection.

Figure 2.5: HSV-1 IE gene expression in SW13 and C33-A cells that do not express BRM and Brg-1 remodeling enzymes. **(A)** BRM, Brg-1 and GAPDH expression in HeLa, SW13 and C33-A cells was analyzed by immunoblotting. **(B, C)** HeLa, SW13 and C33-A cells were infected in parallel at an MOI of 5 pfu/cell (B) or 0.1 pfu/cell (C). IE gene expression (ICP0, ICP4, ICP27) at 2 hpi was analyzed by Q-RT-PCR. IE gene expression in SW13 and C33-A cells is represented with respect to that in Hela cells. The graph shows the average of two independent experiments each done in biological quadruplicate (B) or triplicate (C). Error bars represent the range between the averages of these experiments. Mean values that vary significantly ($p < 0.01$, Student's *t* test) from those obtained from HeLa cells in both of the experiments presented in (B) and (C) are indicated (*). **(D)** SW13 cells were transfected with 4 μg of an empty plasmid or with expression plasmids encoding BRM, Brg-1, dominant-negative BRM (dnBRM) or dominant negative Brg-1 (dnBrg-1) together with 0.5 μg of puromycin selection plasmid. 24 hours post-transfection, media was replaced by puromycin selection media (2.5 μg/ml puromycin). After 2 days of puromycin selection, total protein was isolated and analyzed by immunoblotting against BRM, Brg-1 and GAPDH. **(E, F)** SW13 cells were transfected with the indicated plasmids as in (D) and infected with HSV-1 KOS at an MOI of 0.1 pfu/cell. At 2 hpi total RNA was isolated and, CD44 (E) or IE gene expression (F) was analyzed by Q-RT-PCR. **(G)** Immunoblot showing BRM, Brg-1, CD44 and GAPDH expression in C33-A cells transfected with the indicated plasmids as in (D). **(H, I)** C33-A cells were transfected with the indicated plasmids as in (D) and infected with HSV-1 KOS at an MOI of 0.1 pfu/cell. At 2 hpi total RNA was isolated and CD44 (panel H) and IE gene expression (panel I) were analyzed by Q-RT-PCR. Panels E, F, H, and I are derived from a representative experiment done with biological triplicates; error bars represent standard deviation. Mean values that vary significantly ($p < 0.01$, Student's *t* test) from those obtained from cells transfected with vector plasmid are indicated (*).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relative expression
HeLa SW13 C33-A
C
MOI:0.1 pfu/cell
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Coactivators are not required for VP16-mediated induction of IE gene expression from nucleosomal viral genomes in vitro:

One potential reason why transcriptional coactivators are not required for IE gene transcription during lytic infection is that the viral genomes remain depleted of histones by some undefined mechanism that may bypass the need for coactivators. To explore this question, we designed an experiment in which histones can be more abundantly deposited on viral genomes prior to the introduction of transcriptionally-active VP16. We have shown previously that IE gene expression is dramatically reduced during lytic infection by RP5, a mutant virus that lacks sequences encoding the activation domain of VP16 (66, 76). In addition, recruitment of the p300 and CBP HATs or the BRM and Brg-1 chromatin remodeling enzymes to most IE promoters is also impaired in RP5 infections (20). Furthermore, histones (represented by histone H3) associate with RP5 genomes to a greater extent than with wild-type genomes (20). Therefore, to some extent RP5 infection resembles quiescent or latent infections with respect to defects in IE gene expression and increased histone occupancy on the viral genome.

We first asked whether IE genes in the RP5 genome could be activated by superinfection with HSV-2, which encodes a VP16 protein very similar to that of HSV-1 (9) and which can induce reactivation from quiescence in other contexts (8). HFFs were infected with RP5 at MOIs ranging from 0.0005 pfu/cell to 0.05 pfu/cell, which correspond to approximately 0.5-1 viral genome per cell to 50-100 viral genomes per cell, respectively (data not shown). At 6 hours post-infection, histone deposition on RP5 genomes was dramatically higher than on wild-type genomes (Fig. 2.6A). The RP5-infected cells were then superinfected with HSV-2 strain G at MOIs ranging from 0.1

pfu/cell to 10 pfu/cell. Two hours after initiating the HSV-2 infection, we assayed levels of HSV-1-specific IE gene expression by Q-RT-PCR. As expected, superinfection of RP5- infected cells with HSV-2 activated the expression of ICP4 (Fig. 2.6B) and ICP27 (Fig. 2.6C) in a dose-dependent manner with respect to HSV-2 MOI. These results indicate that the defect in IE gene expression in RP5 infections can be overcome effectively by providing VP16 in *trans* by HSV-2 superinfection. In subsequent assays we have performed RP5 and HSV-2 infections at MOIs of 0.005 pfu/cell and 10 pfu/cell, respectively, to obtain robust IE transcription.

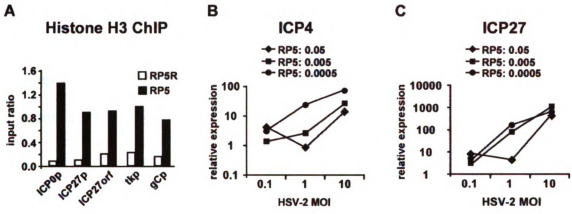
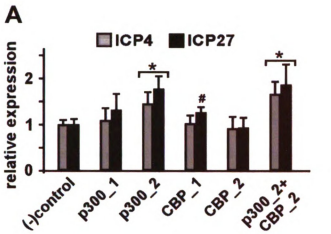


Figure 2.6: HSV-2 superinfection induces IE gene expression in RP5-infected cells. **(A)** HFFs were infected with RP5 or RP5R strains of HSV-1 at an MOI of 0.025 pfu/cell or 5 pfu/cell, respectively. At 6 hpi ChIP was performed assaying the presence of histone H3 on the ICP0 promoter, ICP27 promoter, ICP27 ORF, tk promoter and gC promoter. **(B,C)** HFFs were infected with RP5 strain of HSV-1 at an MOI of 0.05, 0.005 or 0.0005 pfu/cell. At 6 hpi of RP5 infections, superinfection with HSV-2 was performed at MOIs of 0.1, 1 or 10 pfu/cell. ICP4 (B) and ICP27 (C) expression at 2 hpi of HSV-2 superinfection was analyzed by Q-RT-PCR.

To address whether coactivators are required for HSV-2-mediated expression of IE genes from the quiescent RP5 genomes, we disrupted the expression of each coactivator in HFFs by siRNAs, infected these cells with RP5 and then superinfected with HSV-2. Contrary to our hypothesis, IE gene expression from the RP5 genomes was effectively stimulated by HSV-2 superinfection, even in the presence of siRNAs targeting any of the coactivators (Fig. 2.7). If anything, the siRNAs resulted in a modest increase, rather than a decrease, in IE gene expression. These results suggest that coactivators are not required for VP16-mediated induction of IE gene transcription from viral DNA that is abundantly associated with histones.

Figure 2.7: Coactivators are not required for HSV-2-dependent induction of IE gene expression from RP5 genomes. HFFs were transfected with the indicated siRNA duplexes targeting (A) p300 and CBP, (B) PCAF and GCN5, (C) BRM and Brg-1 or a negative control non-targeting siRNA duplex. 48 hours after transfection, HFFs were infected with RP5 at an MOI of 0.005 pfu/cell. At 6 hpi of RP5 infection, HFFs were superinfected with HSV-2 at an MOI of 10 pfu/cell. HSV-1 IE gene expression at 2 hpi of HSV-2 infection was analyzed by Q-RT-PCR. The graph displays the mean of four biological replicates, and error bars denote standard deviations. Mean values that differ significantly from those obtained from cells transfected with negative control siRNA are indicated by (*) for $p<0.01$ or by (#) for $0.01<p<0.05$ as determined by Student's *t* test.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ICP4
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relative expression
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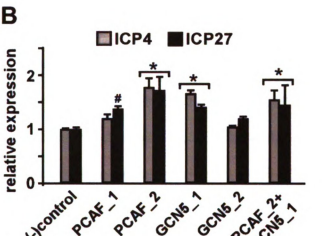
p300_2
CBP_1
CBP_2
p300_2+ CBP_2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PCAF_2
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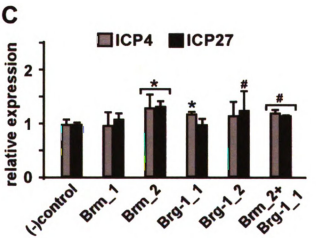
GCN5_2
PCAF_2+ GCN5_1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Brm_1
Brm_2
Brg-1_1
Brg-1_2
Brm_2+ Brg-1_1

5. DISCUSSION

Although a number of prior reports described evidence that the HSV-1 genome remains non-nucleosomal during lytic infection (40, 41, 47), several groups have recently shown that the viral genome is not exclusively histone-free and that acetylated and methylated histones are present on the viral genome during lytic infection, albeit at lower levels than on cellular genes (20, 22, 28, 35, 52). In contrast, during latency, the viral genome is packaged in a manner more nearly resembling host cell chromatin (10). Histones on the latent HSV-1 genomes carry post-translational modifications typical of heterochromatin, with the exception of the actively-transcribed latency-associated transcript gene (32, 71). During reactivation from latency or quiescence, the histones associated with IE gene promoters and other regions of the genome become acetylated, a hallmark of transcriptionally active chromatin (8, 51). These observations all indicate that regulation of chromatin might play a crucial and distinct role in different stages of HSV-1 infection.

We do not yet understand how the viral genome manages to stay predominantly histone-free during lytic infection. Given that VP16 AD interacts with a number of transcriptional coactivators in artificial conditions and that some of these coactivators are recruited to IE gene promoters during lytic infection (20), we hypothesized that these coactivators are involved in establishing a transcriptionally active chromatin state on the viral genome and enable IE gene expression. To this end, we tested whether disrupting the expression of these coactivators would decrease IE gene expression. Reducing the expression levels of p300 and CBP HATs, singly or in combination, using siRNAs had no discernible effect on IE gene expression at different multiplicities of infection (Fig.

2.1), suggesting that neither p300 nor CBP are required for IE gene expression. The modest increase in IE gene expression observed following transfection of one of the p300 duplexes, p300_2, is attributed to an off-target effect of this siRNA duplex, since the other siRNA was just as effective in diminishing p300 protein levels but had no effect on viral IE mRNA levels. An alternative explanation that we cannot exclude is that the diminished expression of p300 seen in the presence of siRNA p300_2 might suppress the host innate immune defense and hence enhance IE gene expression as suggested by others (45). Disruption of other HATs, PCAF and GCN5, by RNAi also had no significant effect on IE gene expression under most conditions tested *in vitro* (Fig. 2.3). Together, these results lead us to reject the hypothesis that histone acetylation is required for IE gene expression during lytic infection *in vitro* in HFFs.

Given that the activation domain of VP16 can stimulate nucleosome eviction by the yeast SWI/SNF remodeling complex (16), we hypothesized that BRM and Brg-1, the mammalian homologues of SWI/SNF, may be required for removing the nucleosomes from the viral genome. However, disruption of BRM and Brg-1, separately or together, did not reduce IE gene expression (Fig.2. 4). Although RNAi of BRM and Brg-1 together increased the expression of most IE genes in some conditions, these results were not supported by our findings in SW13 and C33-A cells. IE gene expression was not impaired in SW13 and C33-A cells, which express neither BRM nor Brg-1 (Fig. 2.5B, C) and restoring BRM and Brg-1 expression in these cell lines did not increase IE gene expression (Fig. 2.5F, I). Collectively, these results indicate that BRM and Brg-1 do not have a substantial role in supporting the VP16-dependent activation of viral IE gene expression during lytic infection *in vitro*.

The results of these experiments suggest that the transcriptional coactivators tested here are not required for IE gene expression during lytic infection, in contrast with our initial hypothesis. We recognize that cell culture models of HSV-1 infection are not necessarily representative of *in vivo* infections in epithelial or neuronal cells. Therefore, future studies will be necessary to test whether coactivators are important in lytic infection *in vivo*.

One potential explanation for the lack of an effect is redundancy or compensation among coactivators, such that the activity lost by disruption of one coactivator is taken up by another coactivator. We have addressed this in part by analyzing IE gene expression in HFFs where two or more coactivators were simultaneously disrupted. However, even when as many as four coactivators were targeted by siRNAs, viral IE gene did not decrease (Fig. 2.3F). Nonetheless, we cannot fully exclude the possibility that other HATs or remodeling complexes are compensating for those disrupted in our experiments.

We used immunoblots of the targeted proteins as an indication of the effectiveness of the siRNAs employed in these experiments. Although the targeted protein levels were substantially diminished (to levels 20% or less relative to control cells), the residual protein may be sufficient for the biological activities we have tested. Unfortunately, in most cases, no suitable positive control genes have been identified; that is, genes that are known to be direct targets of a given coactivator in HFFs. The exception may be the CD44 gene in SW13 and C33-A cells, which clearly responded to the presence of wild-type but not mutant forms of Brg-1 or BRM. In addition, as suggested in a recent study (21), we observed that disruption of p300 and CBP HATs together led to a substantial decrease in H3K18ac (Figure 2.8), suggesting that our RNAi of CBP and

p300 expression was effective. To gain confidence in the outcomes of the siRNA experiments, we also took the complementary approach of testing IE gene expression in mutant cell lines that lack the particular coactivator. The consistency of the results from these two approaches strongly supports our conclusion that these transcriptional coactivators are not intimately involved in HSV-1 IE gene expression.

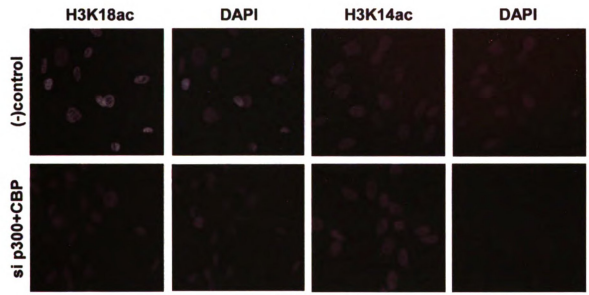

Figure 2.8: H3K18 but not H3K14 acetylation is reduced when p300 and CBP expression is disrupted together. HFFs were transfected with siRNA duplexes targeting p300 and CBP or with a negative contral siRNA duplex as in Figure 2.1. At 2 days post-transfection, immunofluorescence assays were performed as explained before (21).

Although the coactivators tested in this study are not required for IE gene expression, other coactivators might be required for modification of chromatin structure on the viral genome. Employing similar assays, others have shown that the Set1 histone methyltransferase can be recruited by HCF-1 and can contribute to viral gene expression during lytic infection (22). Although disruption of Set1 expression did not significantly affect IE gene expression at early times in infection, at later stages viral gene expression and viral replication were reduced modestly. Similarly, during lytic infection by varicella zoster virus (like HSV-1, a member of the alphaherpesvirus family), Set1 recruitment to the IE62 promoter was correlated with high levels of H3K4me3 (48). However, whether Set1 is required for IE62 expression has not been explicitly tested. Therefore, further studies are necessary to ask whether Set1 or other coactivators are important for IE gene transcription.

A further possibility is that VP16 recruits transcriptional coactivators not for IE gene expression during lytic infection, but during reactivation from latency where the viral genome is nucleosomal (10). We have addressed this in part by testing whether coactivators are required during activation of IE gene expression from histone-laden viral genomes *in vitro*. HSV-1 strain RP5 lacks the VP16 activation domain and the density of histone H3 on the RP5 genome approaches that of cellular genes at later times in infection (Fig. 2.6A). Superinfection by HSV-2 of RP5-infected cells resulted in substantial IE expression from the RP5 template (Fig. 2.6). However, knocking down expression of various coactivators had little or no effect on RP5 IE gene expression (Fig. 2.7), suggesting that VP16-mediated reactivation of IE gene expression does not require the coactivators tested in this study. We recognize that this *in vitro* system is not a

genuine representation of latent infection and that some experiments from others have suggested that VP16 may not be required for reactivation (62). Nonetheless, these experiments do address the role of coactivators for VP16-mediated transcription from a predominantly nucleosomal template. Future studies will be necessary to more directly establish whether coactivators are required in other quiescent infection models or, most importantly, during reactivation from latency *in vivo*.

A final possibility is that these coactivators may not be required at all for HSV-1 gene expression; their apparent presence at IE gene promoters may simply reflect their association with larger complexes of the transcription machinery. Recruitment of a particular transcription factor does not always correspond to a functional requirement for that factor. For instance, although estrogen receptor-α can bind to a large number of cellular promoters, only about 10% of those genes are actually regulated by estrogen (36).

Given that the transcriptional coactivators tested in this study are not required for IE gene transcription, the mechanism by which VP16 stimulates IE gene transcription is still not clear. Although current models suggest that histones are first deposited on the viral genome at early times in infection and then removed, no evidence indicates that histone deposition precedes IE gene transcription. An alternative to the model that histones are deposited, modified and then removed is a model in which deposition of histones on the viral genome is prevented by a yet undefined mechanism. If this is the case, then coactivators would not be required for transcription of IE genes. Alternatively, other mechanisms, such as recruitment of Set1 histone methyltransferase by HCF-1 (22, 48) or disruption of REST/CoREST/HDAC repressor complex by ICP0 (13, 14), may be

the major determinant for histone depletion on the viral genome. In support of ICP0 having a role in chromatin dynamics during HSV-1 infections, a recent study indicated that the absence of ICP0 resulted in an increase in histone occupancy and a decrease in the ratio of acetylated histones on the viral genome during lytic infection (7). Since we have minimized ICP0 protein expression in our assays, it is unlikely that ICP0 will have bypassed the need for VP16 –dependent recruitment of coactivators in the present work.

An alternative mechanism that might lead to histone depletion is high rates of transcription by RNAP II. Several studies in yeast have indicated that histones are depleted from heavily transcribed regions (19, 31, 33, 39, 61). Certain histone chaperones, such as Spt6 and FACT, are components of the RNAP II transcription machinery and facilitate elongation by RNAP II (2, 27). By extension then, VP16, ICP0 and ICP4 might all contribute to effective transcription by RNAP II, which may in turn result in histone depletion from the viral genome. Another histone chaperone potentially involved in histone dynamics on the viral genome is HIRA, which is implicated in replication-independent histone deposition (18, 60) and was shown to be present in PML bodies in senescent cells (77). Future studies should test whether RNAP II or the associated histone chaperones underlie histone depletion from the HSV-1 genome.

Overall, we have shown in this report that various transcriptional coactivators are not required for IE gene expression during lytic infection or during VP16-mediated induction of IE gene transcription from nucleosome-laden HSV-1 genomes in cultured cells. Future studies should address whether other transcriptional coactivators contribute to viral gene transcription during lytic infection. The underlying mechanism for histone deposition on or histone removal from the HSV-1 genome must still be defined. Finally,

the role of chromatin dynamics during establishment and reactivation of latent infections remains an important and incompletely answered question.

6. ACKNOWLEDGEMENTS

BRM, Brg-1, dnBRM and dnBRG-1 expression plasmids were generously provided by Bernard Weissman and David Reisman. pCI-p300 plasmid was obtained from Yoshihiro Nakatani. We thank Rick Hay and Wade Bresnahan for providing SW13 and telomerase-transformed human foreskin fibroblasts, respectively. We thank Dr. Carrie Graveel, Dr. Xu Lu, and Glen Alberts for comments on the manuscript. This research was supported by the Department of Biochemistry and Molecular Biology at Michigan State University, the Van Andel Research Institute and NIH grant AI064634. SK was supported by a special fellowship from the College of Natural Science at Michigan State University and by a predoctoral fellowship from the American Heart Association. SD was supported by a summer research internship funded by the Fred and Lena Meijer Foundation.

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Table 1. siRNA sequences and catalogue numbers used in this study.

siRNA	Catalog #	siRNA sequence
p300_1	1024846	(S) CCC CUC CUC UUC AGC ACC AdTdT* (A) UGG UGC UGA AGA GGA GGG GdTdT
p300_2	SI02626267	(S) GGA CUA CCC UAU CAA GUA AdTdT (A) UUA CUU GAU AGG GUA GUC CdAdA
CBP_1	Custom designed**	(S) AAC UGU CGG AGC UUC UAC GAG dTdT (A) CUC GUA GAA GCU CCG ACA GUU dTdT
CBP_2	SI02622648	(S) CGC AUUGUC GAA CCA UGA AdTdT (A) UUC AUG GUU CGA CAA UGC GdGdG
BRM_1	SI00726747	(S) GCG UCU ACA UAA GGU GUU AdTdT (A) UAA CAC CUU AUG UAG ACG CdCdT
BRM_2	SI03033191	(S) GCC CAU CGA UGG UAU ACA UdTdT (A) AUG UAU ACC AUC GAU GGG CdTdT
BRG-1_1	SI00047579	(S) GCG CUA CAA CCA GAU GAA AdTdT (A) UUU CAU CUG GUU GUA GCG CdGdG
BRG-1_2	SI03098998	(S) GGG CGU ACG AGU UUG ACA AdTdT (A) UUG UCA AAC UCG UAC GCC CdAdG
PCAF_1	SI03038035	(S) AGU CUA CCU CGG UAC GAA AdTdT (A) UUU CGU ACC GAG GUA GAC UdGdT
PCAF_2	SI03048325	(S) CGC CGU GAA GAA AGC GCA AdTdT (A) UUG CGC UUU CUU CAC GGC GdAdT
GCN5_1	SI00426118	(S) CCA UUU GAG AAA CCU AAU AdTdT (A) UAU UAG GUU UCU CAA AUG GdAdG
GCN5_2	SI00426125	(S) GCG GCA UCA UCG AGU UCC AdTdT (A) UGG AAC UCG AUG AUG CCG CdGdG
AllStars control	1027280	Sequence information not provided.

* dN represents the 3' deoxyribonucleic acid overhangs.
** Synthesized by Dharmacon.

Table 2. Oligonucleotides used as primers in quantitative real-time PCR . Other PCR primers used in this study are as explained before (20, 54).

Gene	Oligonucleotide sequence
p300	(F) 5'-CAATGAGATCCAAGGGGAGA (R) 5'-ATGCATCTTTCTTCCGCACT
CBP	(F) 5'-GTGCTGGCTGAGACCCTAAC (R) 5'-GGCTGTCCAAATGGACTTGT
BRM	(F) 5'-CTGAAGATCGTGCTGCTTTG (R) 5'-CCAGTCGCTGTCAAAGATGA
BRG-1	(F) 5'-TCACTGACGGAGAAGCAGTG (R) 5'-TTCTTGCTCTCGTCGTCCTT
PCAF	(F) 5'-GAAACTGGAGAAACTCGGAGTGTAC (R) 5'-TTTCCAGCCATTACATTTACAAGACT
GCN5	(F) 5'-TCCTCACTCACTTCCCCAAATT (R) 5'-TGGAGAGTTTGCCCCATAGATC
CD44	(F) 5'-GAAACTGGAACCCAGAAGCACA (R) 5'-TGATGCTCATGGTGAATGAGGG
ICP27 promoter	(F) 5'-TGGTGTCTGATTGGTCCTTG (R) 5'-CGGGTGGTGGATGTCCTTAT
gC promoter	(F) 5'-TCGGGCGATTGATATATTTTT (R) 5'-TGTCCCCTTCCGGAATTTAT

CHAPTER THREE

Kutluay, Sebla B., Doroghazi, James, Roemer, Martha, Triezenberg, Steven J. (2008). Curcumin Inhibits Herpes Simplex Virus Immediate Early Gene Expression by a Mechanism Independent of p300/CBP Histone Acetyltransferase Activity. Virology. 373(2): 239-47.

Chapter 3

CURCUMIN INHIBITS HSV-1 INFECTION BY A MECHANISM INDEPENDENT OF P300/CBP HAT ACTIVITY

1. ABSTRACT

Curcumin, a phenolic compound from the curry spice turmeric, exhibits a wide range of activities in eukaryotic cells, including antiviral effects that are at present incompletely characterized. Curcumin is known to inhibit the histone acetyltransferase activity of the transcriptional coactivator proteins p300 and CBP, which are recruited to the immediate early (IE) gene promoters of herpes simplex virus type 1 (HSV-1) by the viral transactivator protein VP16. We tested the hypothesis that curcumin, by inhibiting these coactivators, would block viral infection and gene expression. In cell culture assays, curcumin significantly decreased HSV-1 infectivity and IE gene expression. Entry of viral DNA to the host cell nucleus and binding of VP16 to IE gene promoters was not affected by curcumin, but recruitment of RNA polymerase II to those promoters was significantly diminished. However, these effects were observed using lower curcumin concentrations than those required to substantially inhibit global H3 acetylation. No changes were observed in histone H3 occupancy or acetylation at viral IE gene promoters. Furthermore, p300 and CBP recruitment to IE gene promoters was not affected by the presence of curcumin. Finally, disruption of p300 expression using a short hairpin RNA did not affect viral IE gene expression. These results suggest that curcumin affects VP16-mediated recruitment of RNA polymerase II to IE gene promoters by a mechanism independent of p300/CBP histone acetyltransferase activity.

2. INTRODUCTION

Curcumin (diferuloylmethane) is the major component of the curry spice turmeric (*Curcuma longa* Linn.). Curcumin can affect the metabolism of cells and organisms in a number of ways, including apoptosis, cell signaling, inflammation and carcinogenesis [reviewed in (22, 45)]. Various antiviral effects of curcumin have been described, but the biochemical mechanisms of those effects have been incompletely defined (6, 7, 31, 36, 47). Curcumin reportedly inhibits transcription and replication of the human immunodeficiency virus (HIV-1) by blocking Tat-mediated transactivation or by diminishing viral protease and integrase activity (7, 31, 36, 47). Virions of herpes simplex virus type 2 (HSV-2) were rendered less infectious by exposure to curcumin prior to infection of HeLa cells or of mouse genitalia (6). In some cases, the antiviral effects of curcumin arise from inhibition of a cellular process or a transcription factor. For example, curcumin has been shown to suppress transcription activation by the host protein AP-1 (2) leading to diminished HTLV-1 and HPV-mediated cellular transformation (10, 42, 48).

The packaging of eukaryotic DNA in the form of chromatin presents a significant impediment to the transcriptional machinery (30). This barrier can be overcome by the action of coactivator proteins that typically comprise multi-protein complexes with either of two types of enzymatic activities. Some coactivators covalently modify histones by acetylation, methylation, phosphorylation, ubiquitinylation, prolyl isomerization or ADP-ribosylation (17, 21, 33, 37, 40, 51). Other coactivators hydrolyze ATP in the process of remodeling the position of nucleosomes along DNA or in removing nucleosomes from DNA (23, 46). Curcumin inhibits the histone acetyltransferase (HAT) activity of the

closely-related transcriptional coactivator proteins p300 and CBP *in vitro* (IC_{50} of 25 μM) and *in vivo* (3, 34). This inhibition might disrupt expression of genes dependent on those coactivators.

Herpes simplex virus type 1 (HSV-1) is a large DNA virus with a linear double stranded genome of 152 kb. Upon entry by fusion of its envelope with the host cell plasma membrane, the viral capsid is transported to the nuclear pore and the viral genome is released into the nucleus which starts the temporally regulated gene expression cascade involving transcription of immediate-early (IE or α), early (E or β) and late (L or γ) genes. IE gene transcription is strongly stimulated by the virion-borne trans-activator VP16 (8, 53). VP16 comprises a core domain (residues 1-410) and an acidic activation domain (AD) (residues 413-490), which has often been used for creating novel transactivators in heterologous expression systems (44, 49). VP16 is recruited to TAATGARAT motifs on IE gene promoters through interactions of its core domain with two host proteins, HCF and Oct-1, forming the VP16-induced complex (VIC) (55). VP16, mainly by its activation domain, then interacts with various general transcription factors and recruits RNA polymerase II (RNAP II) machinery (13, 18, 25, 32, 52, 56). Although the HSV-1 genome has long been suggested to be non-nucleosomal during lytic infection (28, 29, 38, 39), we and others have recently reported that histone H3 is associated with the HSV-1 genome and that histone tail modifications such as methylation and acetylation can be detected on transcriptionally active viral promoters and ORFs (15, 16, 24). We have also shown that p300 and CBP HATs are recruited to IE gene promoters in a manner dependent on the VP16 AD (15), suggesting that histone acetylation by p300/CBP on the viral genome might be important for IE gene expression.

Since curcumin inhibits p300/CBP HAT activity, we wanted to test whether curcumin could act as a potential anti-herpetic compound by inhibiting IE gene expression. In this report, we show that curcumin treatment of HeLa cells at non-toxic levels slows HSV-1 replication and decreases the ability of cells to support infection by HSV-1. This effect is mediated at least in part by inhibition of IE gene expression, since curcumin reduced the recruitment of RNAP II to IE gene promoters but not to cellular control promoters. However, the concentrations of curcumin required for these effects on viral gene expression were far less than the concentrations required to substantially inhibit global H3 acetylation. Moreover, the occupancy of histone H3 on IE gene promoters and the acetylation of H3 at those promoters did not change significantly in the presence of curcumin. Furthermore, no effect on viral gene expression was observed when p300 expression was disrupted by a short hairpin RNA. These results suggest that curcumin might inhibit recruitment of RNAP II machinery through a mechanism independent of the effect of curcumin on p300 or CBP HAT activity.

3. METHODS

Cell lines, viruses and treatment: HeLa (ATCC, Cat.# CCL-2) and Vero (ATCC, Cat.# CCL-81) cells obtained from the American Type Culture Collection were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). The KOS strain of HSV-1 was used to infect HeLa cells at a multiplicity of infection (MOI) of 1-5 pfu/cell, titered in Vero cells. Curcumin (Sigma, Cat. # C1836) was dissolved in dimethyl sulfoxide (DMSO). Trichostatin A (TSA) (Sigma, Cat. # T8552) was dissolved in ethanol. Cells in normal growth media were treated with curcumin as indicated in figure legends.

Plasmids and transfection: Construction of the pSUPER-p300 plasmid has been previously described (11). Briefly, a 64-mer doublestranded oligonucleotide targeting p300 was cloned into the pSUPER vector (OligoEngine) digested with *Bgl* II and *Hin*d III. The oligonucleotide sequences are as follows, with bold font indicating the p300-specific region:

Forward primer: 5'- GAT CCC C**GT CTT GGC ATG GTA CAA GA**T TCA AGA GA**T CTT GTA CCA TGC CAA GA**C TTT TTG GAA A -3'

Reverse primer: 5'- AGC TTT TCC AAA AA**G TCT TGG CAT GGT ACA AGA** TCT CTT GAA **TCT TGT ACC ATG CCA AGA** CGG G -3'

HeLa cells were transfected with 3 μg of the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions.

Single-step growth curve: To determine growth curves of HSV-1 in the presence and absence of curcumin, Vero cells were plated at a density of 1 x 10^6 cells per 60 mm tissue culture plate. After 24 h, cells were pretreated for two hours with 20 μM curcumin prior to infection at a multiplicity of 1 pfu/cell. Duplicate samples were harvested at 4-h intervals by dislodging cells into the overlying medium with a disposable scraper. The cells were disrupted by sonication and insoluble cell debris was removed by centrifugation. The titer of each sample was determined in triplicate by plaque assay.

Plaque assay: Plaque assays were performed by infecting nearly confluent 60 mm plates of Vero cells in triplicate for each virus dilution. 100 μl of each dilution was added to plates and plates were rocked every 15 minutes for 1 hour. After 1 hour, cells were washed with DMEM lacking FBS and then overlaid with DMEM containing 2% FBS and 0.9% Sea Plaque Agarose. After 3 days, plates were stained with neutral red (0.2 mg/ml in PBS) and incubated one more hour for visualization of plaques. Plaque sizes were measured using a Nikon Eclipse TE300 microscope equipped with a Hamamatsu digital camera, C4742-95, using OpenLab software.

ChIP assay: Chromatin immunoprecipitation (ChIP) assays were performed as described previously (15). Briefly, HeLa cells were fixed with 1% formaldehyde and were collected in a hypotonic buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5 % IGEPAL CA-630 with protease inhibitors). Nuclei were released by dounce homogenizer, collected and sonicated to obtain 200 to 500 bp DNA fragments. After preclearing with protein G-agarose beads, IPs were performed overnight at 4°C. Antigen-antibody complexes were

precipitated by using protein G-agarose beads. Beads were washed and the protein DNA complexes were eluted with 100 µl of 50 mM Tris-HCl (pH 8.0)-10 mM EDTA-1% sodium dodecyl sulfate for 20 min at 65°C. Cross-links in DNA-protein complexes were reversed by overnight incubation at 65°C and then precipitated with ethanol. DNA was then further purified by Proteinase K digestion, phenol:chloroform (1:1) extraction and ethanol precipitation. DNA samples were resuspended in 75 µl of water and subjected to quantitative real-time PCR analysis.

ChIP assays were performed using antibodies or antisera directed against VP16 (50), RNA Pol II (8WG16; Covance), CBP (A-22; Santa Cruz Biotechnology), p300 (N-15; Santa Cruz Biotechnology), histone H3 acetylated at Lys9 (07-352, Upstate), histone H3 acetylated at Lys14 (07-353, Upstate) and a C-terminal epitope of histone H3 (ab1791; Abcam). Control IPs were performed using no antibodies.

RNA isolation, reverse transcription, Q-PCR: Total RNA was isolated by Trizol according to manufacturer's instructions. 1 ug RNA was reverse transcribed using the Reverse Transcription System (Promega). Quantitative real-time PCR analysis for gene expression and ChIP experiments was performed by SYBR Green assay using ABI 7500 real time PCR system (Applied Biosystems). The primer pair for the *c-fos* promoter was: 5'-GAACTGCGAAATGCTCACGAGATTA-3' (forward) and 5'-AGTGTAAACGTCACGGGCTCAA-3' (reverse). The primer pair for the *c-fos* ORF was: 5'-TCAACGCGCAGGACTTCTG-3' (forward) and 5'-GCAGTGACCGTGGGAATGA-3' (reverse). The primer pair for p300 was: 5'-

CAATGAGATCCAAGGGGAGA-3' (forward) and 5'-ATGCATCTTTCTTCCGCACT-3' (reverse). The primer pair for CBP was: 5'-GTGCTGGCTGAGACCCTAAC-3' (forward) and 5'-GGCTGTCCAAATGGACTTGT-3' (reverse). Other primer pairs were previously described (15, 41).

Western blot analysis: Histones from HeLa cells were isolated by acid-extraction. In brief, nuclei were released by Triton extraction buffer (PBS containing 0.5% Triton X-100 (v/v) and PMSF) and collected. Histones were then extracted using 0.2 N hydrochloric acid. Acid soluble proteins were loaded onto a 15% sodium dodecyl sulfate-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane, blocked at room temperature for 1 hr in 5% BSA/TTBS and proteins were identified using antibodies specific to histone H3 (ab1791, Abcam) or H3 acetylated at Lys9 and Lys14 (06-599, Upstate).

For the p300 western blot, total cell lysate was prepared from cells by RIPA buffer and loaded onto a 6% sodium dodecyl sulfate-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and blocked at 4°C overnight in 5% non-fat dry milk-TTBS. p300 was detected by a rabbit polyclonal antibody from Santa Cruz (SC-584). GAPDH was detected by a mouse monoclonal antibody from Chemicon (MAB374).

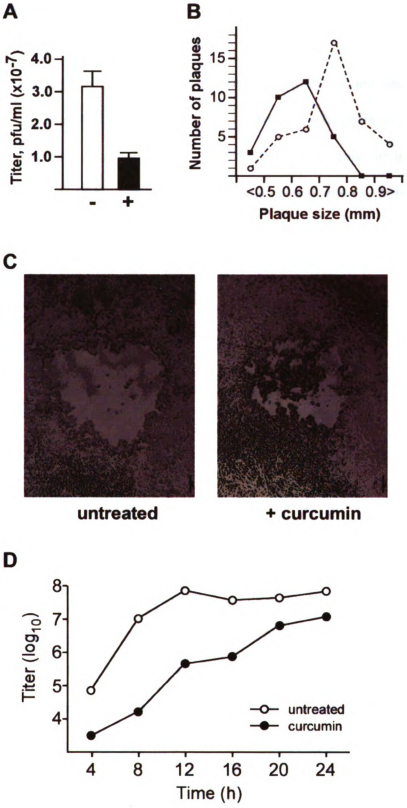
4. RESULTS

Curcumin diminishes HSV-1 infectivity and slows HSV-1 replication:

A previous report indicated that curcumin could reduce the infectivity of HSV-2 virions when the chemical was added to virus preparations prior to infection (6). This observation suggests that curcumin has a biochemical effect on the structure or integrity of the virion itself. Given the evidence that curcumin can also inhibit a range of cellular processes on which viral gene expression and replication might depend, we tested whether the ability of cells to support HSV-1 infection and replication was altered by the presence of curcumin. To address whether curcumin inhibits infection by HSV-1, we performed plaque assays in cells that were treated with curcumin. The presence of curcumin reduced the number of plaques on Vero cell monolayers by approximately three-fold (Fig. 3.1A). The plaque size, which reflects multiple rounds of infection, was also affected by curcumin. After three days, the mean plaque size in the presence of curcumin was 0.62 ($\pm$ 0.10) mm, whereas the mean plaque size in untreated cells was 0.73 ($\pm$ 0.13) mm. The plaque size distributions in treated and untreated cells (Fig. 3.1B) are significantly different ($p \leq 0.00005$ by Wilcoxon rank-sum test). The plaque morphology was also different in treated and untreated cells (Fig. 3.1C). In curcumin-treated cells, although the cytopathic effects of late-stage HSV infection were obvious in the middle of the plaque, the plaque was not cleared as in untreated cells. This effect might also be an indication that the lytic infectious cycle is slowed in the presence of curcumin. These results suggest that curcumin reduces the ability of cells to support HSV infection, and that curcumin slows but does not totally block HSV-1 replication.

We then tested further the effect of curcumin on viral replication kinetics and production of infectious virions. Fig. 3.1D shows the results of a single-step growth curve assay indicating that production of infectious virus was diminished in cells treated with curcumin. In mock-treated Vero cells, production of infectious progeny virus reached a maximum of approximately 10^8 plaque-forming units per ml (pfu/ml) by 12 hours post-infection (hpi). In the presence of curcumin, progeny virus titers were lower throughout the infection period, but did increase steadily to a maximum of approximately 10^7 pfu/ml. We conclude that treatment of cells with curcumin decreases the ability of cells to support HSV-1 infection and slows but does not block the viral replication cycle.

Figure 3.1: Effects of curcumin on HSV-1 plaque formation and viral replication. (A) Plaque assays were performed in Vero cells pretreated (+) with 10 μM curcumin or untreated (-) for 2 hours and infected with serial dilutions of a stock preparation of HSV-1. 10 μM curcumin was also present in the viral inoculums and overlay agar media of treated samples. Error bars represent the standard deviation among triplicate assays at two viral dilutions. (B) Plaque sizes in the absence (open circles) or presence (closed circles) of curcumin were measured by averaging randomly chosen vertical and horizontal diameters from plaques. The numbers of plaques within various size ranges (from less than 0.5 mm to greater than 0.9 mm) are shown. (C) Representative plaques from plaque assays performed in the absence (left panel) and presence (right panel) of 10 μM curcumin. Scale bar: 100 μm. (D) Single-step growth curves were performed in Vero cells infected with HSV-1 at an MOI of 1 pfu/cell. Virus titers in samples collected at 4 h intervals were assayed in triplicate on Vero cells. Closed circles represent curcumin-treated samples; open circles represent parallel cultures of untreated cells.


A
Titer, pfu/ml (x10^{-7})
4.0
3.0
2.0
1.0
-
+
B
Number of plaques
15
10
5
<0.5 0.6 0.7 0.8 0.9>
Plaque size (mm)
C
untreated
+ curcumin
D
Titer (log_{10})
8
7
6
5
4
untreated
curcumin
4 8 12 16 20 24
Time (h)

Curcumin inhibits IE gene expression without affecting the delivery of the viral genome to the nucleus:

We have previously reported that the transcriptional coactivator and histone acetyltransferase proteins p300 and CBP are recruited to IE gene promoters in a manner dependent on the VP16 transcriptional activation domain (15). Curcumin can inhibit the HAT activity of p300 and CBP but not of other mammalian coactivators such as PCAF (3). We hypothesized that if the HAT activities of p300 and CBP are important for IE gene expression and if curcumin inhibits that activity, then curcumin would inhibit IE gene expression. Indeed, in HeLa cells treated with nontoxic levels of curcumin and infected at a multiplicity of 1 pfu/cell, the steady-state mRNA levels for the IE genes ICP4 and ICP27 were significantly reduced (Fig. 3.2A), whereas levels of CBP transcripts (Fig.3.2A) and p300 transcripts (data not shown) were unchanged. This result suggests that curcumin inhibits VP16-dependent transcription activation, potentially by inhibiting p300/CBP HAT activity. Since curcumin accumulates in various cellular membranes (20), we were concerned that it might interfere with viral entry or delivery of the viral genome to the nucleus, which would result in diminished IE gene expression. This concern was alleviated by assaying viral DNA levels in the nuclear fractions of curcumin-treated and mock-treated cells. Quantitative PCR results shown in Fig. 3.2B show no difference in viral DNA levels between treated and untreated cells. We conclude that curcumin treatment does not disrupt the delivery of viral genomes to the infected cell nucleus, and infer that curcumin has a more direct role in inhibiting IE gene transcription.

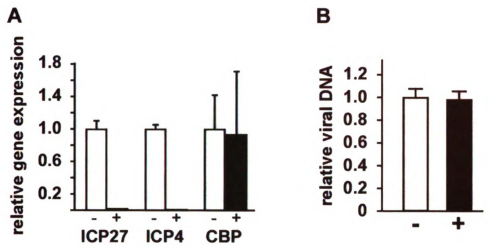


Figure 3.2: Effects of curcumin on HSV-1 IE gene expression. (A) HeLa cells were pretreated with 20 μM curcumin (+, black bars) or DMSO only (-, open bars) for 3 hours and then infected with HSV-1 at an MOI of 1 pfu/cell. Total RNA was isolated at 2 hpi and gene expression was analyzed by Q-RT-PCR, normalized against 18S rRNA levels. Mean values from three experiments for each gene tested are shown relative to values obtained from untreated cells; error bars represent standard deviations. (B) HeLa cells were infected with HSV-1 in the presence (+, black bars) or absence (-, open bars) of 20 μM curcumin. Aliquots of nuclear extracts (as used in ChIP assays, Fig. 3.3) were analyzed by Q-PCR. The amount of viral DNA (represented by the ICP27 gene promoter) was normalized against cellular DNA (represented by the U3 snRNA promoter). Error bars represent the standard deviation among five independent experiments.

Curcumin decreases RNAP II occupancy on IE gene promoters and ORFs but does not affect recruitment of VP16:

One way that curcumin might block IE gene expression is by decreasing the recruitment of VP16 to IE gene promoters. Alternatively, curcumin might affect recruitment of RNAP II without affecting VP16 occupancy. To test these hypotheses, we performed chromatin immunoprecipitation (ChIP) assays to probe the presence of VP16 and RNAP II at IE genes in HeLa cells infected at an moi of 5 pfu/cell. Curcumin had no substantial effect on the recruitment of VP16 to the ICP0 or ICP27 promoters (Fig. 3.3A). The U3 snRNA gene promoter served as a negative control, showing that VP16 occupancy as detected by ChIP assays was specific to viral IE promoters, as previously shown (15). In contrast, curcumin caused a significant decrease in the occupancy of RNAP II on both the promoter and transcribed region (ORF) of ICP27 (Fig. 3.3B). Similar decreases in RNAP II occupancy were observed for the ICP0 gene promoter (data not shown). The modest effect of curcumin on the presence of RNAPII on the U3 snRNA promoter was not observed in other replicates of this experiment (data not shown). We conclude that curcumin decreases VP16-dependent recruitment of RNAP II and hence IE gene expression.

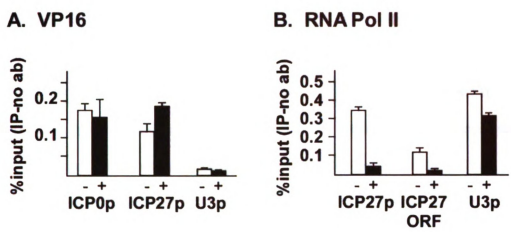


Figure 3.3: Curcumin decreases occupancy of RNA polymerase II but not of VP16 at IE genes. HeLa cells treated with 20 μM curcumin (+, black bars) or DMSO only (-, open bars) were infected with HSV-1 at an MOI of 5 pfu/cell. Chromatin immunoprecipitation from sonicated nuclear extracts was performed using antibodies against VP16 (A) or RNA pol II (B). Samples were analyzed by Q-PCR using primers specific for the ICP0 promoter, ICP27 promoter, ICP27 ORF or the cellular U3 snRNA promoter. Aliquots of samples prior to immunoprecipitation (1%, 0.3%, 0.1%, 0.04% input) were used to create a standard curve of input DNA. This curve was used to determine the relative amounts of a given DNA fragment in immunoprecipitated or control (no antibody) samples. The values for no-antibody controls were subtracted from the values for corresponding IP samples. Error bars represent the range between technical duplicates for a representative experiment.

Curcumin has no significant effect on H3, AcH3, p300 and CBP occupancy on IE gene promoters and ORFs:

One of the potential ways that curcumin might inhibit RNAP II recruitment is by inhibiting p300/CBP HAT activity (3, 34), thus permitting formation of transcriptionally-repressive chromatin on viral DNA. We first tested whether the levels of curcumin (20 μM) that inhibit viral gene expression also affect total acetylation levels of histone H3. An immunoblot using an antibody specific to acetylated Lys9 and Lys14 of H3 revealed no significant change in total histone H3 acetylation after three hours, five hours, or overnight incubation in 20 μM curcumin (Fig. 3.4A). Those times correspond to beginning and end of viral infection, respectively, for the viral gene expression analysis indicated in Fig. 3.2A. A decrease in histone H3 acetylation was observed only after overnight incubation in the presence of higher curcumin concentrations (Fig. 3.4A). We infer that, under the conditions used in viral gene expression assays, curcumin does not significantly inhibit p300/CBP HAT activity.

We have previously shown that HSV-1 IE gene promoters are relatively devoid of histone H3 at 2 hpi, but that H3 and acetylated H3 can be found at IE gene ORFs and also at DE and L gene promoters at that time (15). However, during infection with a mutant virus (strain RP5) lacking the VP16 activation domain, H3 did associate with IE promoters, but acetylated H3 levels at IE ORF and DE or L gene promoters were reduced. On that basis, we hypothesized that p300/CBP HAT activity might be required for covalent modification and subsequent removal of histones from IE gene promoters, to allow recognition of those promoters by RNAP II. That model led to the prediction that curcumin, by inhibiting p300/CBP HAT activity, would diminish levels of acetylated

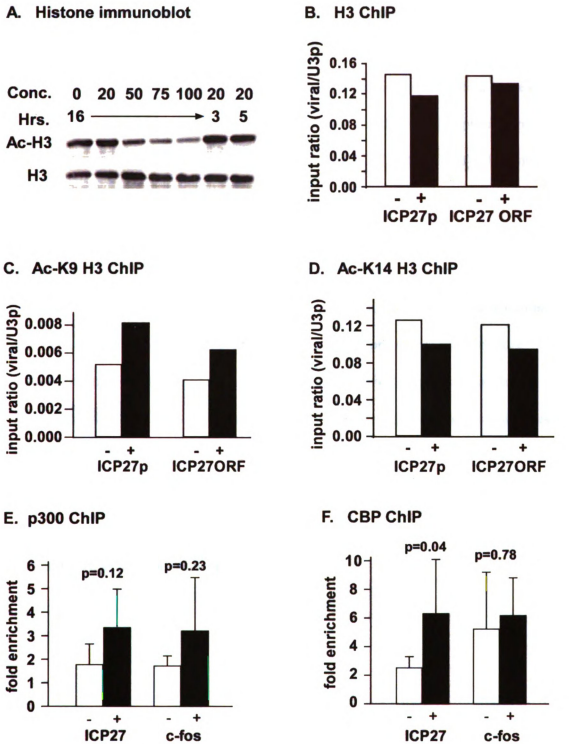
histones at IE gene promoters and ORFs, and would increase the level of H3 at IE gene promoters, as observed during RP5 infection (15).

ChIP assays were performed using antibodies that recognize an H3 carboxyl-terminal epitope unaffected by known modifications or antibodies specific to H3 acetylated at either Lys9 or Lys14. Contrary to our hypothesis, we observed no significant increase in H3 occupancy (Fig. 3.4B) and no significant decrease in K9-acetylated H3 (Fig. 3.4C) or K14-acetylated H3 (Fig. 3.4D) occupancy on the ICP27 promoter or ORF. We conclude that the inhibition of IE gene expression caused by curcumin is not the result of changes in histone occupancy or acetylation at IE gene promoters, and thus the HAT activity of CBP and p300 is not likely to be critical for viral IE gene expression.

A recent report has suggested that relatively high concentrations of curcumin can inhibit the recruitment of p300 to promoter DNA fragments *in vitro* (4). Thus, we considered the possibility that although their HAT activity per se might not be required, p300 and CBP might act as scaffolding proteins at IE gene promoters for the recruitment of other transcription factors. We used ChIP assays to test whether curcumin inhibits the recruitment of p300 and CBP to IE gene promoters. The results (Fig. 3.4E,F) indicate that curcumin did not block the recruitment of p300 or CBP to IE gene promoters nor to the c-fos promoter, which was previously shown to be dependent on p300/CBP (19, 43). Instead, we observed a modest increase in recruitment of p300 and CBP at the ICP27 promoter. One potential explanation for this increase is that curcumin can inhibit the autoacetylation of p300 or CBP and hence inhibit the dissociation of CBP from the IE gene promoter, which would in turn prevent the recruitment of RNAP II. Taken together,

these results suggest that curcumin affects recruitment of RNAP II to the HSV-1 genome but not to cellular control gene promoters, and that this effect is independent of the effects of curcumin on p300 and CBP HAT activity.

Figure 3.4: Curcumin does not affect histone H3, p300, or CBP occupancy, nor H3 acetylation, at IE genes. (A) HeLa cells were treated with curcumin (concentrations given in μM) for various times in the presence of 1.4 μM trichostatin A. Acid extracted histones were analyzed by immunoblotting using antibodies directed against H3 acetylated at Lys9 and Lys14 (top panel) or a carboxyl-terminal epitope of H3 (bottom panel). (B-F). Chromatin immunoprecipitation was performed as described for Fig. 3.3, but using antibodies against the H3 carboxyl-terminal domain (B), H3 acetylated at Lys9 (C), H3 acetylated at Lys14 (D), the coactivator p300 (E), or the coactivator CBP (F). The %input values in B-D were further normalized to U3 snRNA promoter as a means to control immunoprecipitation efficiency. Since the p300/CBP IP efficiencies were low, this data is represented as fold enrichment over no antibody control. Error bars represent the standard deviation among six (for ICP27 promoter) and four (for c-fos promoter) independent experiments (D, E). p-values in (D,E) were determined by Student's-t-test.


A. Histone immunoblot
Conc. 0 20 50 75 100 20 20
Hrs. 16 → 3 5
Ac-H3
H3
B. H3 ChIP
input ratio (viral/U3p)
0.16
0.12
0.08
0.04
0.00
- + - +
ICP27p ICP27 ORF
C. Ac-K9 H3 ChIP
input ratio (viral/U3p)
0.008
0.006
0.004
0.002
0.000
- + - +
ICP27p ICP27ORF
D. Ac-K14 H3 ChIP
input ratio (viral/U3p)
0.12
0.08
0.04
0.00
- + - +
ICP27p ICP27ORF
E. p300 ChIP
fold enrichment
p=0.12
p=0.23
6
5
4
3
2
1
0
- + - +
ICP27 c-fos
F. CBP ChIP
fold enrichment
p=0.04
p=0.78
10
8
6
4
2
0
- + - +
ICP27 c-fos

Diminished expression of p300 does not affect IE gene expression:

In order to validate the conclusion that curcumin inhibits IE gene expression independent of p300 HAT activity, we have analyzed IE gene expression when p300 expression was diminished by RNA interference. Transfection of a plasmid expressing a short hairpin RNA (shRNA) specific for p300 resulted in efficient disruption of p300 expression assessed as either protein (Fig. 3.5A) or as mRNA (Fig. 3.5B). The disruption was specific for p300, since CBP expression was not affected (Fig. 3.5B). However, viral IE gene expression was not significantly affected (Fig. 3.5C), suggesting that p300 is not required for HSV-1 IE gene expression. This result also argues against the possible role of p300 as a scaffolding protein on IE gene promoters. Another possible explanation is that p300 and CBP may act redundantly on viral IE gene promoters and therefore knocking down only p300 does not affect IE gene expression. Another possible explanation is that p300 and CBP may act redundantly on viral IE gene promoters and therefore knocking down only p300 does not affect IE gene expression. We are currently working on a more detailed analysis of coactivator disruption, which will be the subject of another manuscript. Our preliminary results suggest that neither p300 nor CBP are essential for IE gene expression (Kutluay, et al., manuscript in preparation).

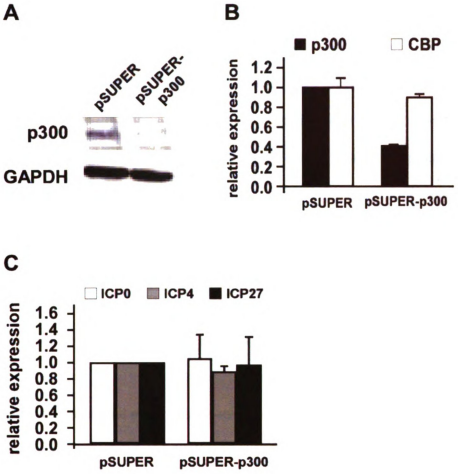


Figure 3.5: Knocking down p300 expression does not affect HSV-1 IE gene expression. HeLa cells were transfected with 3 μg of pSUPER (empty) or pSUPER-p300 plasmid that expresses a shRNA targeting p300. 48 hours post-transfection cells were infected with HSV-1 at an MOI of 5 pfu/cell and total protein and RNA were isolated. p300 knockdown efficiency was analyzed by immunoblotting (A) and Q-RT-PCR (B). IE gene expression was analyzed by Q-RT-PCR as indicated in previous figure legends (C). Data represents the average of three independent experiments done at least in biological duplicates. Error bars represent the standard deviation.

5. DISCUSSION

This study was performed to assess whether curcumin would act as an antiviral agent for HSV-1 infection, based on the hypothesis that curcumin could prevent IE gene expression by inhibiting the p300 and CBP HATs (3) that are recruited to IE gene promoters during lytic infection (15). Our results demonstrate that curcumin-treated cells are indeed diminished in their ability to support HSV-1 infection and replication. The presence of curcumin did not block the delivery of the viral genome to the nucleus, indicating that binding, entry, and intracellular transit of the virus are not substantially affected by curcumin. We did observe a significant decrease in expression of viral IE genes, corresponding to diminished recruitment of RNAP II to IE gene promoters. These outcomes are all consistent with the hypothesis that curcumin specifically affects viral IE gene expression.

However, additional observations suggest that the antiviral effects of curcumin do not arise through the mechanism initially proposed. The effects of curcumin on IE gene expression were evident using concentrations and times much less than those required for significant inhibition of overall histone acetylation. We expected that curcumin, as an inhibitor of p300 and CBP HAT activity would result in diminished histone acetylation and increased histone H3 occupancy at IE gene promoters, but those effects were not observed. Moreover, disruption of p300 protein expression had no significant effect on IE gene expression. We conclude that the HAT activity of p300 and CBP is not essential for histone clearance from HSV IE gene promoters or for IE gene expression, but that curcumin blocks other, yet unknown steps that are required for recruitment of RNAP II and subsequent transcription of IE genes. We cannot yet exclude the possibility that other

HATs such as PCAF and hGCN5 may act redundantly on viral genes, masking any curcumin-dependent changes in histone acetylation (9).

Several potential mechanisms whereby curcumin blocks IE gene expression can be eliminated based on additional ChIP assays. The recruitment of the virion-borne activator protein VP16 to IE promoters was not affected by curcumin. Although a prior report suggested that curcumin can inhibit recruitment of p300 to target gene promoters by the chimeric activator protein Gal4-VP16 in an in vitro experiment (4), we observed no effect of curcumin on p300 or CBP occupancy on IE gene promoters. One possible reason for this discrepancy might be the high concentrations of curcumin (300 μM) used in the *in vitro* system (4), whereas we used 10-20 μM curcumin because of cytotoxicity observed at higher concentrations and long incubation periods.

Our observation that the occupancy of histone H3 and acetylated H3 on IE gene promoters and ORFs did not change upon curcumin treatment leads to the question of why the dramatic decrease in viral gene expression does not correspond to an increase in histone occupancy on the viral genome. Since histones are not packaged with the HSV-1 genome in virion particles (12), one possibility is that the level of histone deposition on the viral genome at 2 hours post-infection is still relatively low compared to cellular genes and hence the histones do not create a substantial barrier for transcription. Another possibility is that the VP16 activation domain recruits other coactivators such as the chromatin remodeling enzymes Brm and Brg-1 (15), independent of the HAT activities of CBP and p300, to keep the viral genome free of histones. In support of the latter hypothesis, VP16 AD has been recently shown to recruit the yeast remodeling complex SWI/SNF to mono- and dinucleosomal arrays to catalyze the eviction of nucleosomes by

SWI/SNF (14).These hypotheses will be tested in the context of viral infection in future experiments.

We note that the relatively modest effects of curcumin on the viral infection and replication (Fig. 3.1) do not seem to reflect the dramatic reduction in IE gene expression (Fig. 3.2A) and in RNAP II recruitment to IE promoters (Fig. 3.3B). We suspect that this discrepancy may be due to the decay of the biological activity of curcumin when exposed to light or to cell culture media (5, 54) in the longer experiments probing viral infection and replication, relative to the shorter experiments used for IE gene expression and ChIP assays.

Others have reported that virions of HSV-2 were rendered less infectious by exposure to curcumin prior to infection of HeLa cells or of mouse genitalia (6). To date, no biochemical mechanism for this virucidal effect has been established. In contrast, the work presented here indicates that curcumin can inhibit viral gene expression and thus replication through mechanisms operative within the infected cells. This is especially evident in our observations that curcumin did not affect delivery of viral DNA to the nucleus, but had a dramatic effect on IE gene expression (Fig. 3.2). Nonetheless, we cannot fully exclude the possibility that the virucidal effects of curcumin might contribute to the diminished replication and plaque formation evident in Fig. 3.1.

Although curcumin has been widely studied in the context of cancer and apoptosis, the current literature does not clearly define the effects of curcumin on RNAPII machinery or transcription factors. One report has shown that curcumin inhibits certain cellular differentiation events by triggering protease-dependent degradation of the transcription factor AP-1 (2). Although our results cannot directly exclude the possibility

that VP16 is degraded similarly, our ChIP data suggests that more or less equivalent amounts of VP16 are immunoprecipitated with IE gene promoters and hence the mechanism whereby curcumin inhibits viral IE gene expression is unlikely to be mediated by degradation of VP16. The ChIP results also suggest that curcumin does not prevent the formation of the DNA-binding protein complex comprising VP16, Oct-1 and HCF. Since VP16 itself cannot bind to DNA with high affinity (1, 26, 35) if curcumin was preventing the interaction between VP16, Oct-1 and HCF, then less VP16 would have been immunoprecipitated to IE gene promoters in the presence of curcumin.

Curcumin is a potent compound with various biological properties. We have shown that curcumin significantly affects HSV-1 IE gene expression which thereby diminishes the ability of the virus to launch the lytic infectious cycle. Whether curcumin can be used as an anti-HSV-1 therapeutic agent will likely be limited by its low bioavailability and high degradation rates when exposed to light. Moreover it is still not known how curcumin is metabolized *in vivo* and how these by-products might affect cellular processes. Therefore understanding the fate of curcumin *in vivo* is key to developing curcumin as an alternative drug for HSV-1 treatment. Hence, our results can be considered as an early step in elucidating the molecular basis of the antiviral activities of curcumin.

6. ACKNOWLEDGEMENTS

This work was supported by the Department of Biochemistry and Molecular Biology at Michigan State University and by the Van Andel Research Institute. SK was supported by a special fellowship from the College of Natural Science at Michigan State University and by a predoctoral fellowship from the American Heart Association. JD was supported in part by summer undergraduate research fellowships from the American Society for Microbiology. We thank Dan Ducat and Dr. Franciso Herrera for preparing the pSUPER-p300 construct. We also thank Dr. Eric Xu, Dr. Xu Lu, and Dr. Francisco Herrera for thoughtful comments on the manuscript.

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CHAPTER FOUR

Kutluay, Sebla, B., Triezenberg, Steven, J. (2009). Regulation of Histone Deposition on the Herpes Simplex Virus Type 1 Genome During Lytic Infection. Journal of Virology,

Chapter 4

MECHANISMS OF HISTONE DEPLETION FROM THE VIRAL GENOME

1. ABSTRACT

During lytic infection by herpes simplex virus type 1 (HSV-1), histones are present at relatively low levels on the viral genome. However, the mechanisms that account for such low levels—how histone deposition on the viral genome is blocked or how histones are removed from the genome—are not yet defined. In this study we show that histone occupancy on the viral genome gradually increased with time when transcription of the viral immediate-early (IE) genes was inhibited either by deletion of the VP16 activation domain or by chemical inhibition of RNA polymerase II (RNAP II). Inhibition of IE protein synthesis by cycloheximide did not affect histone occupancy on most IE promoters and coding regions, but did cause an increase at delayed early and late gene promoters. IE gene transcription from HSV-1 genomes associated with high levels of histones was stimulated by superinfection with HSV-2 without altering histone occupancy or covalent histone modifications at IE gene promoters. Moreover, RNAP II and histones co-occupied the viral genome in this context, indicating that RNAP II does not preferentially associate with viral genomes that are devoid of histones. These results suggest that during lytic infection, VP16, RNAP II, and IE proteins may all contribute to the low levels of histones on the viral genome, and yet the dearth of histones is neither a prerequisite for nor a necessary result of VP16-dependent transcription of nucleosomal viral genomes.

2. INTRODUCTION

During lytic infection of mammalian cells by herpes simplex virus type 1 (HSV-1), virion protein 16 (VP16) triggers the cascade of viral gene expression by stimulating the transcription of immediate early (IE) genes (5). VP16 binds to the cis-regulatory sequences on viral IE gene promoters as part of a protein complex that also includes two host cell proteins, Oct-1 and HCF (67). VP16, through its activation domain (AD), then interacts with various general transcription factors or coactivators and recruits the host RNA polymerase II (RNAP II) machinery (13, 23, 27, 42, 68). In turn, some of the IE proteins regulate expression of the delayed early (DE) and late (L) genes, completing the viral gene expression cascade that results in production of infectious virions.

In eukaryotes, the packaging of DNA by histone proteins presents a significant impediment to the transcriptional machinery (41). This barrier can be overcome by activator-mediated recruitment of transcriptional coactivators that either covalently modify histones (29, 41) or remodel the position of nucleosomes along DNA (11, 56). Many covalent modifications of histones have been identified, including lysine acetylation and methylation (29, 41); the former is generally associated with active transcription and the latter marks either active or inactive transcription depending on which lysine residue is methylated.

Unlike the small DNA tumor viruses of the polyomavirus and papillomavirus families, the large (152 kbp) genome of HSV-1 is packaged in the virion nucleocapsid not with histones but with the polyamine spermine (7, 12, 50, 53). Long-standing evidence suggests that during lytic infection the HSV-1 genome is mainly non-

nucleosomal (39, 40, 46), whereas during latency the viral genome is associated with evenly spaced nucleosomes (10). In support of these earlier findings, chromatin immunoprecipitation (ChIP) assays have indicated that histones, typically represented by histone H3, are present on the viral genome during lytic infection, albeit at relatively lower levels than on cellular genes (20, 21, 26, 35, 50). Furthermore, active transcription marks such as histone H3 acetylated on lysine 9 and lysine 14 (H3K9/K14ac) or trimethylated on lysine 4 (H3K4me3) are also associated with the viral genome during lytic infection (20, 21, 26, 35). In contrast, during latent or quiescent infections, histones associated with the viral genome carry modifications typical of heterochromatin, such as histone H3 trimethylated on lysine 9 (H3K9me3) (8, 66).

An exception to this pattern is the latency-associated transcript (LAT) gene, which during latency is associated with H3K9/K14ac, indicative of its transcriptionally active status (31, 32, 49). Moreover, upon reactivation from latency, lytic genes become associated with acetylated histone H3, and the LAT region becomes depleted of that mark (1, 49). Together, these observations suggest that regulation of chromatin might be an important mechanism for the switch between lytic and latent infections (28).

Although histone H3 occupancy is presumed to represent the existence of the nucleosome core particle on the viral genome, whether other core histones are present on the HSV-1 genome during lytic infection has not been established. This question becomes more compelling in light of the evidence that histone H2A/H2B dimers can be removed from nucleosome core particles, resulting in the presence of "hexasomes", at actively transcribed genes (19, 33, 58). In addition, the mechanisms regulating histone levels on the viral genome during lytic infection have not yet been defined. One key issue

is whether histone deposition is inhibited or deposited histones are removed. Hints of potential mechanisms have arisen in several recent reports. For instance, an IE protein, ICP0, blocks the silencing of HSV-1 DNA by dissociating the histone deacetylase 1 from the REST/CoREST/HDAC repressor complex (14, 15). During lytic infection with a mutant virus that does not express ICP0, histone H3 occupancy on the viral genome increased and the fraction of acetylated histone H3 decreased, suggesting that the disruption of the REST/CoREST/HDAC complex by ICP0 might be relevant (6). Similarly, the tegument protein VP22 was suggested to block nucleosome assembly on the viral DNA (64), but no data yet indicate whether or how histone occupancy on the viral genome changes in the absence of VP22 during HSV-1 lytic infection.

Another factor that may mediate histone levels on the viral genome during lytic infection is VP16. We have previously shown that during lytic infection with a mutant virus that lacks the activation domain of VP16 (strain RP5), higher levels of histone H3 associate with RP5 genomes than with wild-type genomes (20). Moreover, during RP5 infections, transcriptional coactivators—such as p300 and CBP histone acetyl transferases (HATs) or BRM and Brg-1 remodeling enzymes—are not efficiently recruited to IE gene promoters (20). This observation is consistent with prior knowledge about the VP16 AD in artificial experimental contexts, in which VP16 physically interacts with and recruits transcriptional coactivators—such as HATs p300/CBP (3, 17, 24, 34, 62, 65), PCAF (65), and GCN5 (22, 37, 59, 62, 63), as well as ATP-dependent chromatin remodeling enzymes (16, 43, 47, 48)—to potentiate transcription from nucleosomal templates. However, the hypothesis that the transcriptional coactivators recruited by VP16 are required for modifying and removing the histones from the viral

genome, and thereby required for IE gene expression, is contradicted by our recent report that disruption of coactivator expression did not diminish IE gene expression during infection of cultured cells (36). Thus, the impact of histones and chromatin on viral gene expression remains incompletely defined.

In the present report, we show that during lytic infection of cultured cells, all four core histones associate with the viral genome. In the absence of VP16 transcriptional activation (i.e., during infection by the RP5 mutant virus), histone deposition throughout the viral genome increased gradually but dramatically to levels approaching that on cellular genes. Inhibition of RNAP II-mediated transcription by actinomycin D also increased the histone occupancy on the viral genome in a temporal pattern similar to that of RP5 infections. In contrast, inhibition of IE protein expression by cycloheximide had no significant effect on histone association with most of the actively transcribed IE gene promoters and coding regions, but did increase histone occupancy on DE and L gene promoters. To address whether VP16 can stimulate the removal of histones that are already deposited on the viral genome, we asked whether providing wild-type VP16 *in trans* (by HSV-2 superinfection) results in histone depletion from RP5 IE gene promoters. Surprisingly, although HSV-2 superinfection stimulated IE gene expression from the RP5 genome, it did not lead to depletion of histones from IE genes. In addition, active transcription marks, such as H3K9/K14ac or H3K4me3, did not increase on the RP5 IE gene promoters upon HSV-2 superinfection. Sequential chromatin immunoprecipitation (Seq-ChIP) experiments indicated that RNAP II and histone H3 co-occupy RP5 genomes upon HSV-2 superinfection at a level similar to that of a constitutively expressed house-keeping gene, indicating that RNAP II does not

preferentially associate with histone-free viral genomes.

Taken together, our results suggest that the low level of histone occupancy on the viral genome during lytic infection is the result of a complex process that involves VP16, active transcription by RNAP II, and IE proteins. However, histone removal or covalent modification of histones may not be necessary for the VP16-dependent transcription of IE genes from viral genomes heavily associated with histones.

3. METHODS

Cell lines and viruses: HeLa (ATCC# CCL-2) cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) containing 110 mg/l sodium pyruvate and 10% fetal bovine serum (Invitrogen). In some experiments, cycloheximide (Sigma) or actinomycin D (Sigma) were added to the cell culture medium prior to and during infection, as indicated in figure legends. The RP5 strain of HSV-1, which lacks sequences encoding the activation domain of VP16, has been previously described (60). The RP5 and wild-type KOS strains of HSV-1 and G strain of HSV-2 were prepared and titered in Vero cells.

Gene expression assays and Q-RT-PCRs: Total cellular RNA was isolated using Trizol reagent (Invitrogen). Total RNA was reverse-transcribed by random primers using a reverse transcription system (Promega). The synthesized cDNA was used as template in quantitative real-time PCR analysis using SYBR Green Master Mix (Roche) and the ABI 7500 Real-Time PCR System (Applied Biosystems). Gene expression was first normalized against 18S rRNA and then to proper controls by the $2^{-\Delta\Delta Ct}$ method. For chromatin immunoprecipitation assays, data were analyzed using the standard curve method as explained in the following section. Primer sequences for PCRs spanning the ICP27 promoter and gC promoter are as follows:

ICP27 promoter: (F) 5′-TGGTGTCTGATTGGTCCTTG and

(R) 5′-CGGGTGGTGGATGTCCTTAT

gC promoter: (F) 5′-TCGGGCGATTGATATATTTTT and

(R) 5′-TGTCCCCTTCCGGAATTTAT

Other primer pairs used in this study have been previously defined (20, 51).

Chromatin immunoprecipitation and sequential-chromatin immunoprecipitation: Chromatin immunoprecipitation was performed as previously described (20). In summary, confluent plates of HeLa cells were infected in the absence or presence of actinomycin D or cycloheximide as indicated in figure legends. Infections were stopped by addition of formaldehyde to the cell culture plate at a final concentration of 1%. After cells were resuspended in a hypotonic buffer, nuclei were released by Dounce homogenization in order to minimize the background signals from cytoplasmic capsids or membrane-bound virions. Nuclei were collected by centrifugation and then disrupted by sonication using a Branson Digital Sonifier-450 to obtain 200-1000 bp DNA fragments. Protein-DNA complexes were immunoprecipitated using 5-10 μg of antibodies against histone H2A (Abcam, ab18255), histone H2B (Abcam, ab1790), histone H3 (Abcam, ab1791), histone H4 (Upstate, 05-858), RNA polymerase II (Covance, 8WG16), or VP16 [ref. (61)]. Protein-DNA complexes were collected by Protein G-agarose beads (Invitrogen). After several washes, the protein-DNA complexes were eluted and reverse-crosslinked overnight at 65 °C in the presence of 200 mM NaCl and 10 μg RNaseA. Samples were then precipitated with ethanol, digested with proteinase K (Roche) at 42 °C for 2 h, and purified with Qiagen spin columns using the gel extraction protocol. The presence of viral and cellular DNA fragments in the immunoprecipitated material was analyzed by quantitative real-time PCR using SYBR Green Master Mix (Roche) and ABI 7500 Real-Time PCR system (Applied Biosystems). A standard curve using serial 3-fold dilutions of input samples (1%, 0.3%, 0.1%, or 0.04%) was produced to quantitate the signals from immunoprecipitation samples. Background signals obtained from immunoprecipitation reactions performed in the absence of antibodies (no antibody

control) were subtracted from the signals obtained from immunoprecipitation samples [referred as "% input (IP-noab)"]. When necessary, data was further normalized against a cellular control gene (interferon-β promoter or U3 snRNA promoter) by dividing the "% input (IP-noab)" value for the viral DNA to that of the cellular DNA in order to account for the differences in immunoprecipitation efficiencies.

For sequential ChIP assays, after the first immunoprecipitation, protein-DNA complexes were eluted in 100 µl elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) by incubating at 65 °C for 15 min. The eluates were then subjected to the second immunoprecipitation as indicated above, whereby 30-45 µl of eluate was used as input in each immunoprecipitation reaction. The rest of the procedure is the same as the ChIP protocol indicated above.

4. RESULTS

Absence of the activation domain of VP16 causes an increase in histone occupancy on the HSV-1 genome during lytic infection.

We have previously reported that during lytic infection by wild-type HSV-1, the IE gene promoters are relatively free of histones as represented by H3 (20). We have also shown that at immediate early times during infection by strain RP5 (lacking the VP16 activation domain), the HSV-1 genome more abundantly associates with histone H3 but lacks acetylated histone H3, an active transcription mark (20). In order to get a more dynamic and quantitative picture of histone deposition on the viral genome in the presence or absence of the VP16 AD, we have compared histone occupancy on the RP5 and KOS (wild-type) genomes at different stages of infection using ChIP assays coupled with Q-PCR. Parallel plates of HeLa cells were infected with KOS or RP5 viruses at multiplicities of infection such that similar numbers of viral genomes entered cells at the beginning of infection, as shown by quantitative PCR assays of infected cell nuclear extracts (Fig. 4.1). At 2, 4, and 6 hours post-infection (hpi), infections were stopped by formaldehyde cross-linking and the occupancy of histone H2A, H2B, H3, H4, and RNAP II on viral genes was analyzed by ChIP.

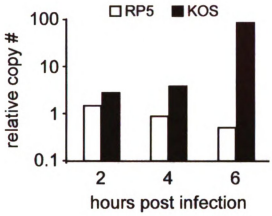


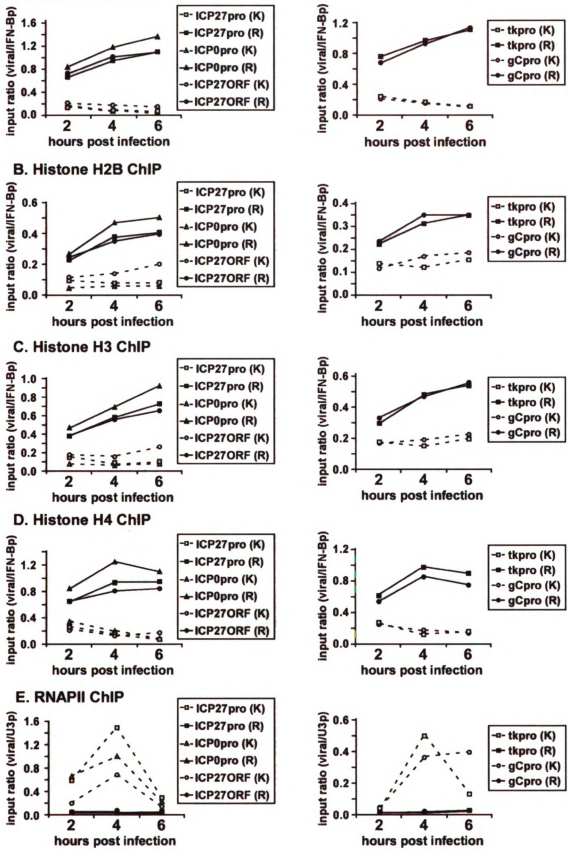
Figure 4.1: Similar numbers of RP5 and KOS viruses enter HeLa cells and RP5 viruses are debilitated in replication. The relative amount of viral DNA in RP5 and KOS infections was compared using the input standard curves from ChIP assays presented in Figure 4.2. The amount of viral DNA (represented by the ICP27 gene promoter) was normalized against cellular DNA (represented by the U3 snRNA promoter).

As indicated in Figure 4.2A, occupancy of histone H2A on the RP5 genome at 2 hpi was higher than on the KOS genome at several viral gene fragments such as IE promoters and ORFs (Fig. 4.2A, left panel) as well as at DE and L promoters (Fig. 4.2A, right panel). Histone H2A occupancy on the RP5 genome continued to increase at 4 and 6 hpi up to a point comparable to that of the cellular gene (i.e., an input ratio of approximately 1). Similar patterns were observed for histone H2B (Fig. 4.2B), H3 (Fig. 4.2C), and H4 (Fig. 4.2D). Occupancy by all core histones throughout the viral genome increased prominently but gradually in RP5 infections, whereas in KOS infections histone occupancy either stayed same or decreased during the later stages of lytic infection. As expected, recruitment of RNAP II to IE promoters, and as a consequence to DE and L gene promoters, was severely impaired in RP5 infections (Fig. 4.2E). These results together suggest that the VP16 AD contributes to keeping the viral genome free of nucleosomes.

This effect could conceivably be mediated directly by the VP16 AD, i.e., by recruiting transcriptional coactivators that modify and remodel the chromatin structure. However, we have recently indicated that transcriptional coactivators recruited by VP16 do not contribute significantly to IE gene expression during lytic infection of human foreskin fibroblasts (36). Alternatively, VP16 may indirectly regulate chromatin, e.g., by stimulating the expression of IE genes that may in turn contribute to lower histone levels on the viral genome. This concept is supported by the observation that histone occupancy on delayed early (tk) and late (gC) gene promoters, where VP16 does not bind directly, also increased substantially in RP5 infections with a kinetics similar to that on IE gene promoters (Fig. 4.2). A further possibility is that active transcription by RNAP II itself

may prevent histone deposition, a mechanism that has been suggested for several actively transcribed genes (4, 19, 33, 55). These last two possibilities are addressed in the next sections.

Figure 4.2: Core histone occupancy on the viral genome increases in RP5 lytic infections. HeLa cells were infected with KOS (K) or RP5 (R) strains of HSV-1 at an MOI of 1 pfu/cell or 0.005 pfu/cell, respectively. Chromatin immunoprecipitation was performed to detect the occupancy of histones H2A (A), H2B (B), H3 (C), H4 (D), and RNA polymerase II (E) on ICP27, ICP0, tk, and gC promoters (pro) and on the ICP27 ORF at 2, 4 and 6 h post-infection. The data were analyzed as explained in Materials and Methods. The graphs show the results of a representative experiment; similar results were observed in replicate experiments.


A. Histone H2A ChIP
B. Histone H2B ChIP
C. Histone H3 ChIP
D. Histone H4 ChIP
E. RNAPII ChIP
Input ratio (viral/IFN-Bp)
Input ratio (viral/U3p)
hours post infection
ICP27pro (K)
ICP27pro (R)
ICP0pro (K)
ICP0pro (R)
ICP27ORF (K)
ICP27ORF (R)
tkpro (K)
tkpro (R)
gCpro (K)
gCpro (R)

Inhibition of transcription increases histone occupancy on the viral genome during lytic infection.

Evidence in yeast has indicated that histones can be depleted from actively transcribed regions (19, 30, 33, 38, 58). Since the increase in histone occupancy on the viral genome in RP5 infections might be dependent on the inability to recruit the RNAP II machinery (Fig. 4.2E), we studied histone deposition on the viral genome during KOS infections under circumstances in which RNAP II-mediated transcription was blocked. To this end, HeLa cells were pretreated with actinomycin D and subsequently infected with KOS in the presence of actinomycin D. Infections in mock-treated cells were carried out in parallel. At 2, 4, and 6 hpi, infections were stopped by formaldehyde cross-linking and ChIP was performed to assay the presence of core histones, RNAP II, and VP16 on the viral genome.

As indicated in Figure 4.3A, the occupancy of histone H2A was not substantially higher on viral genes at 2 hpi in the presence of actinomycin D than in its absence. Similar to RP5 infections, at later times in infection the H2A occupancy throughout the viral genome increased in the presence of actinomycin D and reached a level about half that of the cellular gene.

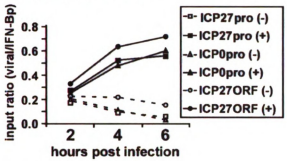
Similarly, the occupancy of histones H2B (Fig. 4.3B), H3 (Fig. 4.3C), and H4 (data not shown) on the viral genome increased in the presence of actinomycin D with a kinetics similar to that observed in RP5 infections, although at 2 hpi, histone deposition on the viral genome was slightly less than that observed in RP5 infections. RNAP II ChIP served as a positive control, showing that actinomycin D effectively blocked the recruitment of RNAP II to the viral genome (Fig. 4.3D). These results suggest that

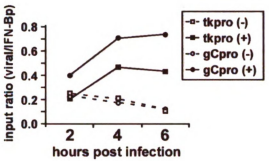
transcription by RNAP II per se may contribute to the depletion of histones from the HSV-1 genome during lytic infection.

Interestingly, actinomycin D treatment also decreased the VP16 occupancy on IE gene promoters, particularly at 4 hpi (Fig. 4.3E). Although this could be due to the fact that actinomycin D is a DNA-intercalating drug and as such might prevent the binding of VP16 to DNA, it is also possible that the increase in histone occupancy on IE gene promoters diminishes the binding of VP16 to these regions.

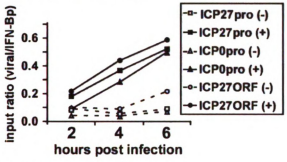
Figure 4.3: Inhibition of transcription by actinomycin D increases the core histone occupancy on the HSV-1 genome. HeLa cells were pretreated with 1 μg/ml of actinomycin D or mock treated (DMSO) for 1 h and then were infected with the KOS strain of HSV-1 at an MOI of 1 pfu/cell in the presence of 1 μg/ml of actinomycin-D. Chromatin immunoprecipitation was performed to detect the occupancy of histones H2A (A), H2B (B), H3 (C), RNA polymerase II (D), and VP16 (E) on the ICP27, ICP0, tk, and gC promoters (pro) and on the ICP27 ORF at 2, 4, and 6 h post-infection. The data were analyzed as explained in Materials and Methods. The graphs show the results of a representative experiment; similar results were observed in independent replicates.

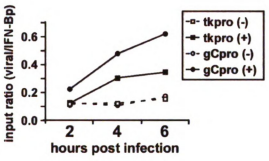
A. Histone H2A ChIP



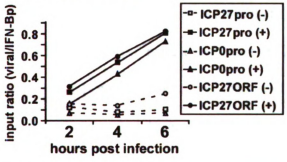


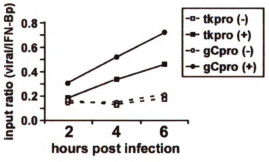
B. Histone H2B ChIP



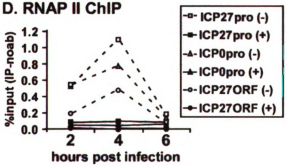


C. Histone H3 ChIP

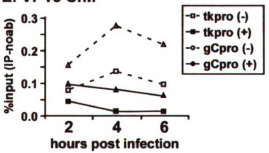




D. RNAP II ChIP



E. VP16 ChIP



IE proteins are not required for inhibition of histone deposition on IE genes during lytic infection.

During RP5 infections or in the presence of actinomycin D, histone occupancy increased not only at IE genes but also at DE and L gene promoters, where VP16 does not bind directly. Given that IE proteins play important roles in transcriptional regulation of DE and L genes, we considered whether IE proteins also contribute to the depletion of histones from these genes. The hypothesis that histones are removed from DE and L genes by IE proteins leads to the prediction that inhibition of IE protein synthesis would increase the occupancy of histones on these promoters but would not affect IE genes, which are actively transcribed by VP16 and RNAP II in the absence of IE proteins. Therefore, we tested whether inhibition of IE protein expression by cycloheximide affected histone deposition on the viral genome during lytic infection.

To this end, HeLa cells were infected with KOS in the presence of cycloheximide to block *de novo* viral protein synthesis. At 2 and 4 hpi, infections were stopped and ChIP assays were performed to analyze histone H3 and RNAP II occupancy on the HSV-1 genome. As expected, histone H3 occupancy on the ICP0 promoter and the ICP0 and ICP4 ORFs was minimally affected by inhibition of IE protein expression at 2 hpi (Fig. 4.4A). H3 occupancy increased modestly on the ICP27 promoter and ICP27 ORF, as well as on the tk and gC promoters (Fig. 4.4A). Similarly, at 4 hpi histone deposition did not change substantially on the ICP0 promoter, ICP0 ORF, and ICP4 ORF (Fig. 4.4B), yet increased dramatically on the ICP27 promoter, ICP27 ORF, and the tk and gC promoters (Fig. 4.4B). These results suggest that IE proteins are not involved in depleting the histones from IE promoters and ORFs, with the exception of ICP27.

Interestingly, the increase in histone H3 occupancy on the ICP27 promoter, ICP27 ORF, and tk and gC promoters corresponded to a significant decrease in RNAP II occupancy (Fig. 4.4C). As expected, RNAP II occupancy on other IE genes either did not change or increased in the presence of cycloheximide (Fig. 4.4C). These results together suggest that during early times in lytic infection, VP16-mediated transcription keeps the IE genes relatively free of histones, and at later stages IE proteins are involved in the removal of histones from other regions of the viral genome.

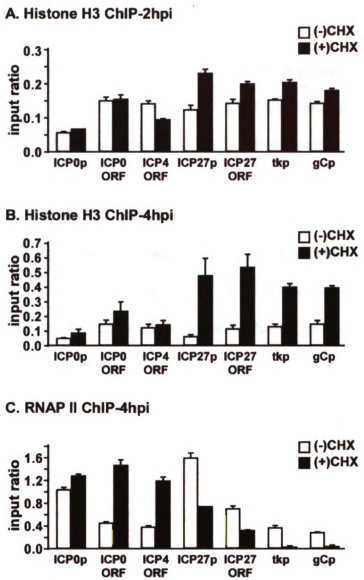


Figure 4.4: Changes in histone occupancy on the HSV-1 genome upon inhibition of IE protein synthesis by cycloheximide. HeLa cells were pretreated with 60 μg/ml cycloheximide for 2 h and then were infected with HSV-1 KOS at an MOI of 1 pfu/cell in the presence of cycloheximide. **(A)** At 2 h post-infection, chromatin was crosslinked and a ChIP assay probing histone H3 on the ICP0 promoter (ICP0p), ICP27 promoter (ICP27p), ICP0 ORF, ICP4 ORF, ICP27 ORF, tk promoter (tkp), and gC promoter (gCp) was performed as indicated before. **(B,C)** At 4 h post-infection, chromatin was crosslinked and a ChIP assay probing histone H3 (B) and RNAP II (C) on the indicated viral gene fragments was performed as indicated above. The data shown represent the average of two independent experiments; the error bars indicate the range between the averages of these experiments.

Histones are neither modified nor removed from the RP5 genome upon superinfection with HSV-2.

We designed an experiment to test whether VP16 can induce the removal of histones that are already deposited on the viral genome. The VP16 proteins of HSV-1 and HSV-2 share 93% sequence similarity (9). Moreover, HSV-2 superinfection can reactivate quiescent HSV-1 infections *in vitro* (8), and the VP16 gene from HSV-2 can function effectively when recombined into the HSV-1 genome (52). These observations indicate that the HSV-2 VP16 protein is capable of activating transcription from the HSV-1 genome. Given that RP5 genomes associate with higher levels of histones at later stages of infection (Fig. 4.2), we asked whether VP16 provided *in trans* by HSV-2 superinfection can overcome the nucleosomal barrier and induce IE gene expression from the quiescent and histone-laden RP5 genome.

We first tested whether VP16 from HSV-2 superinfection could stimulate IE gene expression from RP5 genomes. HeLa cells were infected with RP5 at an MOI of 0.001 pfu/cell. At 6 hpi, when the RP5 viral genomes are highly associated with histones (see Fig. 4.2), the cells were superinfected with HSV-2 at a range of multiplicities. RP5 IE gene expression was analyzed after 2 h of HSV-2 superinfection (Fig. 4.6A). As expected, transcription of the RP5 IE genes ICP4 and ICP27 was induced significantly upon HSV-2 superinfection in a dose-responsive manner with respect to HSV-2 MOI (Fig. 4.6B). RP5 IE gene expression was also stimulated when the HSV-2 superinfections were performed in the presence of cycloheximide (Fig. 4.5), indicating that the induction of IE gene expression is largely attributable to VP16 but does not depend on *de novo* synthesis of HSV-2 IE proteins such as ICP0.These results together indicate that VP16 of

HSV-2 can efficiently stimulate IE gene expression from viral genomes that are predominantly nucleosomal.

We next tested whether VP16 can induce changes in the chromatin structure of RP5 genomes upon superinfection with HSV-2. We hypothesized that if VP16 is involved in the modification and removal of histones, then active transcription marks such as H3K9/K14ac or H3K4me3 would increase and the histone H3 occupancy on the RP5 genomes would decrease upon HSV-2 superinfection.

To address these questions, we performed ChIP assays in cells infected with RP5 and superinfected with HSV-2. HeLa cells were infected with RP5 at an MOI of 0.001 pfu/cell. At 6 hpi, these cells were superinfected with HSV-2 at 10 pfu/cell, which robustly induced RP5 IE gene expression (Fig. 4.6B). Infections were stopped by formaldehyde cross-linking after 2 h of HSV-2 superinfection, and the occupancy of RNAP II, histone H3, H3K9/K14ac, and H3K4me3 on RP5 IE promoters was analyzed by ChIP.

As expected, RNAP II occupancy increased significantly at IE gene promoters and open reading frames upon superinfection with HSV-2 (Fig. 4.6C). Even so, RNAP II occupancy on the IE promoters was still 10–20 % of that observed on the cellular control U3 snRNA promoter, indicating that the relative rate of transcription was lower on IE genes than on the U3 snRNA gene. This result may suggest that only some, but not all, RP5 genomes are transcriptionally active upon HSV-2 superinfection. Interestingly, this increase in RNAP II occupancy did not correlate with a significant decrease ($p > 0.05$, by Student's t-test) in histone H3 occupancy on IE promoters and ORFs (Fig. 4.6D), suggesting that neither the VP16 AD nor RNAP II are sufficient to deplete histones from

the viral genome. No change in the occupancy of H3K9/K14ac (Fig. 4.6E) or H3K4me3 (Fig. 4.6F) on RP5 IE genes was observed after superinfection with HSV-2, indicating that VP16 does not induce major changes in covalent histone modifications on the viral genome. These results collectively suggest that VP16 and RNAP II are not sufficient to alter the chromatin structure on IE promoters. Perhaps more importantly, neither histone removal nor covalent modification of histones seems to be a prerequisite for VP16-mediated transcription from nucleosomal viral genomes.

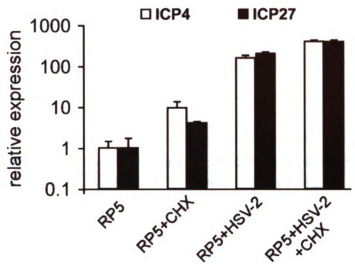
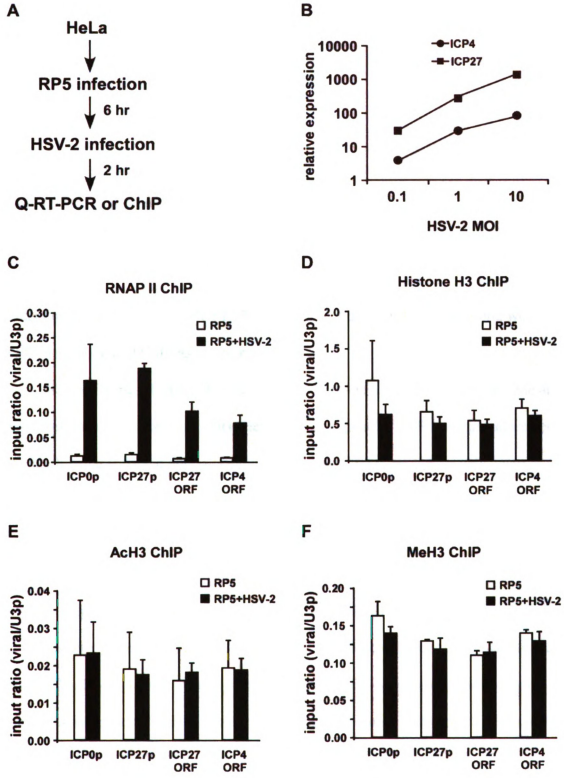


Figure 4.5: HSV-1 IE gene expression is induced by HSV-2 superinfection independent of *de novo* protein synthesis. HeLa cells were infected with the RP5 strain of HSV-1 at an MOI of 0.001 pfu/cell. At 5 hpi, cells were either treated with 60 μg/ml cycloheximide or mock-treated for 1 hr. At 6 hpi, HSV-2 superinfection was started at an MOI of 10 pfu/cell either in the presence or absence of 60 μg/ml cycloheximide. Parallel samples were mock-infected in the presence or absence of cycloheximide. At 2 h after HSV-2 superinfection, RNA was isolated and expression of HSV-1 IE gene ICP4 and ICP27 was analyzed by Q-RT-PCR. The data shown are from a representative experiment performed in biological triplicate. Error bars represent the standard deviation among these samples.

Figure 4.6: HSV-2 superinfection does not cause major changes in histone occupancy or covalent histone modifications on the RP5 genome. **(A)** Summary of the assays performed. **(B)** HeLa cells were infected with RP5 at an MOI of 0.001 pfu/cell and at 6 hpi were superinfected with HSV-2 at MOIs of 0.1, 1, or 10 pfu/cell. At 2 h after HSV-2 superinfection, ICP4 and ICP27 expression was analyzed by Q-RT-PCR. **(C-F)** HeLa cells were infected with RP5 at an MOI of 0.001 pfu/cell and at 6 hpi were superinfected with HSV-2 at an MOI of 10 pfu/cell. At 2 h after HSV-2 superinfection, ChIP was performed assaying the presence of RNAP II (C), histone H3 (D), H3K9/K14ac (E), and H3K4me3 (F) on the ICP0 promoter, ICP27 promoter, ICP4 ORF, and ICP27 ORF. The data shown represent the average of three independent experiments, where "% input" values from viral genes are normalized against the U3 snRNA promoter as indicated before. Error bars represent the standard deviation between these experiments.


A
HeLa
RP5 infection
6 hr
HSV-2 infection
2 hr
Q-RT-PCR or ChIP
B
ICP4
ICP27
relative expression
10000
1000
100
10
1
0.1
1
10
HSV-2 MOI
C
RNAP II ChIP
RP5
RP5+HSV-2
input ratio (viral/U3p)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
ICP0p
ICP27p
ICP27 ORF
ICP4 ORF
D
Histone H3 ChIP
RP5
RP5+HSV-2
input ratio (viral/U3p)
2.0
1.5
1.0
0.5
0.0
ICP0p
ICP27p
ICP27 ORF
ICP4 ORF
E
AcH3 ChIP
RP5
RP5+HSV-2
input ratio (viral/U3p)
0.04
0.03
0.02
0.01
0.00
ICP0p
ICP27p
ICP27 ORF
ICP4 ORF
F
MeH3 ChIP
RP5
RP5+HSV-2
input ratio (viral/U3p)
0.20
0.15
0.10
0.05
0.00
ICP0p
ICP27p
ICP27 ORF
ICP4 ORF

Histones and RNAP II partially co-occupy the RP5 genome upon HSV-2 superinfection.

Although the results in Fig. 4.2 indicated a significant increase in histone occupancy on the RP5 genome at 6 hpi, we cannot be certain that all RP5 viral genomes are occupied by histones (i.e., the input ratio may be somewhat less than 1). In addition, it is likely that not all RP5 genomes were reactivated in response to HSV-2 superinfection, as indicated by the lower occupancy of RNAP II on IE genes than on the U3 snRNA promoter (Fig. 4.6C). These observations raise the possibility that VP16 and RNAP II may preferentially associate with viral genomes that remain non-nucleosomal. This could explain why no changes in histone occupancy or covalent histone modifications were evident on the RP5 genomes after HSV-2 superinfection (Fig. 4.6). Therefore, we performed sequential ChIP assays to probe this issue.

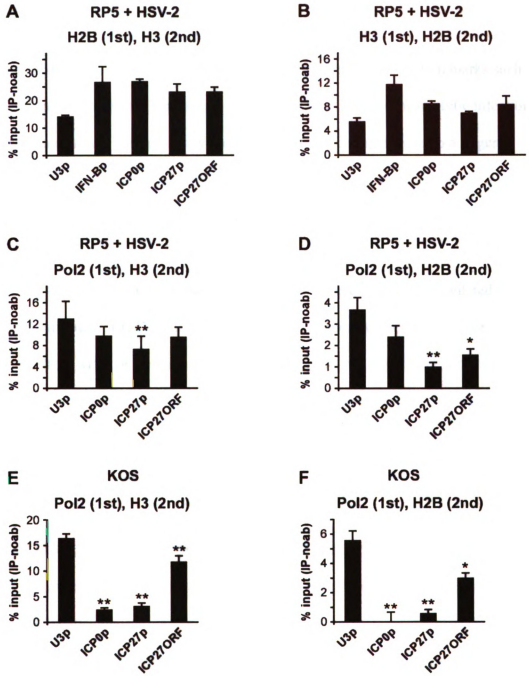
Since histones H2B and H3 are expected to co-occupy a given cellular gene at high levels, as control assays for histone-RNAP II sequential ChIPs we first addressed whether histone H2B and H3 co-occupy cellular and viral genes. HeLa cells were infected with RP5 and superinfected with HSV-2 as in previous experiments. The cross-linked chromatin from infected cells was first subjected to immunoprecipitation using a histone H2B antibody, followed by a second immunoprecipitation with a histone H3 antibody (Fig. 4.7A). As expected, histones H2B and H3 co-occupied both the IFN-β and the U3 snRNA promoters (Fig. 4.7A). We noted that the level of histone co-occupancy on the U3 snRNA promoter was less than that on the IFN-β promoter, which might indicate active transcription on the former promoter. Histones H2B and H3 also co-occupied the viral IE genes at levels similar to those on cellular promoters (Fig. 4.7A).

Similar results were observed when the first IP was performed using H3 antibodies followed by a second immunoprecipitation by H2B antibodies (Fig. 4.7B). These results collectively indicate the feasibility of sequential ChIP assays in this experimental context. Interestingly, the association of H3 and H2B at viral genes was comparable to that at the cellular genes, indicating co-association of these histones on viral DNA. This observation is consistent with the idea that entire nucleosomes form on the viral genome.

Co-occupancies of histone H3 and RNAP II (Fig. 4.7C) and of histone H2B and RNAP II (Fig. 4.7D) were analyzed similarly by sequential ChIP in RP5-infected and HSV-2-superinfected cells. The cross-linked chromatin was first subjected to immunoprecipitation with RNAP II antibodies, followed by a second immunoprecipitation histone H3 or histone H2B antibodies using chromatin material obtained from the first IP. We used the U3 snRNA promoter as a positive control. As expected, RNAP II and histone H3 (Fig. 4.7C) or RNAP II and histone H2B (Fig. 4.7D) co-occupied the U3 snRNA promoter. Interestingly, RNAP II and histone H3 co-occupied the RP5 ICP0 promoter and ICP27 ORF at levels similar to that of the U3 promoter, yet the co-occupancy was significantly lower on the ICP27 promoter (Fig. 4.7C). RNAP II and histone H2B also co-occupied IE genes (Fig. 4.7D), yet the degree of co-occupancy was significantly lower than on the U3 snRNA promoter for some IE genes such as the ICP27 promoter and ICP27 ORF. These results suggest that although RNAP II does not preferentially associate with "histone-free" RP5 genomes upon HSV-2 superinfection, VP16 and RNAP II-mediated transcription may lead to the disruption of nucleosome structure. One possible mechanism is by evicting a histone H2A-H2B dimer from the nucleosome core particle, as observed in other systems (19, 33).

In order to gain more confidence in these results, we analyzed RNAP II and histone co-occupancy on genes that associate with either RNAP II or histones, but not both. The promoter region of the transcriptionally inactive IFN-β gene associates with histones but not RNAP II. As expected, neither of the histones co-occupied the IFN-β promoter with RNAP II, as indicated by the absence of IFN-β-specific amplification in PCR samples parallel to those presented in Figures 4.7C and 4.7D (data not shown). As a second negative control, we analyzed the co-occupancy of RNAP II and histones on genes that are associated with RNAP II but low levels of histones. To this end, we repeated the sequential ChIP assays in KOS-infected cells and asked whether histones and RNAP II co-occupy the viral IE genes. Given that histone levels are low on the KOS genomes (Fig. 4.2, 4.3), we expected that the viral genomes that are associated with RNAP II would be low in histones also. As expected, histone H3 was underrepresented on all viral IE promoters that are associated with RNAP II (Fig. 4.7E). Interestingly, histone H3 was depleted from the ICP27 ORF to a lesser extent than from IE promoters. Similarly, the co-occupancy of histone H2B and RNAP II on IE promoters and the ICP27 ORF was significantly lower than on the U3 snRNA promoter (Fig. 4.7F). These results together reinforce our conclusions from sequential ChIP assays performed in RP5-infected and HSV-2-superinfected cells.

Figure 4.7: Histone and RNAP II co-occupancy on RP5 IE genes upon HSV-2 superinfection. HeLa cells were infected with RP5 at an MOI of 0.001 pfu/cell and at 6 hpi were superinfected with HSV-2 at an MOI of 10 pfu/cell. At 2 h after HSV-2 superinfection, seq-ChIP was performed assaying the co-occupancy of histone H3, histone H2B, and RNAP II on the U3 snRNA promoter, IFN-β promoter, ICP0 promoter, ICP27 promoter, and ICP27 ORF. **(A)** Histone H2B (first immunoprecipitation) and H3 (second immunoprecipitation) co-occupancy. **(B)** Histone H3 (first immunoprecipitation) and H2B (second immunoprecipitation) co-occupancy. **(C)** RNAP II (first immunoprecipitation) and H3 (second immunoprecipitation) co-occupancy. **(D)** RNAP II (first immunoprecipitation) and H2B (second immunoprecipitation) co-occupancy. **(E)** HeLa cells were infected with KOS at an MOI of 1 pfu/cell and seq-ChIP was performed at 2 hpi as in (A-D). RNAP II (first immunoprecipitation) and H3 (second immunoprecipitation) co-occupancy. **(F)** Seq-ChIP was performed in HeLa cells infected as in (E). RNAP II (first immunoprecipitation) and H2B (second immunoprecipitation) co-occupancy. The data shown in (A, C, D) represent the averages of three independent experiments, with error bars representing the standard deviation between these experiments. The data in (B) represents the average of two independent experiments, with error bars representing the range between these experiments. The data in (E, F) represents three independent IPs done in parallel, where the error bars represent the standard deviation. Samples with mean values that vary significantly ($p \leq 0.01$ or else $0.01 \leq p \leq 0.05$ by paired student's *t* test) from the U3 snRNA promoters in (C-F) are indicated by (**) and (*), respectively.


A
RP5 + HSV-2
H2B (1st), H3 (2nd)
% input (IP-noab)
U3p
IFN-Bp
ICP0p
ICP27p
ICP27ORF
B
RP5 + HSV-2
H3 (1st), H2B (2nd)
% input (IP-noab)
U3p
IFN-Bp
ICP0p
ICP27p
ICP27ORF
C
RP5 + HSV-2
Pol2 (1st), H3 (2nd)
% input (IP-noab)
U3p
ICP0p
ICP27p
ICP27ORF
D
RP5 + HSV-2
Pol2 (1st), H2B (2nd)
% input (IP-noab)
U3p
ICP0p
ICP27p
ICP27ORF
E
KOS
Pol2 (1st), H3 (2nd)
% input (IP-noab)
U3p
ICP0p
ICP27p
ICP27ORF
F
KOS
Pol2 (1st), H2B (2nd)
% input (IP-noab)
U3p
ICP0p
ICP27p
ICP27ORF

5. DISCUSSION

An increasing amount of evidence indicates the important role of chromatin regulation during the lytic and latent stages of HSV-1 infection. Recent studies have shown that histone H3 associates with the HSV-1 genome at lower levels than with cellular genes during lytic infection (20, 21, 26, 35, 50). Active transcription marks such as H3K9/K14ac and H3K4me3 are also present on the viral genome during lytic infection (20, 21, 26). In contrast, during latency the viral genome is chromatinized to a larger extent, and the histones associated with the viral genome carry inactive chromatin marks with the exception of the actively transcribed LAT gene (10, 32, 66). In addition, during reactivation from latency and quiescence, acetylated histones associate with IE gene promoters (8, 49). These results all indicate that chromatin is dynamically regulated during different stages of HSV-1 infection and that the transcriptional status of the viral genome correlates with the type of covalently modified histones associated with it. On the other hand, the mechanism of how the viral genome stays predominantly non-nucleosomal during lytic infection has not been studied in detail.

In this study we addressed potential mechanisms to account for the dearth of histones on the viral genome during lytic infection. We have previously analyzed the role of transcriptional coactivators that are recruited by VP16 in this process and showed that disrupting the expression of a number of coactivators did not reduce IE gene expression during lytic infection, suggesting that these coactivators are not likely involved in modulating histone occupancy on IE genes (36). We thus turned our attention to other possible mechanisms and analyzed whether the VP16 AD, transcription by RNAP II per se, or IE proteins affect histone levels on the viral genome.

Our results show that the activation domain of VP16 contributes to low histone levels on the HSV-1 genome, as the absence of the VP16 AD resulted in a dramatic increase in the occupancy of all core histones on the RP5 genome (see Fig. 4.2). An indirect mechanism whereby the VP16 AD might mediate the removal of histones from the viral genome is simply by recruitment of host RNAP II machinery, as it has been shown that histones are underrepresented on actively transcribed genes (4, 19, 44, 45, 55). In support of this notion, histone occupancy dramatically increased throughout the viral genome in the presence of the RNAP II inhibitor actinomycin D (see Fig. 4.3). This result indicates that transcription *per se* reduces histone occupancy on viral genes. This result might be further interpreted to mean that the VP16 AD and whatever coactivators it recruits are not sufficient to maintain low histone levels on the viral genome without active transcription by RNAP II. This interpretation is complicated by the observation that VP16 occupancy on IE promoters at later times post-infection was also reduced by actinomycin D. The loss of VP16 binding might be a consequence of increased histone occupancy on these promoters, blocking access by the VP16-induced protein complex. An alternative possibility is that actinomycin D directly prevents the binding of VP16 and its partners to IE promoters, which may in turn lead to an increase in histone occupancy.

IE proteins themselves may also affect histone levels on the viral genome, as indicated by the increase in histone occupancy on DE and L gene promoters in the absence of IE protein expression (see Fig. 4.4). As expected, inhibition of IE protein expression did not affect histone occupancy on most IE genes; the exception was the ICP27 gene, both the promoter and ORF. We currently do not have a clear explanation of why the ICP27 gene is regulated differently than other IE genes. An important point to

note is that RNAP II occupancy on the ICP27 gene was reduced substantially, much like on the DE and L promoters in the presence of cycloheximide, in contrast to the enhanced RNAP II recruitment to other IE genes (see Fig. 4.4C). Therefore, it is likely that *de novo* IE protein synthesis is necessary for the maximal transcription of ICP27, but not of the other IE genes.

One mechanism by which IE proteins might mediate histone depletion is the recruitment of RNAP II and the induction of active transcription. Alternatively, IE proteins might be directly involved in regulating histone deposition on the viral genome. For instance, ICP0 was shown to disintegrate the REST/CoREST/HDAC repressor complex (14, 15) and thus was suggested to prevent the formation of inactive chromatin on the HSV-1 genome. Cliffe and Knipe (6) have recently shown that this function of ICP0 may be relevant during lytic infection, as in the absence of ICP0, histone H3 occupancy increased and the fraction of acetylated histones on the viral genome decreased significantly. Whether the REST/CoREST/HDAC complex is the only target of ICP0 and whether the changes in chromatin on the viral genome in the absence of ICP0 are directly mediated by REST/CoREST/HDAC still need to be determined.

Although our results indicate that VP16 and RNAP II contribute to the dearth of histones on IE gene promoters and ORFs during lytic infection, reduced histone levels are not required for IE gene transcription. Histones were neither modified on nor depleted from RP5 genomes upon superinfection with HSV-2 (see Fig. 4.6), in spite of the induction of RP5 IE gene expression by HSV-2 (see Fig. 4.6B). These results suggest that the presence of histones may not interfere with the ability of VP16 and RNAP II to initiate transcription from the viral genome.

A potential complication in these assays is the possibility that not all viral genomes are activated by HSV-2 superinfection, as indicated by the low levels of RNAP II occupancy on IE genes (Fig. 4.6C). This might explain why no change in histone occupancy or modifications was observed on the RP5 genome after HSV-2 superinfection. We think that similar problems might be faced when studying reactivation from latency *in vivo*, where only a fraction of latent viral genomes might get reactivated.

Given the possibility that not all RP5 genomes become transcriptionally active upon HSV-2 infection and that some RP5 genomes may escape histone deposition, we hypothesized that the RP5 genomes that are activated by HSV-2 superinfection might actually be the ones that escape nucleosome deposition. However, sequential ChIP assays showed that RNAP II has no clear preference for histone-free RP5 genomes (see Fig. 4.7C, D). We conclude that removal of histones from viral templates is not a prerequisite for transcriptional activation by VP16. Interestingly, histone H3 and H2B were significantly underrepresented in some RNAP II-associated IE genes (Fig. 4.7C, D), suggesting that nucleosomes might be partially disrupted at actively transcribed IE genes. A similar phenomenon was suggested for transcriptionally active eukaryotic genes (19, 33) (30, 38, 58).

Another important question addressed in this study is the nature of chromatin on the viral genome during lytic infection. We suggest that the presence of all core histones on the viral genome during KOS and RP5 infections is indicative of formation of canonical nucleosomes on the viral genome. This conclusion is supported by our findings in sequential ChIP assays, which indicated that histones H2B and H3 co-occupied the RP5 genomes in HSV-2 superinfected cells (see Fig. 4.7A, B).

A crucial question is whether the low histone occupancy on the HSV-1 genome during lytic infection matters for transcription. A related issue is whether the dearth of histones on the viral genome during lytic infection is due to prevention of histone deposition or effective removal of histones that are deposited. In contrast to a recent report (6), we have noted that even at very early times in infection (30 min to 1 h), the amount of histones on the viral genome is much lower than on cellular genes (data not shown), suggesting that histones may not be deposited at all on most viral genomes during lytic infection.

One possible model is that efficient transcription, which starts soon after the viral genome is released into the nucleus, might block histone deposition on the HSV-1 genome. If transcription does not start efficiently, as in the case of RP5 infections or in the presence of actinomycin D, then the histones are deposited on the viral genomes rather slowly as indicated by the time-course assays in Figures 4.2 and 4.3. Therefore, we propose that histones are prevented from being deposited on the viral genome, rather than being deposited first and then actively removed. If this is the case, then the small amount of histones on the viral genome may not matter for viral transcription during lytic infection. In this scenario, the small amount of histones might represent randomly positioned histones on the viral genome. A second alternative is that during wild-type lytic infection, a small fraction of viral genomes are silenced by deposition of nucleosomes. In either case, histones may not matter for the outcome of lytic infection, as a large fraction of actively transcribed viral genomes stay non-nucleosomal.

Given that histones are not present in the HSV-1 nucleocapsid (7, 50, 53) and are underrepresented on the HSV-1 genome during lytic infection, it is tempting to speculate

that histone chaperones and assembly factors may be involved in regulating the viral chromatin structure. Some of the candidate chaperones include Spt6 and FACT, which associate with RNAP II and enable its elongation on nucleosomal templates (2, 25). Interestingly, the histone chaperone HIRA, which is involved in replication-independent histone deposition (18, 57), was shown to be present in PML bodies in senescent cells (69) and as such is likely to be involved in chromatin assembly on the viral genome. A recent study suggested that disruption of HIRA expression reduces both the association of histone H3.3 with the HSV-1 genome and the viral gene expression at later stages of infection, concomitant with a reduction in viral replication (54). On the other hand, it is not clear whether HIRA contributes to viral gene expression at early times in infection.

Nucleosome assembly on the viral genome at early times may be blocked by VP22, a tegument protein that interacts with TAF-I, a homologous protein to histone chaperone NAP-1 (64). However, our results (see Figures 4.2 and 4.3) suggest that VP22 delivered by the incoming virion is not sufficient for histone depletion, because the levels of VP22 are presumably unaffected by the absence of the VP16 AD or the presence of actinomycin D. Given these findings, it will be important to address whether histone chaperones are modulated in a way that leads to low levels of histones on the HSV-1 genome during different stages of infection.

Overall, we show that during lytic infection of HSV-1, histones are found at low levels on the viral genome; the cause of such levels may involve active transcription by RNAP II, as well asVP16 and IE proteins. Future studies should address the details of the underlying molecular mechanism in terms of histone deposition on or histone removal from the HSV-1 genome. In addition, our results suggest that IE proteins also contribute

to keeping other regions of the viral genome free of nucleosomes at later stages of lytic infection. Therefore, it will be crucial to define the cellular factors involved in depositing the histones on the viral genome. Lastly, it will be interesting to see whether and how chromatin assembly factors are regulated during establishment of and reactivation from latency of HSV-1.

6. ACKNOWLEDGEMENTS

This research was supported by the Van Andel Research Institute and by NIH grant AI064634. SK was supported by a predoctoral fellowship from the American Heart Association. We thank Martha Roemer for initiating the chromatin immunoprecipitation analyses of core histones. We also thank David Nadziejka for assistance in preparing the manuscript and members of the Triezenberg lab for their comments on this work.

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Chapter 5

SYNTHESIS AND FUTURE DIRECTIONS

This dissertation contributes significantly to our understanding of how the HSV-1 genome stays predominantly free of nucleosomes during lytic infection. Although the molecular details remain to be elucidated, histones are likely prevented from being deposited on the HSV-1 genome during lytic infection by a complex mechanism that involves VP16, RNAP II and IE proteins (Chapter 4), rather than being deposited on the viral genome first and then being removed from it. This mechanism explains why the transcriptional coactivators that are recruited by VP16 are not required for IE gene expression during lytic infection (Chapter 2). A number of issues that arise from these observations are discussed below.

1. Potential mechanisms leading to the lack of histones on the HSV-1 genome during lytic infection

Although VP16, RNAP II and IE proteins contribute to keeping the viral genome relatively free of histones during lytic infection, the mechanistic details of this process remain unknown. In this section I will discuss the potential molecular mechanisms of how these factors may lead to the dearth of histones on the viral genome during lytic infection.

Although our initial studies using KOS and RP5 viruses indicated that VP16 AD contributes to keeping IE, DE and L genes depleted of histones (17), it is now clear that the role of VP16 in this process is restricted mainly to IE genes, indicated by the increase in histone occupancy on DE and L, but not IE genes, in the presence of cycloheximide. One way that VP16 may block histone deposition on the viral genome is by recruitment

of RNAP II machinery. Alternatively, VP16 may recruit other factors, which in turn block histone deposition on or induce rapid removal of histones from the viral genome. Some of these factors, such as transcriptional coactivators and proteins associated with the RNAP II machinery, that interact with VP16 AD have been identified *in vitro* (see Chapter 1). However, some of these interactions may not be relevant during HSV-1 lytic infection as evidenced by the lack of change in viral gene expression in infected cells when the expression of transcriptional coactivators that interact with VP16 AD *in vitro* is disrupted (Chapter 2). Interestingly, a yeast-two-hybrid system using Gal4-VP16 AD fusion as bait and a human cDNA library as prey identified a number of interaction partners for VP16 AD, such as proteins without any known functions in addition to proteins associated with RNAP II machinery (18). On the other hand, in this same study, known *in vitro* interaction partners of VP16 AD were not identified, suggesting that VP16 may interact with a different set of proteins *in vivo*. As such, it will be important to identify the *in vivo* interaction partners of VP16 AD during genuine HSV-1 lytic infection, which might clarify the mechanistic details of how VP16 activates IE gene expression and how it regulates the structure of chromatin on IE genes. Identifying the *in vivo* interaction partners of VP16 during infection, however, is a challenging task given the need for large volumes of cells and viruses to isolate VP16 containing protein complexes by mass-spectrometry applications.

Although hypothetical, another mechanism that VP16 may prevent histone deposition is by directing the viral genomes to microdomains in the nucleus that may be rich in RNAP II and other transcription factors but poor in histones. This model would explain why DE and L genes that are not transcribed at early times in infection still

remain relatively free of histones. In this scenario, RNAP II recruitment might still be the rate-limiting step, which might be overcome by transcriptional activators, such as VP16 at early times and ICP4 at later times in infection.

The idea of the presence of microdomains surrounding the viral genome is not unprecedented; upon the entry of the HSV-1 genome to the nucleus, PML bodies form around the viral genome as part of the innate immune response. Although the association of the viral genomes with PML bodies is thought to result in transcriptional silencing of the viral genome (10, 28), not all components of PML bodies may be inhibitory for viral gene expression. For instance, transcriptional coactivators such as p300 and CBP HATs are also present in ND10 structures (23, 24, 26), although it is now clear that these HATs are not necessary for viral gene expression (Chapter 2). Interestingly, when plasmid DNA was artificially targeted to PML bodies, transcription was either inhibited or induced in a promoter-dependent manner (2), supporting the idea that PML bodies comprise factors that can both inhibit or activate transcription. Therefore, although PML bodies may be formed around HSV-1 DNA as a host defense, HSV-1 may employ some of the PML-associated factors for initiating viral transcription.

Whether VP16 has any role in the association of the HSV-1 DNA with PML bodies is currently not known. Interestingly, the functional homologue of VP16 in human cytomegalovirus, pp71, leads to dissociation of ATRX, a protein containing an ATPase domain found in chromatin remodeling enzymes, from the PML bodies at early times in infection (25). In this case, ATRX contributes to inhibition of IE gene expression and hence its dissociation from PML bodies by pp71 potentiates IE gene expression (25). Although VP16 does not share sequence homology with pp71, it is possible that VP16

also contributes to early changes in PML bodies to stimulate viral transcription. It will therefore be important to address whether VP16 is involved in localization of the viral genomes to PML bodies and whether it alters components of the PML bodies for optimal viral gene expression during lytic infection. To this end, fluorescence in situ hybridization and immunofluorescence assays in infected cells can be employed.

One important finding in line with the potential importance of nuclear architecture for HSV-1 gene expression is the interaction between PML and special AT-rich sequence binding protein 1 (SATB1) (22), which regulates higher-order chromatin structure and transcription by attaching actively transcribed genes to the nuclear matrix and inducing the formation of DNA loops that emanate from matrix associated regions. Therefore, it would be interesting to address whether SATB1 also colocalizes with the incoming DNA and tethers it to the nuclear matrix, which might be important for the initiation of viral gene expression. Another intriguing question then would be whether VP16 has a role in this process. To address whether the incoming viral genomes are directed to the nuclear matrix, one could employ the "in situ hybridization to nuclear halos" assay (5, 11). In this assay, cells are permeabilized by Triton X-100 treatment, and histones and soluble nuclear proteins are extracted by high-salt treatment. 4,6-Diamidino-2-phenylindole (DAPI) staining of these nuclei show that the regions that associate with nuclear matrix form a DAPI-dense region, surrounded by a relatively faint DAPI staining (halo) which represents the histone-rich heterochromatin regions. Florescence in situ hybridization of these nuclei reveals the association of a given DNA sequence with either heterochromatic regions or the nuclear matrix. One could therefore use this assay in cells infected with wild-type or the VP16 AD-deleted viruses to address whether the activation domain of

VP16 is involved in directing the viral genome to the nuclear matrix potentially rich in transcription factors and poor in histones. One would then ask whether SATB1 is involved in mediating this attachment by performing similar assays in SATB1 depleted cells. Further assays would address whether VP16 and SATB1 interact to direct the viral genome to distinct regions in the nucleus.

In support of the role for nuclear architecture for regulation of viral gene expression and histone deposition on the viral genome, a recent study indicated that absence of lamin A, a major structural component of the nuclear lamina, leads to defects in viral gene expression and replication as well as a significant increase in heterochromatin formation on the viral genome (36). It will be important to elucidate the underlying molecular mechanism and whether VP16 has any role in this process, i.e. by localizing the incoming viral genomes in distinct regions in the nucleus by interacting with lamin A.

The mechanism of how IE proteins prevent histone removal is currently not substantiated, yet current evidence indicates an important role for ICP0 in this process. One way that ICP0 may block histone deposition on the viral genome is by overcoming a general silencing mechanism by disrupting the PML bodies (9). Interestingly, blocking the disruption of PML bodies by overexpressing a structural component had no effect on viral gene expression (24), indicating that disruption of PML bodies may not be necessary for escaping from silencing by PML. Another relevant function of ICP0 is its interaction with the REST/CoREST/HDAC1 repressor complex, which results in dissociation of HDAC1 from the complex (12, 13). Whether this mechanism is relevant to viral lytic infection is currently not known. It is interesting, however, that during lytic

infection by a mutant virus that lacks ICP0, the amount of histone H3 on the viral genome increases significantly and the fraction of acetylated histones is reduced (6), suggesting that ICP0 may indeed be regulating histone occupancy and histone acetylation on the viral genome during lytic infection. Interestingly the requirement for ICP0 during lytic infection is both dependent on the cell type and the multiplicity of infection, suggesting that ICP0 itself may not be necessary, at least in certain experimental settings, to prevent chromatin formation on the viral genome. Finally, another mechanism that ICP0 may be contributing to histone depletion is simply by allowing active transcription by RNAP II, as explained below.

Active transcription by RNAP II may also lead to partial or complete histone depletion from gene promoters or transcribed regions (16, 21, 40). In addition, recent *in vitro* evidence indicated that passage of RNAP II leads to the elimination of an H2A-H2B dimer from nucleosomes (20, 38). 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 (8). One hypothesis posits that the density of RNAP II molecules on a given template may itself block nucleosome formation (21). Although we have shown in Chapter 4 that VP16, ICP0 and transcription by RNAP II all contribute to the dearth of histones on the viral genome, it is important to address more extensively whether active transcription of the viral genome correlates with the level of histones during lytic infection by ChIP assays. A number of mutant viruses that are debilitated in expression of different temporal classes of viral genes can be employed to address whether presence of RNAP II on a given viral gene correlates with histone depletion from that gene. For instance, viruses that are deleted for DNA polymerase gene are debilitated

for L gene expression, but can express IE and DE genes. Therefore, during lytic infection with these viruses, one would expect to observe an increase in histone occupancy on L genes, but not on IE or DE genes.

Since a number of histone chaperones and chaperone-like proteins, such as FACT and Spt6, associate with the elongating RNAP II, it is attractive to suggest that RNAP II-associated chaperones might regulate chromatin formation on the viral genome during lytic infection. Although hypothetical, given that HSV-1 infection dramatically changes the phosphorylation of the C-terminal domain of RNAP II (32), it is possible that a number of cellular chaperones (or other cellular proteins) might differentially interact with this form of RNAP II and thus contribute to histone depletion from the viral genome.

2. Transcriptional coactivators and HSV-1 infection

A surprising finding in our studies was that transcriptional coactivators which are recruited by VP16 AD are not important for IE gene expression during lytic infection (see Chapter 3). As it is likely that histones may be prevented from being deposited on the viral genome rather than being deposited first and then removed from it, coactivators may not be important for viral gene expression during lytic infection. This, however, does not exclude the possibility that transcriptional coactivators are required in other contexts, i.e. during reactivation from latency.

It is well established that the viral genome is nucleosomal during latent infections (7) and the histones associated with the viral genome carry inactive transcription marks (39). During reactivation from latency, HSV-1 transitions from a nucleosomal state to a non-nucleosomal one. Although there is conflicting evidence whether VP16 is required

during reactivation from latency (14, 34), it may well be the case that recruitment of coactivators by VP16 is important during this process. Interestingly, using the *in vitro* "quiescent infection" model (see Chapter 2) we have shown that coactivators may not be required during induction of IE gene expression by VP16 from heavily nucleosomal templates, yet this system may not be representative of *in vivo* latent infections. Addressing whether transcriptional coactivators are required during reactivation from latency *in vivo*, however, is challenging given that the absence of most coactivators result in embryonic lethality in mouse (3, 4, 29, 41, 42), a widely used animal model for HSV-1 latent infection.

As explained in Chapter 1 in detail, during latency in sensory neurons the HSV-1 genome is associated with histones that carry inactive transcription marks. Upon reactivation from latency by stress stimuli, some of the viral genomes become transcriptionally active leading to their replication and production of infectious virions that are released by anterograde transport at the epithelial cells, the sites of lytic infection. An important question regarding the state of viral chromatin during this process is at what stage and by what mechanism the histones on the viral genome are depleted, such that no histones are incorporated in the viral capsids produced in the sensory neurons. Although there is evidence that reactivation from latency correlates with the appearance of active transcription marks, such as H3K9/K14ac, on the transcriptionally active ICP0 promoter (1), it is currently not known whether histones are removed simultaneously with their covalent modification or whether histone depletion is a later event following replication. If the former model is the case, then one would expect blocking viral replication would still lead to histone depletion on the actively transcribed viral genes. With regards to the

latter model, given that CAF-1, the replication-dependent nucleosome assembly factor, interacts with the DNA polymerase clamp, proliferating cell nuclear antigen (PCNA) (35), it would be informative to address whether CAF-1 also interacts with the HSV-1 processivity factor, UL42. The lack of such interaction would support the model that upon replication of reactivated viral genomes, the newly replicated viral DNA would be prevented from being nucleosomal and marked for packaging into the viral capsid. One difficulty of testing these models is that only a small fraction of latent viral genomes are reactivated, and as such the large fraction of viral genomes that are still nucleosomal upon reactivation stimuli may hinder observing the changes in histone levels on the reactivated viral genomes.

Although a number of coactivators tested in this thesis (see Chapter 2) are not required for the transcription of IE genes, a number of studies indicated a role for Set1 histone methyltranferase for optimal expression of viral genes and replication (19, 27). Set1 is recruited to IE gene promoters by interacting with HCF-1, a component of VP16-induced complex. Interestingly, although the disruption of Set1 by RNAi did not cause a substantial decrease in IE gene expression at early times, it did cause a decrease in ICP0 and VP16 steady-state mRNA levels at late times in infection, concomitant with replication (19). Hence, future studies will be necessary to address whether Set1 is required for IE gene expression. In addition, if Set1 is also required for the expression of other temporal classes of HSV-1 genes, it will also be important to address the mechanism of how Set1 would be recruited to other regions of the viral genome. On the other hand, given that histones may not be deposited at all on the HSV-1 genome, we think it is more likely that Set1 is also not essential for viral gene expression.

3. Role of histone chaperones in HSV-1 infections

The assembly of histones and histone variants into nucleosomes requires the activities of a number of proteins and protein complexes (15, 33). 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 is involved in both replication-dependent and -independent histone assembly.

Given that during lytic infection histones are likely prevented from being deposited on the viral genome, it is possible that chromatin assembly by histone chaperones may be blocked by viral proteins. Interestingly, HIRA is a component of PML bodies, which are disrupted by ICP0 during lytic infection. Therefore, an attractive model is that HIRA may be included in PML bodies to silence the incoming viral genome, yet its activity or targeting may be blocked by viral factors such as ICP0, which disrupts PML bodies (9). However, a recent report suggested that HIRA and histone deposition on the viral genome may actually contribute to viral gene expression and replication, rather than inhibiting it (30). On the other hand, in this same study, no significant change in expression of certain viral genes was observed at early times in infection. These results are also contradictory with the fact that most of the viral genomes remain free of histones during lytic infection. In other words, if histone deposition on the viral genome was necessary for viral gene expression, one would expect to observe more histones on the actively transcribed viral genome. This, however, does not seem to be the case as evidenced by our sequential-ChIP assays in KOS-infected cells (see Chapter 4), where the actively transcribed IE genes are depleted of histones. Therefore, future studies

will need to address in more detail whether the presence of low amount of histones is important for viral gene expression and whether the nucleosome assembly machinery in host cells is altered in a way to prevent histone deposition on the viral genome. One way to address the latter possibility is by checking whether HSV-1 infected cells are debilitated in nucleosome assembly *in vitro* by DNA supercoiling assays (31, 37), which allow testing both replication-dependent and –independent nucleosome assembly. Alternatively, RNAi and overexpression of histone chaperones and chromatin assembly factors may be employed to address the necessity of these factors for viral gene expression.

4. Concluding Remarks

Chromatin on the HSV-1 genome is clearly regulated differentially during lytic and latent stages of infection. In this thesis, I tried to elucidate the mechanisms that lead to dearth of histones on the viral genome during lytic infection and indicated a role for VP16, IE proteins and RNAP II-mediated transcription in preventing histone deposition on the viral genome. This mechanism points to an important concept, that is, regulation of chromatin itself is not likely to determine whether the virus will initiate lytic infection or stay latent. It is more likely that changes in the chromatin state of the viral genome is a result of transcriptional activity mediated by VP16, IE proteins and RNAP II, and possibly other transacting factors that still need to be determined. Therefore, identifying these factors and the mechanisms of their action is crucial for our understanding of regulation of chromatin during HSV-1 infections.

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