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VP16-DEPENDENT ASSOCIATION OF CHROMATIN-MODIFYING COACTIVATORS AND UNDER-REPRESENTATION OF HISTONES AT IE GENE PROMOTERS DURING HSV-1 INFECTION

By

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ABSTRACT

VP16-DEPENDENT ASSOCIATION OF CHROMATIN-MODIFYING COACTIVATORS AND UNDER-REPRESENTATION OF HISTONES AT IE GENE PROMOTERS DURING HSV-1 INFECTION

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During infection by herpes simplex virus type 1 (HSV-1), the virion protein VP16 activates the transcription of viral immediate early (IE) genes. Genetic and biochemical assays have shown that the potent transcriptional activation domain of VP16 can associate with general transcription factors and with chromatin-modifying coactivator proteins of several types. The latter interactions are particularly intriguing because previous reports indicate that HSV-1 DNA does not become nucleosomal during lytic infection.

In the present work, chemical crosslinking and immunoprecipitation assays were used to probe the presence of activators, general transcription factors, and chromatin modifying coactivators at IE gene promoters during infection of HeLa cells by wildtype HSV-1 and by RP5, a viral strain lacking the VP16 transcriptional activation domain. The presence of VP16 and Oct-1 at IE promoters did not depend on the activation domain. In contrast, the associations of RNA polymerase II, TATA-binding protein, histone acetyltransferases (p300 and CBP) and ATP-dependent remodeling proteins (BRG-1 and hBRM) with IE gene promoters were observed in wildtype infections but were absent or reduced in cells infected by RP5. Contrary to the previous evidence for

non-nucleosomal HSV-1 DNA, histone H3 was found associated with viral DNA at early times of infections. Interestingly, histone H3 was under-represented at the IE gene promoters in a manner dependent on the VP16 activation domain. Thus, the VP16 activation domain is responsible for recruiting general transcription factors and coactivators to IE promoters and also for the reduced levels of histones present at those promoters.

To my Family

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LIST OF ABBREVIATIONS

aa	amino acid
Ab	antibody
AcH3	aceylated histone H3
AD	activation Domain
ARC	activator-recruited cofactor
ATP	adenosine 5'-triphosphate
BRM	Brahma
BRG-1	Brahma-related gene 1
CBP	CREB-binding protein
ChIP	chromatin immunoprecipitation
CMV	cytomegalovirus
DE gene	delayed early gene
EBV	Epstein-Bar virus
gC	glycoprotein C
GTF	general transcription factor
HAT	histone acetyl transferase
HIV	human immunodeficiency virus
hpi	hours post-infection
HSV-1	herpes simplex virus type 1
ICP	infected cell polypeptide
IE gene	immediate early gene

IFN-β	interferon beta
IP	immunoprecipitation
KSHV	Kaposi's sarcoma-associated herpes virus
L gene	late gene
MOI	multiplicity of infection
ORF	open reading frame
PCR	polymerase chain reaction
PIC	pre-initiation complex
pfu	plaque forming unit
qPCR	quantitative polymerase chain reaction
RNA Pol II	RNA polymerase II
RT-PCR	reverse transcriptase PCR
SNF	sucrose non-fermentable
SWI	mating-type switching
TAF	TBP-associated factor
TBP	TATA-binding protein
TFII	transcription factor, RNA polymerase II
TK	thymidine kinase
UAS	upstream activating sequences
VP	virion protein

Chapter I
Literature Review

1.1. Transcriptional activation in eukaryotes[1].

The genome of an organism not only encodes the information to produce thousands of proteins but also regulatory sequences that dictate when each of these genes is to be expressed. In case of protein-encoding genes, these regulatory sequences are recognized by *trans*-factor proteins that are able to modulate the activity of the RNA polymerase II (RNA Pol II). The interplay of *trans*-factors and regulatory sequences is a critical step in regulating gene expression and therefore is important for cellular process including cell homeostasis, growth, differentiation and diseases.

The *cis*-acting regulatory sequences for genes transcribed by RNA Pol II typically comprise a combination of core promoter elements and upstream activating or repressing sequences [reviewed in (262)]. The core promoter serves as the binding site for the basal transcriptional factors and defines the transcriptional startpoint. The upstream activating or repressing sequences (UASs and URSs) are recognized by transcriptional activators and repressors that modulate the activity of core promoters, helping to control the transcription of genes.

[1] Part of this chapter was published in "Herrera FJ, Shooltz D and Triezenberg SJ (2004). Mechanisms of Transcriptional activation in eukaryotes. Handbook of Experimental Pharmacology. Vol 166:3-31".

1.1.1. Promoter architecture.

1.1.1.1. Core promoters.

One of the more prominent features in core promoters is the TATA box, typically located about 30 bp upstream of the transcription start. Although initial models proposed that the TATA box was necessary for transcription of RNA Pol II genes, analysis of a subset of promoter sequences (205 sequences) from *Drosophila* showed a 42% prevalence of TATA box containing promoters (160). Similarly in a subset of human promoters (1031 sequences) the prevalence of TATA containing promoter was 32% (274). These results reveal that although the consensus TATA box is present in many gene promoters, it is not a necessary feature of every RNA Pol II promoter. Surrounding the start site itself may be found an initiator (Inr) element involved in defining the correct start site for transcription. Two other core promoter motifs present downstream of the transcription start site have been identified in *Drosophila* and humans. The downstream promoter element (DPE) is present at +28 to +32 relative to +1 start site and is involved in TFIID binding (125). The motif ten element (MTE) is located at +18 to +27 and its activity is dependent on Inr but independent of TATA box and DPE (171). Furthermore, a TFIIB recognition element (BRE), flanking the TATA box, has been described in some organisms [reviewed in (262)]. Differences among core promoters of various genes, with respect to the presence and strength of these core elements, have pronounced effects on how those promoters respond to particular transcriptional activators. This specificity may allow a particular enhancer to differentially regulate various target genes, and may allow

a particular core promoter to selectively respond to different enhancers. For example, the Sp1 activation domain strongly activates core promoters containing TATA elements, Inr elements, or both, whereas the Gal4-VP16 activator is most effective at a core promoter with both TATA and Inr (68). In an "enhancer trapping" study in *Drosophila*, many enhancers were able to drive expression from both TATA dependent and DPE dependent promoters, but some enhancers preferentially activated either one or the other core promoter (36). The mechanistic differences leading to enhancer-core promoter specificity may involve the recruitment of transcriptional cofactors that display core promoter specificity. For example, the transcriptional cofactor NC2 represses transcription from TATA dependent promoters, but activates transcription from DPE dependent promoters (307). Thus, either direct or indirect recruitment of transcriptional cofactors by an activator may differentially regulate transcription from different core promoters, leading to activator-promoter specificity.

1.1.1.2. Proximal promoter and enhancers.

In addition to the core promoter elements, transcription of many genes depends on *cis*-acting regulatory elements termed enhancers or upstream activating sequences (UAS), which provide the binding sites for transcriptional activators. These DNA elements can vary in sequence and affinity for a particular DNA binding domain, and may exist in promoter-proximal locations or hundreds to thousands of basepairs upstream or downstream of a promoter.

Combinations of activator binding sites may be clustered into more complex regulatory elements. In such cases, cooperative binding of activators can lead to the formation of large DNA-protein structures termed enhanceosomes, resulting in synergistic effects on transcription. In the prototypical virus-inducible IFN-β enhanceosome [reviewed in (196)], a cluster of three different activator binding sites direct the expression of IFN-β in response to viral infection. However, none of the activator binding sites act alone; only the combination of all three activator binding sites recapitulates the logic necessary to drive proper expression and specificity of the IFN- β gene. In this model system, the enhancer represents not simply the sum of individual activator functions, but rather an integration of inputs from different sources interpreted by the particular combination of transcription factors present at the enhancer.

Taking this organizational theme one step further, many genes may have multiple enhancers, each of which is poised to respond to particular developmental, growth, or environmental signals. Each of these independent enhancers may be simultaneously signaling to the core promoter either to stimulate or repress transcription, depending on the signal inputs received by the regulatory proteins that bind there. This diverse and sometimes conflicting information must be integrated and interpreted at the promoter to make a final decision on whether or not transcription is to proceed. This "information display" model for genes with multiple enhancers has arisen from studies of developmentally related genes in *Drosophila* (152), but will likely be relevant for many hormonally or pharmacologically regulated genes in humans as well.

With the increasing availability of genomic sequences for prominent experimental organisms, computational analysis for identifying *cis*-acting regulatory sequences has become a powerful tool. In some cases, these searches focus on a particular *cis* regulatory element, such as the estrogen response elements in mammalian genomes (11). Other programs are designed for broader application, searching for sites corresponding to any of the transcription factors in the TRANSFAC database and combining site searches to increase the likelihood of identifying legitimate regulatory regions rather than idiosyncratic consensus sequence matches (22, 89, 132). These computational approaches yield results that still must be validated by direct evidence of the function of putative elements in gene regulation and their interaction with specific transcription factors. Although this is often done on a case-by-case basis, either by mutational analysis of the *cis* elements or by *in vitro* binding assays, more global assessments are also now possible. For several transcription factors in yeast (241) and in mammalian cells (240, 301), genomic mapping of transcription factor binding sites has been accomplished by combining chemical crosslinking and immunoprecipitation (chromatin IP or "ChIP" assays) with DNA microarrays comprising intergenic or putative regulatory sequences (so-called "ChIP on chip" assays).

1.1.2. Transcriptional activators.

The dual functions of a transcriptional activator protein, *cis*-element recognition and transcriptional activation, are typically fulfilled by distinct regions of the protein's

primary structure. For example, the DNA binding domain of the yeast Gal4 protein resides within the amino-terminal 100 amino acids, whereas the major transcriptional activation domain resides within the carboxyl-terminal 120 amino acids. This modular design seems advantageous both for evolutionary and technological appropriation. In the latter sense, a fusion protein linking the DNA-binding domain of Gal4 with the transcriptional activation domain of the VP16 protein from herpes simplex virus (250) is widely used in both *in vitro* and *in vivo* investigations into the mechanisms of transcriptional activation. A second example, comprising a fusion of the DNA binding domain of the tetracycline repressor with the VP16 activation domain, allows the regulation of DNA binding by the presence or absence of the tetracycline ligand (86). This regulatable artificial activator can function in a wide range of eukaryotes ranging from plants to mammals (85, 87, 302).

1.1.2.1. DNA binding domains.

The DNA binding domains of a large number of eukaryotic transcriptional activator proteins have been extensively characterized by genetic, biochemical, and structural approaches. Recent reviews catalog the known structures and specificities (182) and highlight the common themes in structure and recognition (77). In many cases, the binding activity or specificity of a DNA binding domain may be modulated by ligand binding, dimerization with other DNA binding proteins, or by association with other factors [reviewed by (189)].

The principles of protein:DNA interaction have now been established to a sufficient degree to permit the design of DNA binding modules of engineered specificity (16, 70). This is particularly true for the zinc-finger families of transcription factors (51, 127, 257, 310). This ability to tailor novel chimeric transcriptional activators for recognition of DNA sequences that might not serve as native regulatory elements has profound implications for potential technological application (172, 174, 327).

1.1.2.2. Activation domains.

In contrast to DNA binding domains and despite substantial research, relatively little is known about the structures of transcriptional activation domains (TADs). By analysis of primary sequence, TADs have been broadly classified based on the abundance of particular amino acids, resulting in acidic, glutamine rich, proline rich, and other classes (200, 282). Despite these amino acid preferences, however, careful mutational analyses have indicated that the most critical elements of activation domains are frequently the patterns of hydrophobic and aromatic amino acids (6, 38, 54, 113, 282).

Less is known about the secondary and tertiary structures of activation domains. A number of biophysical analyses of various regulatory proteins have shown that activation domains are largely unstructured in solution under physiological conditions (56, 221, 254, 259). However, key amino acids in an activation domain can become conformationally constrained upon interaction with a target protein, suggesting that the

most promising targets for structural studies will be binary complexes between activators and targets. For example, circular dichroism spectra indicate that the c-Myc transactivation domain is induced to form a helical structure upon binding to TATA binding protein (TBP) (192). The activation domains of VP16 and of the estrogen receptor also become conformationally constrained upon interaction with TBP (260, 299). Furthermore, the VP16 activation domain appears to become helical upon binding a TBP associated factor, human TAF9 (284). An amphipathic helix from the p53 activation domain fills a hydrophobic cleft in the MDM2 oncoprotein (159). An amphipathic helix structure is also seen in the interface between the activator CREB and its coactivator protein CBP (236). These examples support the model that an activator target provides a folding template for an unstructured activation domain, which might allow activation domains to interact with a number of different target proteins.

In some cases the tables may be turned: that is, an activation domain may provide the folding template for a potential target. Nuclear hormone receptors have a conserved C-terminal activation domain, known as AF-2. Ligand binding leads to a conformational change in the activator that opens a hydrophobic groove for interaction with transcriptional co-activators. In this case, the unstructured LxxLL peptide motif present in several coactivators (101) folds into an amphipathic helix upon binding the AF-2 region of a hormone receptor [reviewed in (298)].

Although in many cases the activation domains seem to adopt α-helical structures, that rule is not universal. Mutational and biophysical analysis of the Gal4 activation

domain suggested that it might form a β-strand instead (287). The activation domain of E2F-2, upon interaction with the Rb tumor suppressor protein, assumes a combination of helical and β-strand conformations (164). Together, these observations suggest that activators and their target proteins bind to each other using a highly diverse set of interaction surfaces.

1.1.3. Actions of activators at promoters.

Once localized to a promoter, a transcriptional activator can interact with a number of different targets, including RNA Pol II, the basal transcription factors, the mediator complex, coactivators, and chromatin-remodeling machinery. A common theme in models of activation is recruitment, where a promoter-bound activator localizes either a component of the transcriptional machinery or a transcriptional cofactor. This model is supported by evidence of direct physical interactions of activators with basal transcription factors, and by activator bypass experiments [reviewed in (231)]. In the latter experiments, a component of the transcriptional machinery is fused directly to a DNA binding domain, and this artificial recruitment serves to activate transcription. Conceivable, in a variation of recruitment, a transcriptional activator may modulate the activity of components of the transcriptional machinery, facilitating the assembly of the preinitiation complex.

1.1.3.1. Stepwise recruitment of basal transcription machinery.

Transcription of protein-coding genes requires the assembly of a preinitiation complex (PIC) comprising RNA Pol II, the general transcription factors (GTFs), and a number of associated factors. In the stepwise model of PIC assembly, the TATA-binding protein (TBP)-containing TFIID complex binds to a promoter, followed by TFIIA, TFIIB, TFIIF and RNA Pol II, and TFIIE and TFIIH (34). TFIID is the only general transcription factor that can specifically bind a promoter in the absence of interactions with other GTFs, suggesting that it nucleates the assembly of the PIC. Any step of PIC assembly might be rate limiting, and the recruitment of GTFs by association with activators may facilitate assembly.

1.1.3.1.1 TFIID, TBP and TAFs.

The TFIID protein complex comprises the TATA binding protein (TBP) and several TBP-associated factors (TAFs) (35, 232). TBP binds selectively to the TATA core promoter element, while the TAFs extend the footprint to include the Inr and DPE elements. Studies *in vitro* show that although TBP is sufficient for basal transcription, the TAFs are required for activated transcription (61, 190, 253).

TBP can bind directly to transcriptional activation domains, as demonstrated by *in vitro* binding assays using a wide range of activator proteins, and mutations in activators that weaken activation also weaken interaction with TBP (111, 213, 260). While these results might suggest that activators simply recruit TBP, other evidence suggests that the

mechanism is more complicated. The acidic activator Gal4 binds TBP competitively with TATA DNA (317) and TBP and Gal4 do not bind cooperatively to promoters (318), suggesting that competition for the DNA binding domain of TBP may be involved in activation mechanisms.

The TFIID complex associates with TATA box DNA more slowly than does isolated TBP, implying that the TAFs contain inhibitory functions (143). Some activators, including VP16 and the Zta protein of Epstein-Barr virus, can stimulate the assembly of a TFIID-TFIIA-DNA complex (141, 169), but do not stimulate a ternary complex when TBP is used instead of TFIID, suggesting that activators may counteract inhibitory functions of the TAFs. This ability of activators to stimulate ternary complex assembly appears to be relevant for activation, since mutations in the activation domain that reduce transcriptional activation potential *in vivo* also diminish the *in vitro* D-A assembly function (142).

Other TAFs have a direct affinity for certain transcriptional activators, suggesting a more direct role in recruitment or modulation of activity. For example, the glutamine-rich activators Sp1, NFAT, and CREB interact with the TAF4 protein from *Drosophila* or human cells (46, 72, 83, 137, 247, 311) and the acidic VP16 and p53 activation domains can interact with TAF9 (84, 140, 284). These interactions cannot always be interpreted to imply an effect on TFIID, since a significant number of these TAF proteins are present in protein complexes lacking TBP that nonetheless influence transcriptional activation (91, 305).

1.1.3.1.2. TFIIA, TFIIB, TFIIF and TFIIH.

TFIIA is a positive cofactor in PIC assembly, as it binds cooperatively with TFIID at TATA DNA elements. TFIIA also functions as an antirepressor, inhibiting the TBP-DNA destabilizing actions of Mot1 and NC2 [reviewed in (232)]. The formation of the ternary TFIID-TFIIA-DNA complex is a rate-limiting step in PIC formation, and activators can enhance this step (141, 169). Some evidence points to direct association of the VP16 AD with subunits of TFIIA (141, 142).

TFIIB also stabilizes the TBP-TATA complex, and serves as a docking site for other components of the PIC. Several activators including the VP16 TAD have been shown to bind TFIIB with high affinity (173), and the TAD-TFIIB connection has been implicated in transcriptional activation (245). Interaction with the VP16 TAD has been shown to alter the conformation of TFIIB, possibly priming it for incorporation in the PIC (100, 244) or altering TFIIB-DNA contacts (69). Although this evidence is intriguing in pointing to TFIIB as a potential activator target, other reports have failed to find evidence supporting this association (84, 260).

TFIIH, which contains both protein kinase and nucleic acid helicase activities, also appears as a target for activation domains. The activation domains of VP16, p53, and E2F1 can interact with TFIIH (223, 315) and recruitment of IIH may stimulate promoter escape (153), but no clear evidence exists that activators stimulate the enzymatic activities of TFIIH. TFIIF and TFIIE might also be targets for activation domains. The serum response factor (SRF) interacts with the RAP74 subunit of TFIIF

(121), and Fos-Jun dimers can interact with both TFIIF and TFIIE (191). Although the mechanistic implications of these interactions have not yet been fully developed, the dual role of TFIIF as both an initiation and elongation factor suggests the possibility that activators might modulate promoter escape or elongation in addition to assembly of the preinitiation complex.

1.1.3.2. Holoenzyme recruitment.

Many of the models described in preceding sections are predicated on the premise that transcriptional activation involves a sequential recruitment of the basal transcription factors and RNA Pol II to form the PIC at the target promoter. This premise was challenged, however, by the biochemical purification from yeast cells of an extraordinarily large protein complex comprising RNA Pol II stably associated with a subset of GTFs together with additional polypeptides (the Mediator proteins, described below) (138). Certain transcriptional activators were shown capable of recruiting this "holoenzyme" to a promoter in a manner sufficient to achieve transcriptional activation *in vitro* (103, 138). The model arising from these observations is that rather than separately and sequentially recruiting each general transcription factor, activation might more simply involve recruitment of the distinct TFIID and holoenzyme complexes. A similar RNA Pol II holoenzyme complex has been also purified from human cells (144), suggesting that the recruitment of the holoenzyme by activators might be an evolutionarily conserved mechanism [reviewed by (95)]. Kinetic and thermodynamic questions arising from the two competing models have not been fully resolved. How

could the stepwise assembly occur quickly enough (given diffusion parameters for each component) for efficient transcriptional activation? And yet, how can a complex the size of the holoenzyme be translocated to specific genes at specific times quickly enough to respond to transcriptional activation?

1.1.3.2.1. Mediator complex.

The mediator complex, first identified as a component of the yeast RNA Pol II holoenzyme (138), is composed of ~20 subunits forming three major domains (Gal 11, Med9/10 and Srb modules) that wrap around the RNA Pol II [reviewed by (25, 209)]. Homologues of the yeast mediator subunits and similar protein complexes have since been identified in a wide range of organisms. Mammalian protein complexes resembling yeast mediator were described independently by several laboratories using biochemical purifications of proteins stably bound to different activator proteins (reviewed by (185, 235). These different purifications lead to very similar complexes (variously termed TRAP, DRIP, ARC, CRSP, SMCC and PC2) suggesting that different activators bind the same or highly related human mediator complexes. The slight differences in protein compositions of these human mediators might represent variations due in differences in the biochemical purifications or might represent different forms of the mediator complex that associate with different activators.

Several activators are known to interact physically and functionally with the mediator complex and the particular mediator subunits involved in these interactions are

being identified. For example, the p53 tumor suppressor protein interacts with the TRAP80 subunit of the human mediator complex whereas the thyroid hormone receptor and PPARγ2 interact with TRAP220 (79, 112). The VP16 activation domain may associate either with TRAP80 or with ARC92 (112, 201). Interferon-stimulated transcription depends on an interaction of STAT3 with the DRIP150 mediator component (163). These and other examples indicate that the mediator complex can be considered as a modular interface connecting activators with RNA Pol II allowing the integration of different signals during transcriptional activation.

1.1.4. Chromatin and Chromatin-modifying coactivators.

Transcription activation must overcome the physical barriers presented by the packaging of eukaryotic DNA into chromatin. It is now clear that histones are not only a static scaffold for the compaction of DNA but rather they participate actively in the regulation of gene expression. Transcriptional activators affect chromatin with at least the assistance of two general classes of coactivator proteins. Some of these coactivators are enzymes that covalently modify amino acids within the histones themselves. These modifications then either directly alter chromatin structure, or serve as recognition signals for binding additional proteins that modulate that structure (110). Other coactivators use the energy of ATP to remodel chromatin by sliding or removing nucleosomes.

1.1.4.1. Covalent modifications of histones.

Covalent modifications that have been identified in histone proteins include acetylation, methylation, phosphorylation, ubiquitinylation, and ADP- ribosylation [reviewed by (18)]. The panoply of such modifications on the various histones has been likened to a "code" (119, 269) that, when deciphered and integrated, signals whether and how strongly a given gene is to be expressed. This code might be considered in several levels. First, any given modification at a given position on a given histone might either be present or absent. This implies the existence of complementary enzymes that either put on or take off the modifying mark. Second, at any given nucleosome or at several nucleosomes in any given promoter, various sets of such signals might be present. Third, the signals might occur sequentially – that is, one modification might serve as a signal to permit or stimulate another, or conversely one modification might block or inhibit the deposition of another. In recent years much effort has been dedicated to correlate specific histone modifications with the levels of gene expression of particular loci. The mechanisms involved in "reading" this histone code might involve the binding of proteins to a particular histone modification or to specific combinations of these modifications. For instance, bromodomains and chromodomains, present in many transcription factors, can bind acetylated and methylated histones respectively. The bromodomain of the histone acetyltransferase GCN5 can itself bind to acetylated histone H3 peptides (108), which might contribute to the stability of these complexes at promoters of actively transcribed genes (98). The heterochromatin protein HP1, through its chromodomain, binds methylated H3-K9, a modification prominent in heterochromatin regions. In

contrast, the chromodomain present in the Polycomb protein binds to methylated H3-K27, demonstrating that different chromodomains can bind different methylated residues in the histone proteins (74, 197).

1.1.4.1.1. Acetylation

Hyperacetylation of the amino-terminal tails of histones (especially of H3 and H4) is generally correlated with gene activation, and conversely hypoacetylation is associated with gene repression [reviewed by (248)]. The acetylation of the ε-amino group of lysine residues by HATs neutralizes the positive charge of the lysine side chain, which may affect the affinity of histones for DNA, altering the packaging of the chromatin, or may affect protein-histone interactions contributing to the recruitment of specific transcription factors to active promoters. An important connection between histone modification and transcriptional activation was made when genetic evidence for transcriptional coactivator proteins was linked to biochemical evidence for histone acetylation (31, 188). The first of these enzymes identified was GCN5, a histone acetyltransferase (HAT) present in the yeast multiprotein complexes termed SAGA and ADA. GCN5 is recruited to promoters by activators and contributes to the hyperacetylation of histones associated with the promoter region during transcriptional activation (156, 162). Homologs of the yeast GCN5 in other organisms play similar roles in transcription regulation, implying conserved mechanisms of regulation through evolution (37, 158, 292).

Several distinct families of evolutionarily conserved HATs have been identified in a wide range of organisms [reviewed by (41)]. These groups include the GNAT family (GCN5-related N-acetyltransferase), the MYST family (MOZ, Ybf2/Sas3, Sas2 and Tip60), the highly related p300 and CBP coactivators, and the basal transcription factors TAF1 and TFIIIC. A major challenge is to define the roles for each family and each member of those families, including which sites on which histones serve as substrates; which genes are affected by acetyltransferase activity; and which regulatory proteins establish those effects. This challenge is made more difficult because the various HATs likely have both unique and overlapping activities.

Intriguingly, many of these HAT enzymes can also acetylate substrates other than histones and might thereby alter (either positively or negatively) the function of the target protein. Known non-histone substrates include activator proteins such as p53, c-Myb, E2F, GATA-1, and MyoD; the general transcription factors TFIIE and TFIIF; and the high-mobility-group (HMG) chromatin-associated proteins [reviewed by (268)]. Thus the relevant *in vivo* substrates of a particular HAT might not necessarily be restricted to the histones.

1.1.4.1.2. Methylation.

Methylation of histones occurs on both Arg and Lys residues, most prominently in histones H3 and H4 [reviewed by (145)]. Individual Lys residues can accommodate one, two or three methyl groups, and each isomer can exert a distinct and separate downstream effect. In contrast to histone acetylation, which typically is associated with

transcriptionally active genes, methylation of certain histone amino acids corresponds to active loci whereas methylation of other residues is associated with inactive genes or even heterochromatic regions (96). Thus, methylation of Arg3 of histone H4 is associated with transcriptional activation, as is methylation of lysines 4, 36 and 79 of histone H3. In contrast, methylation of lysines 9 and 27 of histone H3 or of Lys20 of histone H4 are correlated with transcriptional repression or silencing. Interestingly, histone methylation is not associated solely with nucleosomes near the promoter, but extends throughout the coding region of the gene. For instance, trimethylation of H3-K4 by the Set-1 enzyme is a mark of the early phase of transcriptional elongation (218) whereas Set-2 methylation of H3-K36 seems to mark subsequent stages of elongation (150).

Discrete enzymes are responsible for methylating different amino acids in the histones. The Arg methyltransferases best known for their transcriptional role are CARM1 and PRMT1, both especially prominent as coactivators for nuclear hormone signaling (319). The Lys-specific histone methyltransferases typically possess a conserved catalytic domain known as the SET domain originally found in the SUV39, E(Z) and trithorax proteins of *Drosophila* (161). Curiously, whereas the enzymes that attach other covalent modifications all have complimentary removal enzymes (histone deacetylases or phosphatases, for example) no histone demethylase have yet been described (13). Interestingly, an alternative activity has been recently described that might serve as the enzyme responsible for Arg demethylases. The human enzyme peptidylarginine deiminase 4 (PAD4/PADI4) can catalyse the deimination of methylated

arginines to produce citrulline resulting in the removal of the methylation mark (55, 297). The fate of the citrulline-containing nucleosomes is still unknown.

1.1.4.1.3. Phosphorylation.

Phosphorylation of histones, most notably at Ser10 of H3, has been correlated with both mitotic condensation of chromosomes and activation of gene expression in yeast, insects and mammals (45, 176, 220). For transcriptional activation, this modification stimulates the subsequent acetylation of H3 Lys14 (177). Several kinases responsible for the phosphorylation of H3 Ser10 have been identified. In yeast, SNF-1 phosphorylates H3-S10 during transcription activation of genes involved in the biosynthesis of inositol (176). In mammalian cells, the immediate early response to EGF through the MAP kinase pathway results in the phosphorylation of H3 at the c-fos and c-jun gene promoters by the MSK1 and MSK2 kinases (45, 264, 281). The Ikk-α is the kinase responsible for the phosphorylation of H3 at promoters of NF-kB-regulated genes during cytokine-induced gene expression (7, 321). The Aurora family of H3 kinases is responsible for mitotic phosphorylation (222). Histone phosphatases have also been identified, most notably the Glc7 protein (73), but their roles in chromatin modification and gene regulation are relatively poorly defined at this point.

1.1.4.1.4. Ubiquitinylation.

Ubiquitinylation is another covalent modification observed in histones. Histones H2A and H2B are most often the targets of this modification, although ubiquitinylation of H3 and H1 has also been described. In contrast to the ubiquitin-proteasome proteolytic

pathway, in which multiple ubiquitin moieties might be added, histone ubiquitinylation typically comprises addition of a single group and does not seem to be associated with protein degradation [reviewed by (207)].

Monoubiquitinylation of histones might affect higher-order chromatin folding or might affect histone-protein interactions by creating binding sites for particular transcription factors [reviewed by (328)]. In yeast, transcriptional activation by Gal4 depends (in part) on both ubiquitinylation of H2B (by the ubiquitin ligase Rad6) and on subsequent removal of the ubiquitin by a Ub-specific protease, Ubp8. This latter enzyme is a component of the SAGA complex which also contains the GCN5 histone acetyltransferase, suggesting an intimate relationship between histone ubiquitinylation and acetylation. Failure either to add or remove ubiquitin results in diminished gene activation and in altered levels of gene-associated methylation of Lys4 and Lys36 of histone H3, both modifications linked to transcription activation (104).

1.1.4.2. ATP-dependent chromatin remodeling complexes.

The second general class of chromatin-modifying transcriptional coactivators is composed by the ATP-dependent chromatin remodeling complexes. These multiprotein complexes use the energy from ATP to "remodel" chromatin by mechanisms that include alterations of DNA-histone contacts. The ATPase subunits of these complexes belong to the SNF2-like family of ATPases and can be classified in different subfamilies according

to the presence of other protein motifs such as bromodomains, chromodomains and SANT domains [reviewed by (64, 212)]. These complexes have both common and distinctive biochemical characteristics and are involved not only in transcription activation but also in other cellular functions that involve unwrapping of DNA such as DNA repair, homologous recombination and chromatin assembly [reviewed by (184)].

ATPases of the SNF2 subfamily contain a bromodomain and in general are part of multiprotein complexes of approximate 10-12 subunits designated SWI/SNF and RSC in yeast and Brahma in *Drosophila*. SWI/SNF subunits were identified in yeast genetic screens focused on mating type switching (SWI alleles) or sucrose metabolism (sucrose non-fermentable alleles, SNF). These protein complexes can relocate nucleosomes, alter DNAase accessibility patterns and alter the superhelicity of DNA *in vitro* [reviewed by (212)]. The human genome encodes two SNF2 homologs, BRG-1 and BRM, present in similar but distinct protein complexes. These complexes regulate different set of genes as demonstrated by the variations in mutant phenotypes. In yeast, SWI/SNF seems to control approximate 5% of yeast genes whereas RSC seems to play a more global effect in gene regulation (217, 270). In mice, null mutations of the Brg-1 gene result in death of homozygotic embryos during the peri-implantation stage, whereas BRM-/- mutant mice develop normally although cell proliferation seems to be misregulated (33, 242). The recruitment of SWI/SNF to gene promoters by activators has been observed *in vivo* and *in vitro*, in biological systems ranging from yeast to human. Mammalian activators including nuclear receptors, erythroid Kruppel-like factor, C/EBPβ, c-Myc, MyoD, HSF-

1 and viral activators such as EBNA2 and VP16 are known to recruit human SWI/SNF complexes during transcriptional activation [reviewed by (212)].

Members of the ISWI subfamily of ATPases contain a SANT domain and are present in complexes comprising 2-4 subunits. These complexes include NURF, CHRAC and ACF from *Drosophila*, and vertebrate complexes such as RSF, hACF, hCHRAC and NoRC. Biochemical characterizations indicate that several of these complexes are involved in the assembly of chromatin [reviewed by (184)]. Members of the CHD1 subfamily of ATPases are characterized by the presence of a chromodomain and are present in complexes including NurD and Mi-2. These complexes also contain HDAC activities and are known to be recruited by certain repressor proteins (136, 255). The yeast Ino80 and Swr1 ATPases comprise another subfamily. Ino80 is present in a large multiprotein complex that also contains DNA helicase activity. This complex is involved in transcription of genes encoding enzymes for phospholipid biosynthesis and also of genes such as PHO5, GAL1, CYC1 and ICL1 involved in unrelated pathways (63). Besides its role in transcription, this complex seems to play a role in DNA damage repair (261). The Swr1 ATPase is present in a complex that is able to exchange histone H2A variants (202). Other ATPases known to have chromatin-remodeling activities include the Arabidopsis DDM1, involved in maintaining normal levels of DNA methylation (117); Rad54, involved in homologous recombination (5); and CSB (Cockayne Syndrome protein B) involved in DNA excision repair (49).

The mechanisms of these remodeling activities are not yet fully understood but include local and stable alterations of the DNA-histone contacts leading to sliding of nucleosomes along the DNA or transfer of nucleosomes from one DNA to another in *trans*. These protein complexes can also alter the superhelicity of DNA *in vitro* [reviewed by (212)]. Repositioning of nucleosomes may alleviate chromatin-mediated transcription repression, for example by exposing DNA binding sites for additional activators or by exposing core promoter elements that might be critical for the binding of general transcription factors and the formation of the pre-initiation complex.

1.1.4.3. Chromatin dynamics.

Chromatin remodeling occurring during transcription activation might not only involve covalent modifications of histones and localized modifications of histones-DNA contacts, but also seems to involve more dramatic histone dynamics. Accumulating evidence indicates that histones might be actively exchanged during activation of genes. The histone H3 is exchanged with the histone variant H3.3 during transcription activation in *Drosophila* and human cells by DNA replication-independent mechanisms (3, 116, 275). The histone H2A-H2B dimers are destabilized by the elongating RNA Pol II *in vitro* in a process facilitated by the FACT complex (17, 139). Collectively this evidence indicates that the histone content or composition at a particular locus can vary during transcriptional activation and outside of S-phase.

The loss of histones during transcription activation has been observed at the Pho5 promoter in *Saccharomyces cerevisieae* (23, 239). The displacement of histone H3 occurs upon transcriptional activation and is reversed during transcriptional repression (2). The mechanism for the loss of histones in this promoter involve histone disassembly rather than histone sliding (24) and requires the activity of the histone chaperone Asf-1 (2). Genome wide analyses of yeast promoters have recently revealed that nucleosome depletion is a wide spread phenomena occurring at the promoters of active genes (21, 165).

1.1.5. Post-initiation effects of activators.

Although most studies of transcriptional activation have focused on recruitment and initiation, subsequent steps including promoter escape and elongation can also be stimulated by activator proteins. Several lines of evidence indicate that activators might also work in post-initiation steps. One well-characterized example corresponds to the human and *Drosophila* gene encoding heat shock protein 70 (hsp70). The uninduced hsp70 gene contains a paused polymerase near the 5' end of the gene. In response to heat shock, not only does the transcriptional initiation rate increase, but the pausing time is dramatically reduced (29, 249). Transcriptional activators can also stimulate rates of transcriptional elongation. For example, the heat shock factor-1 (HSF-1) involved in the activation of hsp70 gene and the viral activators VP16 and E1A can stimulate elongation by mechanisms that apparently different from that of stimulation of initiation (30, 324).

The interaction of VP16, HIV tat, c-myc, and NF-kB with the elongation factor P-TEFb (a kinase that modifies the carboxyl-terminal tail of RNA Pol II) further illustrates this link (14, 47, 128, 129, 157). These post-initiation effects of activators might be regulated by covalent modification occurring during transcriptional activation. The ubiquitinylation of LexA-VP16 enhances the interaction with P-TEFb and rescues the elongation defect of the mutant LexA-VP16F422A indicating that ubiquitylation of activators might help stimulating transcription elongation (157).

Transcription and RNA processing have often been considered as separate and sequential events, but a more recent perspective views these as a single integrated pathway [reviewed by (230)]. Capping, splicing and polyadenylation are tightly coupled to RNA Pol II through the carboxyl-terminal domain of the largest subunit (reviewed by (186). Selection of splice sites in the nascent RNA can be influenced by promoter elements in a manner that is independent of the promoter strength, suggesting that activators might also regulate alternative splicing decisions (53) (9) (219).

1.2. Transcriptional activation of herpes simplex virus genes.

1.2.1. Herpes viruses.

Viruses from the Herpesviridae family infect most animal species and those that infect humans include pathogens such as herpes simplex virus type 1 (HSV-1), herpes

simplex virus type 2 (HSV-2), varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpes virus (KSHV). Viruses from the Herpesviridae family are formed by a double-stranded DNA genome enclosed in an icosahedral capsid of approximately 100-110 nm in diameter. The capsid is contained within a lipid envelope of cellular origin that contains glycoprotein spikes on its surface. Between the capsid and envelope is the tegument, which is the structure containing viral proteins involved in the initial steps in the subsequent infection (246)

Viral genomes from the Herpesviridae family encode a large number of enzymes involved in nucleic acid metabolism and DNA synthesis including DNA polymerases, helicase and primase. The synthesis of the DNA and assembly of the capsid occur in the nucleus of the cell. Herpesviruses are able to establish lytic and latent infections in their natural host. The production of the newly synthesized virion particles during lytic infection results in the destruction of the infected cell. The specific cell type that is infected during lytic and latent cycles varies among different herpesviruses. The Herpesviridae family is divided into three subfamilies according to different biological properties: Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae (246).

1.2.2. Herpes simplex virus 1.

HSV-1 is a prototypical virus in the subfamily Alphaherpesvirinae. This subfamily is characterized by broad host range, relatively short reproductive cycle, rapid

spread in culture, efficient destruction of infected cells and capacity to establish latent infections in sensory ganglia (20).

Primary infections by HSV-1 usually occur at mucosal and skin surfaces. The histopathologic characteristic of a skin lesion caused by HSV infections include alterations of the cell membranes and changes in the chromatin structure within nuclei followed by degeneration of nuclear structures. Infection by HSV-1 also results in the fusion of plasma membranes leading to the formation of multinucleated cells or polykaryocytes. After cell lysis, clear fluid containing viruses, cell debris, inflammatory cells and multinucleated cells accumulate between the epidermis and dermal layer forming vesicles (243).

HSV-1 can enter the sensory nerves near primary infection sites and establish latency by unknown mechanisms. In latently infected neurons, the genome remains mostly silent expressing only the latent associated transcript (LAT). During latency the viral genome stays as a circular and extrachromosomal DNA packaged into nucleosomal structures (60, 194). Reactivation of the latent virus can be induced by several stimuli through molecular mechanisms not well characterized and seems to require the IE protein ICP0 and LAT [reviewed by (20)].

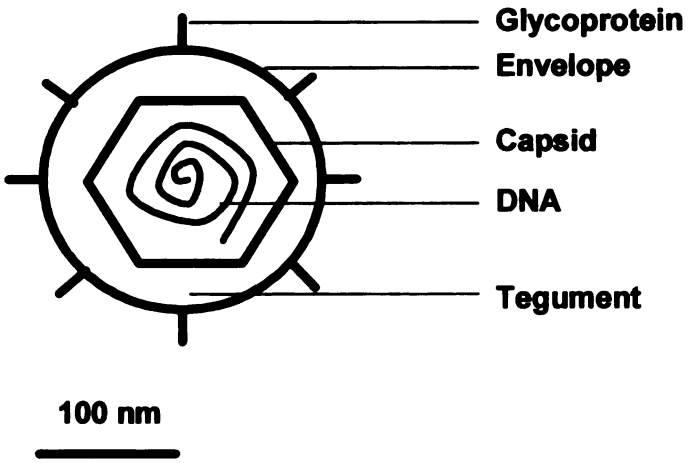
1.2.2.1. Virion structure.

The HSV-1 virion particle contains approximately 30 different types of proteins. About 10 of these proteins are membrane proteins present in the envelope of the virus (Figure 1A). These membrane proteins are usually glycosylated and include proteins known to be involved in attachment of the virus to the cell surface (such as glycoprotein C) and for the viral entry (such as glycoprotein B, H and L) [reviewed by (265)]. The capsid is formed by four predominant viral proteins, VP5, VP19C, VP23 and VP26 where VP5 is the major structural component of the capsomers [reviewed by (106)]. The remaining virion proteins are present in the tegument. These proteins are delivered to the newly infected cells helping to start the new infection cycle. Among these tegument proteins delivered to the infected is the viral trans-activator VP16, VP13-14, virion host shutoff (VHS) and VP1-2.

1.2.2.2. Viral genome.

HSV-1 is a relatively large DNA virus encoding genes involved in regulation of gene expression, DNA replication, virion structure and assembly. The herpes virus contains a linear double-stranded DNA genome of about 150 kbp and is formed by two unique segments of DNA referred as unique long (U_L) and unique short (U_S) (Figure 1B). These unique fragments are flanked by inverted repeated DNA segments (R_L and R_S). The inverted repeat sequences of R_L are designated ab and a'b' and the repeats of RS a'c' and ca. The viral DNA is linear in the virion particle and whether it becomes circular

A.



B.

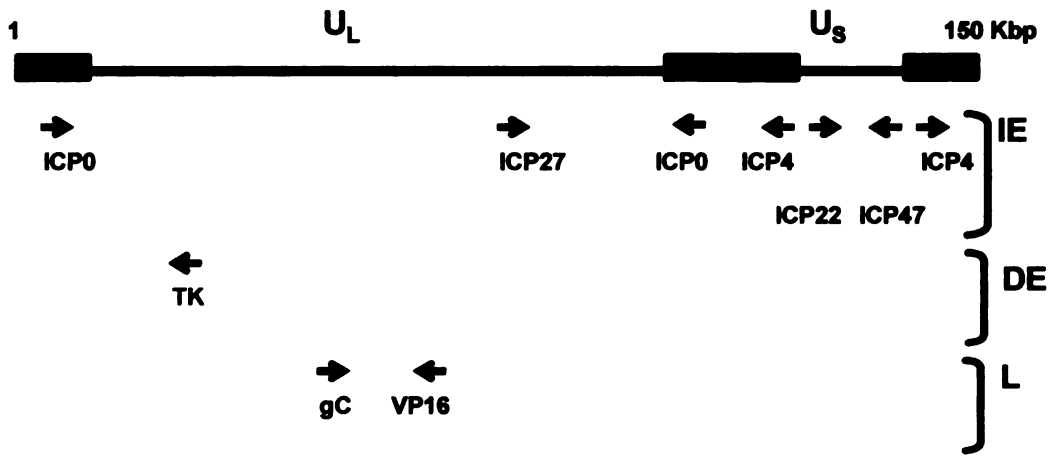


Figure 1. HSV-1 structure. (A) HSV-1 virion particle is formed by a double-stranded DNA genome enclosed in the capsid. The capsid is contained within a lipid envelope. The structure between the capsid and the envelope is referred as the tegument. (B) HSV-1 viral DNA is composed of two unique sequences (U_L and U_S) flanked by inverted repeats R_L and R_S (closed boxes). The approximate location and orientation of the IE genes and selected DE and L genes are shown by arrows.

upon entry to the nucleus remains controversial (114, 226, 252). The HSV-1 genome codes for approximately 80 polypeptides expressed in a temporal cascade during lytic infection [reviewed by (303)]. The viral DNA stays episomal and seems to be primarily non-nucleosomal during lytic infection. Nuclease assays have shown that little or none of the viral DNA delivered to infected cells was digested to fragment sizes consistent with nucleosomes (166, 167, 205). Electron microscopy studies showed the accumulation of non-nucleosomal DNA in infected cells (206). Viral DNA was localized to an interchromosomal space that excludes cellular chromatin (233) and does not incorporate histone H2B (203). Moreover, the viral DNA in the virion particle is not associated with histones but with polyamines spermidine and spermine (82, 225)

This mostly non-nucleosomal character of the DNA is restricted to lytic infection since during latent infections herpes simplex resides as a episomal circular DNA associated with histones (60). A large part of the evidence indicating HSV-1 as non-nucleosomal has been obtained at later times during infection, reflecting the nucleosomal status of the progeny or newly synthesized DNA.

1.2.2.3. Cascade of viral gene expression.

The temporal cascade of viral gene expression can be broadly divided into three main classes: immediate early (IE), delayed-early (DE) and late (L) genes. The IE genes namely ICP0, ICP4, ICP22, ICP27 and ICP47 were initially identified as viral genes

expressed upon infection in the absence of *de novo* protein synthesis [reviewed by (303)]. ICP4 is essential for virus replication in experimental conditions and is required for the efficient expression of DE and L genes. The ICP4 activation domain and DNA-binding domain have been mapped, however no clear consensus binding site has been identified on DE and L gene promoters. The ICP4 activation domain can interact with general transcription factor TFIID providing an explicit mechanism by which ICP4 might be helping the recruitment of the preinitiation complex at viral promoters (40, 92).

ICP0 is known to transactivate viral and cellular gene promoters. No *cis* element has been identified as responsible for the recruitment of this protein to target promoters. ICP0 is a RING-finger containing protein and possesses ubiquitin conjugating enzyme activity (E3)(303). During infection, ICP0 is required for the disassembly of nuclear domain ND10, a process that involves the degradation of specific proteins such as PML and Sp100 through the ubiquitin-proteasome system. These observations connect the E3 ligase activity of ICP0 with a known early event in viral infection requiring the Ub-proteasome pathway (94). The increasing evidence connecting transcriptional activation with the Ub-proteasome pathway in eukaryotes and the known role of certain E3 ligases as coactivators suggest that ICP0 might be a coactivator during viral gene expression.

ICP27 is another IE gene essential for viral replication. Current evidence indicates that ICP27 regulates gene expression at the post-transcriptional level. ICP27 contains an RNA binding domain and has been shown to regulate 3' RNA processing, inhibit RNA splicing and stimulate the nuclear export of intronless viral mRNAs. The roles of ICP22

and ICP47 protein are by comparison less understood. ICP22 is involved in the efficient expression of a subset of late genes and it is known to alter the phosphorylated status of RNA Pol II. ICP47 is known to inhibit the process of presenting antigenic peptides on the cell surface [reviewed by (94, 303)]. The IE promoters are the most complex herpes gene promoters in terms of *cis* elements that are required for proper temporal expression. Each of the IE promoters contains a different arrangement of VP16 responsive *cis* elements (TAATGARAT), TATA box, and binding sites for cellular activators Sp1 and GABP (Figure 2).

The DE genes are the second group of genes to be expressed during lytic infection. Abundant levels of mRNA for DE genes are detected at 3-4 hours post-infection [reviewed by (303)]. The promoter region of the thymidine kinase (TK) gene, one of the prototypical DE genes, contains Sp1 binding sites, CAAT elements and a TATA box element (Figure 2). The expression of the DE genes requires IE proteins although the binding sites for viral activator such as ICP4 have not been identified.

The third group of viral genes to be expressed are the L genes. This group can be subdivided into leaky-late and true-late genes depending on whether viral DNA replication is absolutely required for their expression. True-late gene promoters usually contain a TATA box and Initiator (Inr) element and sometimes downstream promoter elements (Figure 2). Leaky-late genes contain additional *cis* elements upstream of the core promoter. VP16 is an example of leaky-late genes whereas gC is a true-late gene. L gene expression is readily detected about 6-8 hours post infection. The mechanisms

linking DNA replication with late gene expression are unknown. The different promoter architecture of viral genes may result in the differential requirement of cofactors that are necessary for viral gene expression. The increasing simplicity of viral promoter from IE to L suggests that fewer transcription factors may be necessary later in infection in order to activate the expression of viral genes.

1.2.3. Activation of viral IE genes during infection.

1.2.3.1. Virion protein 16.

The virion protein 16 (VP16) is part of the tegument in the virion particle and is present at an estimated number of 1000-1500 copies per virion (102, 266). VP16 translocates to the nucleus by uncharacterized mechanisms where it activates the transcription of the IE genes. The activation of the IE genes triggers the cascade of viral gene expression resulting in lytic infection. VP16 binds the IE promoter in a complex with two cellular proteins, Oct-1 and HCF-1, at the TATGARAT elements present at the IE promoters. This ternary complex formed at the IE gene promoters is referred as the VP16 induced complex (VIC) (147, 229, 267, 316).

VP16 does not have a DNA binding domain and does not bind DNA in a sequence-specific manner. However, basic residues on the surface of VP16 apparently make DNA contacts in the context of the VP16 induced complex (10). VP16 is a 490 aa protein comprising a core domain (1-410 aa) and an acidic activation domain at the C-

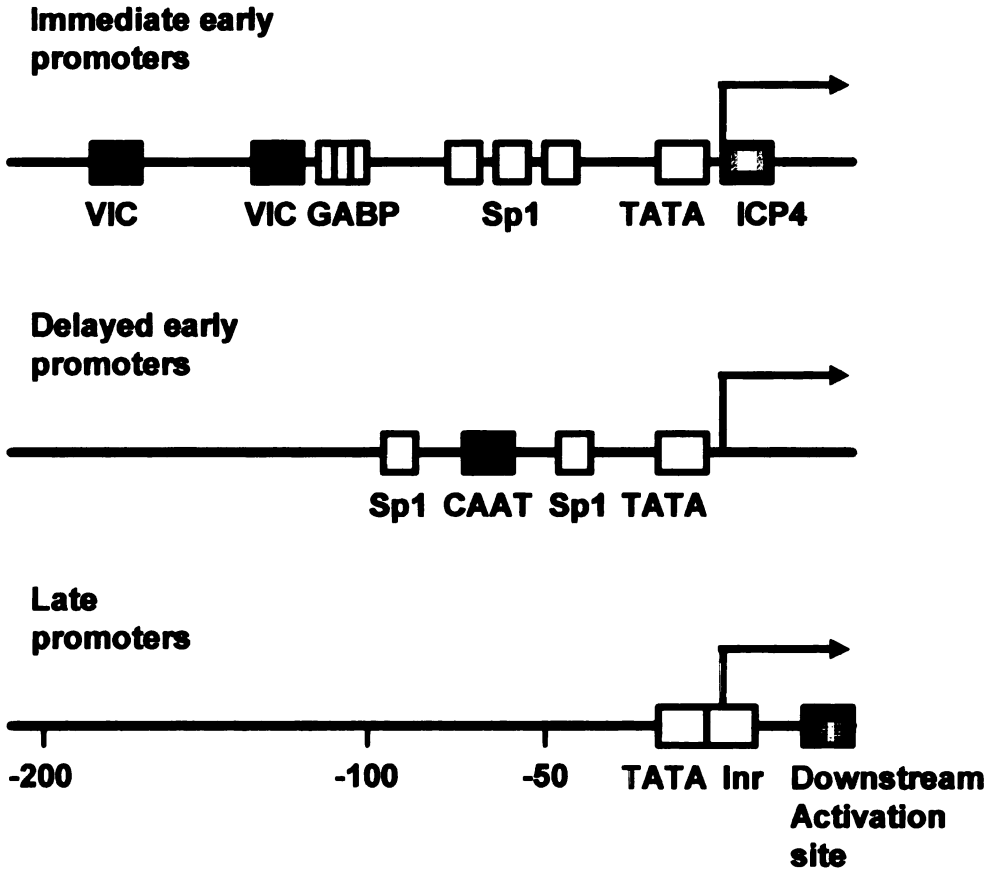


Figure 2. Schematic representation of three temporal classes of HSV-1 gene promoters. Immediate early (IE) gene promoters contain binding sites for the VP16-induced complex (VIC) shown in closed boxes. IE gene promoters also have a TATA box element and binding sites for cellular activators GABP and Sp1 and viral activators ICP4. Delayed early (DE) gene promoters contain typically contain a TATA box element and binding sites for cellular activators such as Sp1 binding sites and CAAT elements. Late (L) gene promoters usually contain only the core promoter elements TATA box, Initiator (Inr) and downstream activation site. Figure modified from J. Weir (2001) Gene 271: 117.

terminal end (aa 413-490). The core domain is involve in the interaction with HCF-1 and Oct-1 in the VIC and the activation domain of VP16 is required for the efficient activation of the IE genes both in transfection experiments and during HSV-1 infection (1, 276, 283, 295).

A virus carrying a 12-base pair DNA insertion in the VP16 gene shows reduced levels of IE expression and results in a high particle to plaque forming unit ratio at low multiplicity of infection (1). This DNA insertion results in additional 4 amino acids in the VP16 core domain and abolishes the interaction with Oct-1 *in vitro* (1). The 8MA HSV-1 virus strain with the VP16 gene replaced by the Lac-Z gene is not viable and must be grown on a complementary cell line expressing VP16. In the absence of VP16 delivered in *trans*, the 8MA virus does not replicate, but shows the accumulation of immature virion particles indicating a role of VP16 in virion assembly and maturation (300). A viral strain carrying temperature sensitive mutations on VP16 gene (C78A, C102A and C176A) also resulted in low yield of infectious particles at non-permissive temperature apparently due to problem in virion maturation (228). These experiments revealed that VP16 also plays a role in virion assembly and maturation (300). The HSV-1 viral strain RP5 contains a deletion in the VP16 activation domain and shows reduced and altered levels of IE gene expression and high ratio of particle to plaque forming unit (263, 276, 323). Collectively, this evidence indicates that VP16 is required for the efficient activation of IE genes early in infection as well as for the correct virion assembly during late times of infection.

1.2.3.2. VP16 induced complex.

Oct-1 confers most of DNA binding capability to the VP16 induced complex. The Oct-1 DNA binding is formed by a POU-specific domain and POU-homeo domain that binds the TAATGARAT element on IE gene promoters [reviewed by (313)]. This bipartite DNA binding domain not only is present in Oct-1 but also in other transcription factors including Pit-1, Oct-2 and Unc-86 and thus its denomination as the POU domain. The POU-homeo domain of Oct-1 also interacts with VP16, helping in the formation of the VIC at the responsive elements present in the IE gene promoter (267).

Host cell factor 1 (HCF-1) was identified as a VP16 interacting protein and is required for the stability of the VP16 induced complex (80). HCF-1 is a 2035 aa protein cleaved post-translationally into two subunits ($HCF\text{-}1_N$ and $HCF\text{-}1_C$) that remain associated through non-covalent interactions (148, 308). Several domains have been mapped in HCF-1 including the Kelch domain required for VP16/Oct-1 interaction, SAS domains (self association sequences), a basic and acidic region and an activation domain that cooperates with VP16 to activate transcription (179, 313). HCF-1 has been shown to interact with several other protein including cellular activators GABP, Sp1 and LZIP and with coactivator proteins. The basic domain of HCF-1 interacts with Sin3 histone deacetylase complex and the Kelch domain with Set1/Asha histone methyltransferase complex (314). Besides its role in IE gene expression during infection, HCF-1 seems to be involved in promoting cell growth and division (88, 313). The molecular mechanism involved in controlling cell growth might include HCF-1 as coactivator of cellular

activators LZIP and GABP, and as corepressor of the cell cycle arrest inducer Miz-1 [reviewed by (313)].

1.2.4. VP16 as a model transcriptional activation domain.

The activation domain of VP16 (VP16AD) has been extensively studied as a model of acidic transcriptional activators, usually fused to a heterologous DNA-binding domain (e.g. Gal4-VP16AD). Different studies have shown the interaction of VP16AD with general transcription factors, including TFIIA, TFIIB, TFIIH and TBP. This suggests that VP16AD may activate transcription by increasing the affinity of RNA Pol II to a particular promoter through its direct interaction with general transcription factors (84, 111, 140, 213, 260, 315). Consistent with this model, *in vitro* experiments have demonstrated the ability of VP16AD to promote the formation of the ternary complex formed by TFIIA, TFIID, and TATA box (141).

VP16AD can also interact with coactivator, adaptor and mediator proteins. Mutation of the Ada2 gene relieves the toxicity produced by overexpression of Gal4-VP16AD in yeast, suggesting functional interaction between VP16AD and the yeast HAT-containing coactivator complexes ADA/SAGA (19). Moreover, the VP16AD can interact physically and functionally with SAGA/ADA complex *in vitro* (98, 291). Ada2 protein and more recently Tra1, components of the HAT-containing coactivator complex SAGA/ADA and NuA4, have been implicated as direct VP16AD targets (15, 28).

VP16AD also interacts physically and/or functionally with another yeast HAT-containing coactivator NuA4 complex (285, 291).

In mammalian cells, Gal4-VP16AD interacts with two highly related HAT coactivators CBP and p300. *In vitro*, Gal4-VP16 requires p300 to activate transcription from chromatin templates and in transfection experiments Gal4-VP16 activity is augmented by increasing amounts of CBP and p300 encoding plasmids (146, 154, 296). Moreover, GST pull-down assays have shown the direct interaction of VP16AD with CBP and p300 (296). The activity of Gal4-VP16AD in transfection experiments is also augmented by the human homologues of yGCN5 (hGCN5 and PCAF) and yADA2 proteins (37, 296). Moreover, targeting the VP16AD to an heterologous locus in mammalian cells result in the recruitment of CBP, p300, PCAF and GCN5 (195).

VP16AD also interacts with TAF9, another type of coactivator protein present in TFIID and SAGA complexes (140, 284). Another type of coactivators are the ATP-dependent chromatin remodeling complexes. The Gal4- VP16AD also have been shown to interact *in vitro* with the yeast SWI/SNF complex (97, 214) and *in vivo* LexA-VP16AD recruits mammalian SWI/SNF to an heterologous promoter integrated into condensed chromatin (195).

The mediator complex also has been proposed as the direct target of VP16AD in the cellular transcriptional machinery. Trap80 (MED17) component of the mediator-like TRAP/SMCC complex was the first mediator subunit identified as VP16AD target by

testing a limited mediator subunit set using GST pull-down assays (112). Recently, more extensive analyses of VP16AD interactions with the mediator complex identified the ARC92 (MED25) subunit as direct target of VP16 in the mediator complex (201, 322).

The VP16AD can be divided into two sub-domains each capable of activating transcription when artificially targeted to a promoter. These two sub-domain, namely VP16N (aa 410 to 456) and VP16C (aa 450 to 490) seems to utilize different mechanism to activate transcription as suggested by their functional and structural differences. VP16C but not VP16N can interact with TFIIA and TAF9 in *in vitro* assays (84, 140) and can stimulate TFIIA-TFIID complex assembly (142). Moreover, the different patterns of acidic amino acids surrounding the critical phenylalanines residues of VP16N and VP16C are consistent with distinct mechanism of action (54, 238, 272). The presence of these two sub-domains in the VP16AD might be in part responsible for the ability of VP16 to interact with multiple targets.

The large list of putative targets for the VP16AD raises the question of which interactions are relevant or most important *in vivo*. The association of chromatin-modifying complexes with VP16AD in heterologous systems is particularly intriguing since the viral DNA does not seem to be packaged in nucleosomes during lytic infection (for references see section 1.2.2.). Thus, there would seem to be no reason for VP16 to recruit an ATP-dependent chromatin remodeling complex such as SWI/SNF or a HAT-containing protein complex such as human GCN5 to a non-nucleosomal IE promoter. Several alternatives may explain these apparently contradictory observations. VP16

might require chromatin-modifying coactivators during activation of IE genes to serve roles other than chromatin remodeling. Perhaps some of these coactivators are essential structural components of the preinitiation complex. Alternatively, some of the enzymatic activities of these chromatin remodeling complexes could use substrates other than histones. For instance, several HATs are known to acetylate general transcription factors, activators or HMG proteins. Thus in some cases the relevant *in vivo* target for the acetylase activity may not be histones.

The association of VP16 with chromatin-modifying coactivators might indicate that histones are associated with viral DNA and thus chromatin remodeling might play a role during transcriptional activation of viral genes. The failure to effectively detect nucleosomes on viral DNA might be a result of low levels of histone occupancy on viral DNA or from the difficulties of detecting a small fraction of the incoming DNA being associated with histones.

Another alternative is that VP16 might not require chromatin-remodeling coactivators to activate transcription from a non-nucleosomal viral DNA template. Then the interactions of VP16 with chromatin remodeling complexes observed in heterologous or *in vitro* might not reflect the *in vivo* mechanisms used by VP16 during infections.

1.3. Main hypothesis and experimental setting.

The main objective of this doctoral thesis research is to explore the role of chromatin modifying coactivator proteins in activation of IE genes by VP16 during herpes simplex virus infection. To that end, chromatin immunoprecipitation assays were adapted to study the recruitment of cofactors to the IE gene promoters during herpes simplex infection of cultured cells. Using this approach, the presence of selected activators, general transcription factors and coactivators proteins on IE gene promoters was tested. The dependence on the activation domain of VP16 for the recruitment of different transcription factors was tested by using the RP5 viral strain lacking the VP16 activation domain. The association of histones (histone H3) with incoming viral DNA was also tested using ChIP assays. The functional role of selected chromatin remodeling complexes was explored by trying to alter their expression levels with overexpression and RNAi strategies followed by HSV infections and RT-PCR to quantify IE gene expression. The results of these experiments indicate that, contrary to prevalent models, chromatin transactions might play an important role during the expression of viral genes at early times during infection.

Chapter II

Recruitment of transcription factors to IE viral promoters during HSV-1 infection[2]

2.1. Introduction.

The activation domain of VP16 (VP16 AD) from herpes simplex virus type I (HSV-1) has been widely used as a model for the study of transcriptional activation in eukaryotes. During infection, VP16 triggers the cascade of viral gene expression by activating transcription of the viral immediate-early (IE) genes (303). VP16 forms a DNA-binding complex with the cellular proteins Oct-1 and HCF-1 at specific *cis* elements present in the IE gene promoters (147, 229, 267, 316). The potent activation domain of VP16 (52, 283), often artificially fused to a heterologous DNA-binding domain (250), can activate transcription in a wide range of organisms including yeast, insects, plants and mammals (19, 250, 286, 306) indicating that mechanisms of transcriptional activation are broadly conserved through evolution.

Interactions of the VP16 AD with general transcription factors (GTFs) including TFIIB, TFIIH, TBP and TBP-associated factors (TAFs) suggest that the VP16 AD might activate transcription by stimulating the assembly of an RNA polymerase II (RNA Pol II) pre-initiation complex (84, 111, 140, 173, 284, 315). Consistent with this model, *in vitro* experiments have demonstrated the ability of the VP16 AD to promote the formation of the ternary complex formed by TFIIA, TFIID, and TATA box DNA (142). Other

[2] Part of this chapter was published in "Herrera FJ and Triezenberg SJ (2004). VP16-dependent association of chromatin-modifying coactivators and underrepresentation of histones at immediate-early gene promoters during herpes simplex virus infection. Journal of Virology 78(18): 9689-9696".

potential targets of the VP16 AD include chromatin-remodeling coactivator or adaptor proteins. The VP16 AD can interact physically or functionally with histone acetyltransferase (HAT) proteins including the yeast ADA/SAGA and NuA4 complexes and the human coactivators CBP, p300 and hGCN5 complex (15, 19, 146, 154, 195, 285, 296). Acetylation of nucleosomal histones near gene promoters is generally correlated with increased transcription (110, 268). In particular, CBP and p300 are very similar and ubiquitously expressed coactivators involved in cell cycle control, differentiation and apoptosis, with histone acetyltransferase (HAT) and factor acetyltransferase (FAT) activities (293). Despite the sequence similarity of CBP and p300, gene deletion experiments suggest that the two proteins serve nonredundant but overlapping functions (131, 237, 277, 325).

The VP16 AD can also interact *in vitro* with the ATP-dependent chromatin remodeling complex SWI/SNF (97, 214). This protein complex can relocate nucleosomes, alter DNAase accessibility patterns and alter the superhelicity of DNA *in vitro* [reviewed by (212)]. The mechanism by which SWI/SNF acts in transcriptional activation requires the DNA-dependent ATPase activity of the largest subunit known as SWI2/SNF2. The repositioning of nucleosomes in *cis* (through tracking or sliding of nucleosomes along DNA) or the transfer of histone octamers to different DNA segments in *trans* might alleviate chromatin-mediated repression and therefore might contribute to transcriptional activation (224, 304). BRM and BRG-1 (BRM related gene-1) are the mammalian homologs of the ATPase subunit of yeast SWI/SNF complex. These ATPases have high sequence similarity but play different biological roles as indicated by

the phenotypes of mutant mice (33, 242), and are differentially recruited to various gene promoters during cellular proliferation and differentiation (123).

The large list of putative targets for the VP16 activation domain raises the question of which are more relevant for the *in vivo* function of VP16. The main purpose of this thesis work is to explore the *in vivo* function of some of the VP16 putative targets during the activation of HSV-1 viral genes. To that end, we developed chromatin immunoprecipitation assays (ChIP assays) to test for the presence of VP16 and Oct-1 and for selected general transcription factors at the IE gene promoters during HSV-1 infection of cultured cells. The dependence on the activation domain for the recruitment of these factors was tested using the RP5 viral strain lacking the VP16AD.

The association of chromatin-modifying coactivators with the VP16 AD in heterologous systems is particularly intriguing since previous evidence indicates that HSV-1 DNA is not packaged in nucleosomes during lytic infection (for further details see Chapter 3). Thus, the purpose for recruiting chromatin-remodeling coactivators to viral IE gene promoters remains enigmatic. Given that the association of VP16 with such coactivators has arisen solely from artificial or heterologous experimental contexts, we tested whether the chromatin-modifying coactivator proteins were associated with viral IE gene promoters during infection.

Using ChIP assays, we found that HATs (p300 and CBP) and also ATP-dependent chromatin remodeling enzymes (BRG-1 and BRM) were present at viral IE promoters. The recruitment of the HATs and general transcription factors (TBP and RNA

Pol II) was fully dependent on the VP16 activation domain, whereas the recruitment of the SWI/SNF components was only partially dependent on VP16. The ARC92 mediator subunit was also detected at one IE gene promoter (ICP27) and its efficient recruitment was also dependent on VP16AD. These results uncover some of the transcription factors recruited to IE gene promoters during infection and suggest that chromatin-modifying coactivators are among the proteins involved in the activation of these genes.

2.2. Materials and Methods

2.2.1. Cells and viruses.

HeLa and Vero cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Stocks of wildtype HSV-1 (strain KOS) and the VP16 truncation mutant RP5 (276) were prepared in Vero cells and titered by plaque assays. For gene expression and ChIP assays, HeLa cells (approx. 3 x 10^7) were infected with KOS at a multiplicity of infection ranging from 1 to 10 pfu/cell in different experiments. Infections with RP5 were performed using comparable virion numbers (approximately 100-fold lower pfu than in KOS infections). In some experiments, cycloheximide (60 μg/ml) was added to the medium for two hours prior to and during infection to inhibit protein translation.

2.2.2. Chemical crosslinking and immunoprecipitation assays.

To crosslink protein-DNA complexes, formaldehyde was added to the medium overlaying infected cells to a final concentration of 1% for 15 min. The crosslinking reactions were quenched by adding glycine to a final concentration of 125 mM. Cells were collected, resuspended in a hypotonic buffer, and dounce homogenized to release nuclei which were collected by centrifugation. Nuclear pellets were sonicated to obtain DNA fragments with an average length of 300-400 basepairs. Aliquots corresponding to 10% of the input material were reserved.

Prior to immunoprecipitation, samples were precleared using protein-G agarose beads (Upstate, Charlottesville VA). Immunoprecipitations were performed using specific antibodies at concentrations ranging from 10 to 50 μg/ml at 4°C overnight. Antigen-antibody complexes were precipitated using protein-G agarose beads. The beads were washed extensively before protein-DNA complexes were eluted using 100 μl of 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS for 20 min at 65 °C. A second eluate, using 150 μl TE with 0.67% SDS, was added to the first. The combined eluates are referred to as the pellet sample. Crosslinks were reversed by adding NaCl to 200 mM and 10 μg of RNAase A and incubating at 65°C overnight. After ethanol precipitation, samples were digested with proteinase K (Boehringer) at 42°C for 2 hours and then extracted with phenol/chloroform. After another ethanol precipitation, DNA samples were resuspended in 75 μl TE pH 8.0.

ChIP assays were performed using antibodies or antisera directed against VP16 (283), Oct-1 (a gift from W. Herr, Cold Spring Harbor Laboratory), TBP (a gift from R. W. Henry, Michigan State University), RNA polymerase II (8WG16, Covance), CBP (A-22, Santa Cruz Biotechnology), p300 (N-15, Santa Cruz Biotechnology), Brm (N-19, Santa Cruz Biotechnology), BRG1 (H-88, Santa Cruz Biotechnology) or ARC92 (a gift from A. Näärs, Harvard University). Control IPs using pre-immune sera exhibited essentially the same results as mock IPs using no antibody (data not shown).

Semi-quantitative polymerase chain reactions (PCRs) were performed to detect specific viral or cellular gene fragments in the immunoprecipitated samples. These fragments included the promoters of the HSV IE genes (ICP0, ICP27, and ICP4); the coding region of the ICP27 gene; the promoters of viral DE (TK) and L (VP16, glycoprotein C) genes; and the promoters of cellular genes U3 snRNA and IFN-β (See details in appendix A). Parallel PCRs were routinely performed on serial dilutions of input samples (typically corresponding to 0.5%, 0.1%, and 0.02% of the total material) to confirm that observed signals were within linear range of the assay and were comparable between different set of primers. Standard PCR conditions included 0.25 μM of each primer, 2.5 U *Taq* DNA polymerase (Invitrogen), 0.1 mM each deoxynucleotide triphosphate, 2 mM $MgCl_2$, 10% Enhancer solution (Invitrogen), with incubation at 95°C for 5 min followed by 30 to 35 cycles of 95°C for 30 sec, 65°C for 30 sec and 72°C for 1 min and ending with 5 min at 72°C. The Enhancer solution was omitted from reactions amplifying the ICP27 ORF, U3 snRNA promoter, and IFN-β promoter fragments. Annealing of primers for amplification of the IFN-β promoter and the GAPDH ORF was

performed at 55 °C. Negative images of ethidium bromide-stained gels of representative experiments are shown.

Quantitative real time PCR (qPCR) analysis was also used to quantify specific viral and cellular DNA fragments in the immunoprecipitated samples. qPCR were performed on a ABI Prism 7700 Sequence Detection System using SYBR Green Core Reagents (Applied Biosystems). Standard PCR conditions included 3 μL of template (IP sample or input dilution), 0.25 μM of each primer, 0.75 U Taq DNA polymerase (Applied Biosystems) and 2-3 mM $MgCl_2$ in 30 μL final volume. Parallel PCRs were routinely performed on serial dilutions of input samples (3%, 1%, 0.3%, 0.1%, 0.03%, 0.01% of the input material) to obtain standard curves for each PCR. The standard curves were used to determine the percentage of input present in each IP sample. The enrichment of a particular promoter over background levels (No Ab sample) was determined using $2^{-\Delta Ct}$ (see appendix A for further details). Error bars shown in figures represent range of the technical duplicates.

2.2.3. Gene expression assays.

The steady-state levels of viral mRNAs (ICP27, TK, and VP16) expressed in infected HeLa cells were determined using reverse-transcriptase PCR assays. Total RNA was isolated from infected cells using TRI-Reagent following the directions provided by the manufacturer (Molecular Research Center). cDNA was prepared using 1 μg total RNA and a randomly-primed reverse transcription system (Promega). Viral gene cDNAs

encoding ICP27, TK and VP16 were amplified using PCR conditions described previously (44) and analyzed on ethidium bromide stained agarose gels. Negative images are shown.

cDNA levels were also analyzed using quantitative real time PCR (qPCR). Appendix B describes in detail the protocol used in reverse transcriptase qPCR analysis (qRT-PCR). In brief, viral gene fragments were amplified using SYBR Green Core reagents and the ABI Prism 7700 Sequence Detection System (Applied Biosystems). PCR conditions included 0.1-0.25 μM each primer and 2-3 $MgCl_2$ in 30 μL final volume. Relative levels of viral gene expression were obtained with the $2^{-\Delta\Delta Ct}$ method (175) using wild type infections as standard and 18S rRNA as endogenous control. Bars represent the average expression levels of 3 independent infections each measured in duplicate. Errors bars indicate the range of the biological triplicates.

2.2.3.1. Analysis of viral gene expression in cells expressing a dominant negative form of ARC92.

The VP16 binding domain (VBD) on ARC92 mediator subunit (MED25) resides between amino acids 402 and 590 and shows a dominant negative effect on Gal4-VP16AD activation of plasmid-based reporter gene (322). To explore the role of mediator complex and in particular the role of ARC92 subunit we tested whether ARC92 VBD interferes with IE gene expression during infection. Approximately 4 x 10^6 HeLa cells were transfected with a plasmid expressing the ARC92 VBD or the empty vector as

a control. To select the cells that received the plasmid, the samples were co-transfected with pBABE plasmid encoding a puromycin resistant gene and grown on media containing puromycin 2 μg/ml. Two days after initial transfection of cells, control plates (mock transfections) did not show cell survivors indicating that the puromycin treatment efficiently selected transfected cells. This selection of transfected cells is of importance since subsequently all cells are infected with HSV-1. Low levels of transfections might reduce any potential effect on viral gene expression of the peptide delivered by transfected plasmids.

The selected cells from both samples (empty vector and ARC92 VBD) were collected and seeded to grow overnight on media without puromycin. Infections were performed in duplicate for each condition (empty vector or ARC92 VBD) with wild type HSV-1 viral strain KOS at MOI of 1 pfu/cell. Total RNA and proteins were isolated at two hours post-infection. To quantify viral gene expression, RNA was reversed transcribed and used as a template for quantitative PCR (qRT-PCR). cDNA levels were obtain using $2^{-\Delta\Delta Ct}$ analysis and 18S rRNA as endogenous control (see appendix B for details).

2.3. Results.

2.3.1. VP16 associates with IE gene promoters with or without its transcriptional activation domain.

The VP16 protein comprises a core domain (encompassing amino acids 1-410) and a transcriptional activation domain (amino acids 413-490) (Figure 3A.). The core domain interacts with two cellular proteins, Oct-1 and HCF-1, to form a DNA-binding complex at specific *cis* regulatory elements in the viral IE gene promoters (147, 229, 267, 316). The VP16 AD is required for efficient transcription of viral IE genes both in transfection experiments and during HSV-1 infection (276, 283, 295). Infection of HeLa cells by RP5 (Figure 3B), a mutant viral strain lacking sequences encoding the VP16 AD results in little IE gene expression (276, 323).

Chemical crosslinking and immunoprecipitation (ChIP) assays were adapted to detect the association of specific proteins with viral promoters during lytic infection by HSV-1. HeLa cells were fixed with formaldehyde at 2 hours post-infection, when transcription of IE genes is robust. Sonicated nuclear lysates were immunoprecipitated with antisera directed against VP16 or Oct-1, or with protein-G agarose beads alone (no antibody). PCR products representing the promoters of the IE genes ICP0 and ICP4 were more abundant in reactions using the VP16 and the Oct-1 IPs than in the samples lacking primary antibodies (Figure 4), fulfilling the expectation that these proteins are associated with IE promoters during infection. Little or no PCR product corresponding to the VP16 promoter was detected in the VP16 and Oct-1 IPs, as expected for a late gene promoter. The cellular U3 snRNA gene contains an Oct-1 binding site and is activated upon HSV-1 infection in the absence of the *novo* protein synthesis and by VP16 delivered by transfection (133, 271).

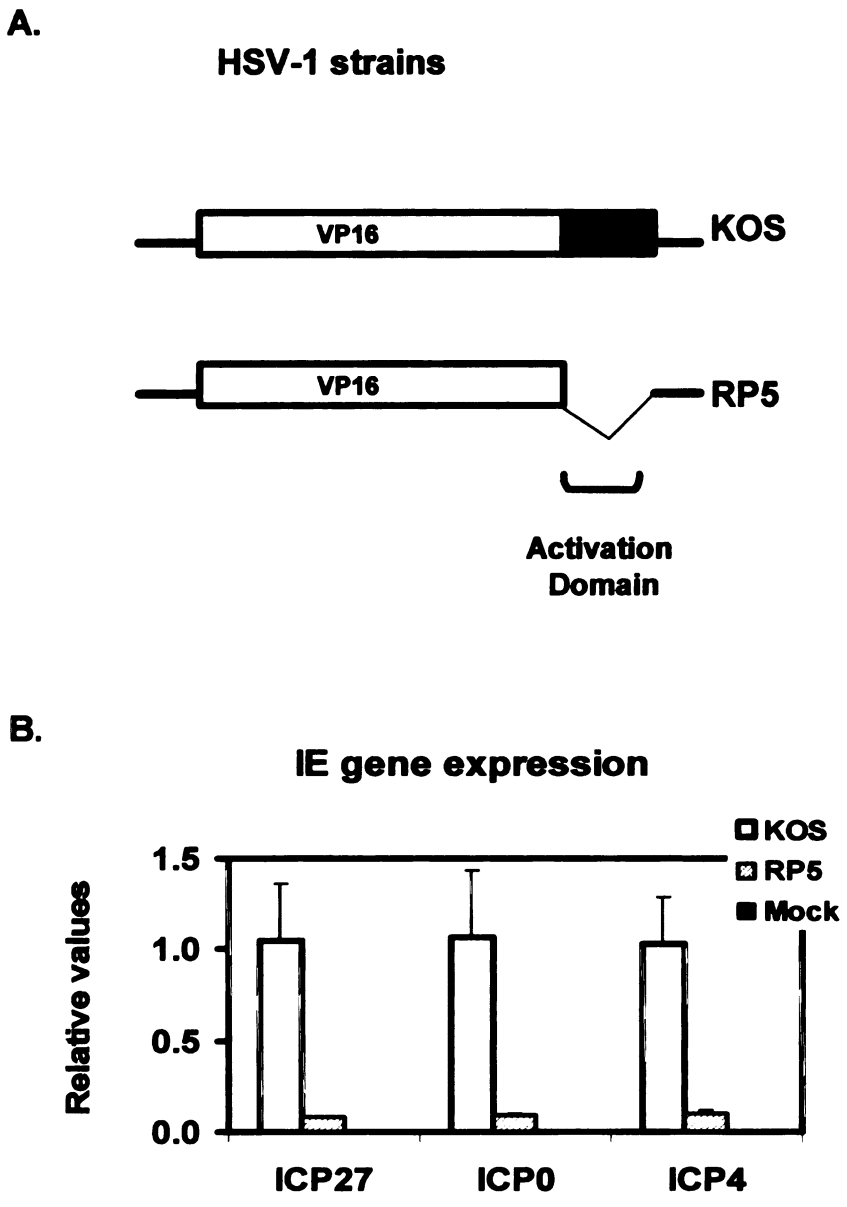


Figure 3. KOS and RP5 viral strains. (A) Schematic representation of the VP16 protein encoded by the HSV-1 strains KOS (wildtype) and RP5 (lacking sequences encoding the VP16 AD). (B) qRT-PCR analysis of steady-state mRNA levels of viral IE (ICP27, ICP0 and ICP4) genes in cells infected by KOS, RP5 or mock infected for 2 h. Relative values were obtained with $2^{-\Delta\Delta Ct}$ method using 18S rRNA as endogenous control. Errors bars indicate range of biological triplicates.

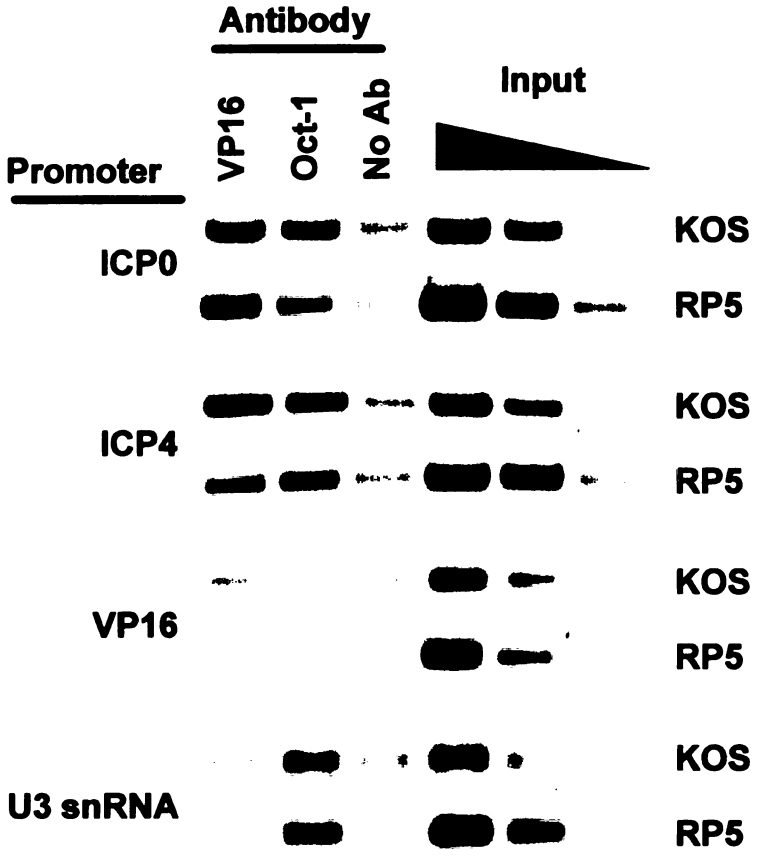


Figure 4. Detection of VP16 and Oct-1 at viral IE promoters during infection. Cross-linking and immunoprecipitation (ChIP) assays using antibodies against VP16 or Oct-1 in lysates of cells infected for 2 h by KOS or RP5. Controls include precipitations performed without specific antisera (No Ab) and aliquots of samples prior to precipitation (0.5%, 0.1%, 0.02% input). Samples were analyzed using PCR detecting viral IE gene promoters (ICP0, ICP4), a viral L gene promoter (VP16) and the promoter of the cellular U3snRNA gene. Negative images of ethidium bromide-stained gels are shown.

The presence of the octamer sequence at the U3 snRNA promoter suggests that VP16 might bind to the U3 snRNA promoter through interactions with Oct-1. Thus the presence of Oct-1 and VP16 at the U3 snRNA during infection was tested using ChIP assays. Figure 4 shows that anti-Oct-1, but not anti-VP16, immunoprecipitated the promoter region of the cellular U3 snRNA gene. This result does not support the hypothesis that VP16 directly activates the U3 snRNA.

To test whether the VP16 AD was required for association with the IE promoters, parallel ChIP assays were performed using cells infected with the VP16 truncation mutant RP5. The binding of VP16 and of Oct-1 to the IE promoters was not altered by the absence of the VP16 AD (Figure 4). The comparable signals arising from the input samples of the two infections ensures that comparable amounts of viral DNA were present in the nuclear extracts. The association of Oct-1 with the U3 snRNA promoter was likewise unaffected. Therefore, the reduced expression of IE genes in RP5 infections does not result from a failure of the activator to associate with its target genes, but likely arises from a defect in transcriptional activation *per se*.

The DNA immunoprecipitated by anti-VP16 and anti-Oct-1 serum in ChIP assays of cells infected with wild type virus (KOS) was also analyzed using quantitative real time PCR (qPCR). The ChIP assays were performed as in figure 4 but the immunoprecipitated samples were analyzed using $2^{-\Delta Ct}$ analysis to quantify the differences between the IP samples and the "No Ab" sample (for details see appendix A). The enrichment of IE gene promoters ICP0 and ICP27 was detected using both

antibodies as observed previously on ethidium bromide-stained gels for ICP0 and ICP4 gene promoters (Figure 5). The VP16 promoter region was not significantly immunoprecipitated above background levels ("No Ab" sample) in either anti-VP16 or the anti-Oct-1 IP samples. As observed previously, the cellular U3 snRNA gene promoter was immunoprecipitated with antibodies against Oct-1 but not with antibodies against VP16.

Input dilutions were used in parallel PCR reactions during qPCR analysis to quantify the percentage of input present for a given viral DNA fragment in each IP sample (see appendix A for further details). ChIP assays using VP16 and Oct-1 typically resulted in the immunoprecipitation of approximately 0.2 - 0.1% of the input sample used in each IP (data not shown). The results obtained with quantitative real time PCR are consistent with the results obtained with the semi-quantitative analysis using ethidium bromide-stained gels. Thus, these results confirmed that ChIP assays using antibodies against VP16 and Oct-1 result in the specific enrichment of IE gene promoters during early times of infection.

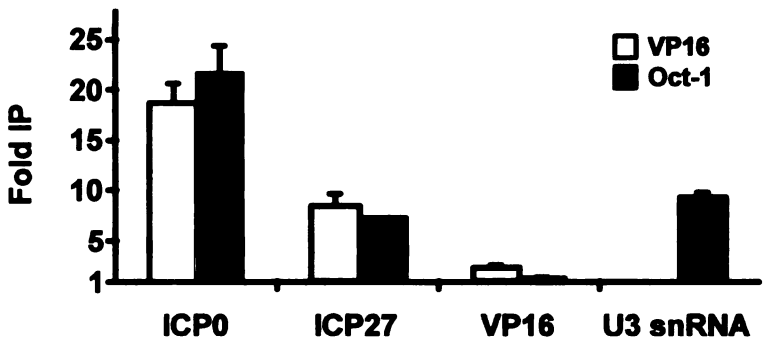


Figure 5. Detection of VP16 and Oct-1 at viral IE promoters during infection using ChIP followed by quantitative PCR analysis. ChIP assays were performed using antibodies against VP16 or Oct-1 in lysates of cells infected for 2 h by KOS. Samples were analyzed using real time PCR detecting viral IE gene promoters (ICP0, ICP27), a viral L gene promoter (VP16) and the promoter of the cellular U3snRNA gene. Relative IP values were obtained comparing values of immunoprecipitations samples with precipitations performed without specific antisera (No Ab) using $2^{-\Delta Ct}$ analysis. Error bars indicate the range of the technical duplicates for the qPCR analysis.

2.3.2. The VP16 AD is required to recruit general transcription factors to IE promoters during infection.

Transcription of HSV-1 genes depends on general transcription factors and RNA Pol II from the infected host cell (303). During infection of HeLa cells by wildtype virus (KOS), IE gene mRNAs were readily detected at 2 hpi (Figure 3B). In contrast, infection by RP5 (lacking the VP16 AD) resulted in a dramatic reduction in viral gene expression, consistent with prior reports (276, 323). To test whether this decreased expression corresponded to a failure to recruit the general transcription machinery to IE gene promoters, ChIP assays were performed using antibodies directed against the general transcription factor TBP and RNA Pol II. As shown in figure 6, PCR products corresponding to the promoters of the IE genes ICP0, ICP4 and ICP27 were readily detected in the TBP and RNA Pol II IPs from cells infected with wildtype virus at 2 hpi.

In contrast, these PCR products were not detected in parallel IPs from cells infected with RP5. The presence of the U3 snRNA promoter fragments in the IPs from both KOS- and RP5-infected cells demonstrates that the IP reactions were successful. We conclude that the recruitment of TBP and RNA Pol II (and also TFIIF, data not shown) to IE gene promoters require the VP16 AD, and that the lack of IE gene transcription observed in RP5 infections correlates with the absence of GTFs at those promoters.

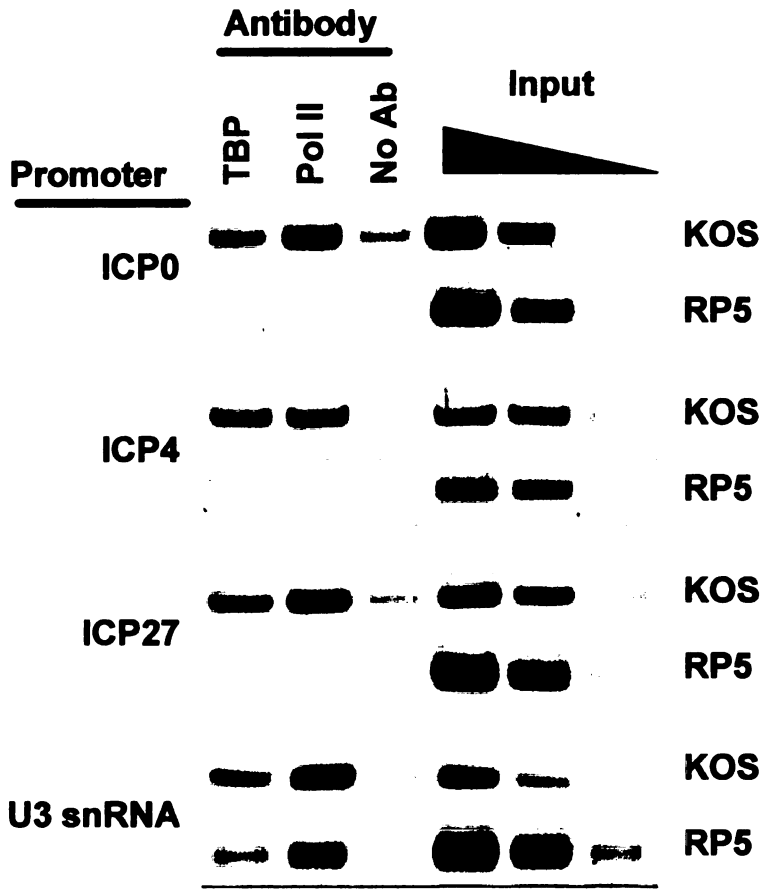


Figure 6. General transcription factors are recruited to IE promoters by the VP16 AD. ChIP assays using antibodies against TBP and Pol II in lysates of cells infected for 2 h with KOS or RP5. Immunoprecipitated samples were analyzed using PCR detecting viral IE gene promoters ICP0, ICP27, ICP4, and the cellular U3snRNA promoter. Controls include precipitations performed without specific antisera (No Ab) and aliquots of samples prior to precipitation (0.5%, 0.1%, 0.02% input). Negative images of ethidium bromide-stained gels are shown.

In the course of these experiments, we noted that DE and L gene promoters were also associated with RNA Pol II at 2 hpi (Figure 7B), even though transcription of these genes is not readily detected until 4 hpi (Figure 7A). Because IE gene products themselves are transcriptional regulatory proteins that can further stimulate expression of IE genes (as a positive feedback loop) as well as DE and L genes, the observation of TBP and RNA Pol II at the IE promoters may arise from the action of IE proteins rather than of VP16.

To test this hypothesis, cells were infected by KOS in the presence of cycloheximide to inhibit IE protein synthesis. This treatment effectively blocked the cascade of viral gene expression, as no DE or L gene mRNAs were detected at 2 or 4 hpi in the presence of cycloheximide (Figure 7A). ChIP assays of cycloheximide-treated cells detected RNA Pol II at the ICP0 promoter albeit to an apparently reduced level (Figure 7B). This result suggests that recruitment of RNA Pol II to IE promoters arises directly from VP16 activity and not by action of IE proteins themselves. In contrast, the association of RNA Pol II with DE and L genes (TK and VP16, respectively) was abolished in the presence of cycloheximide. Therefore, the presence of RNA Pol II at DE and L genes does indeed depend on IE protein synthesis, whereas its presence at IE gene promoters does not.

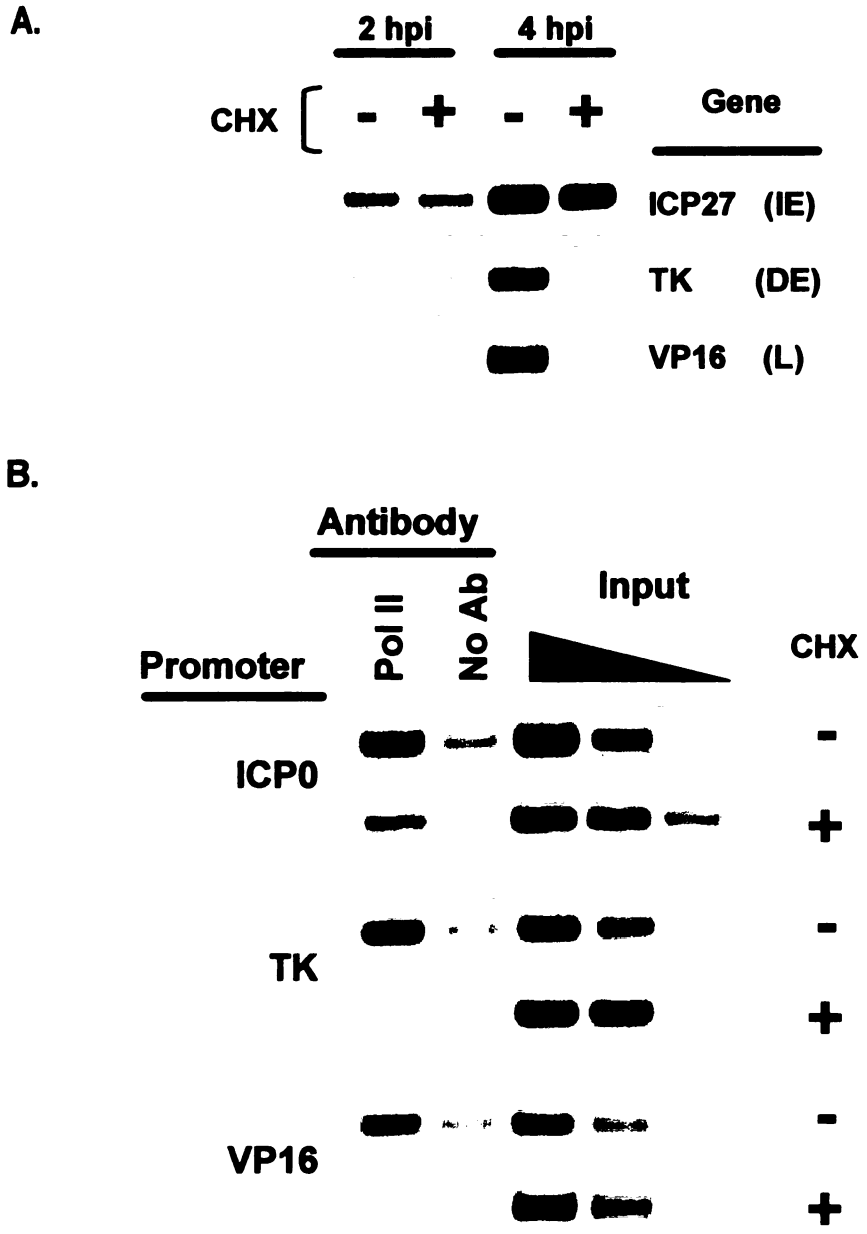


Figure 7. Recruitment of RNA Pol II at different classes of viral promoters. (A) RT-PCR analysis of steady-state mRNA levels of viral IE (ICP27), DE (TK) and L (VP16) in cells infected with KOS in the presence or in the absence of cycloheximide. (B) ChIP assay performed using antibodies against Pol II in lysates for KOS infections in the presence or absence of cycloheximide, using PCR to detect promoters of the viral genes ICP0, TK and VP16. Controls include precipitations performed without specific antisera (No Ab) and aliquots of samples prior to precipitation (0.5%, 0.1%, 0.02% input). Negative images of ethidium bromide-stained gels are shown.

2.3.3. The VP16 AD recruits chromatin-modifying coactivators to IE gene promoters during infection.

The association of chromatin-modifying coactivators with the VP16 AD in various heterologous systems led us to ask whether some of these factors are present on active IE gene promoters during HSV-1 infection. ChIP assays were performed on extracts of HeLa cells infected with KOS or RP5, using antibodies specific to the closely related histone acetyltransferases CBP and p300. In extracts from KOS-infected cells, antibodies directed against CBP or against p300 immunoprecipitated the promoters of the ICP0, ICP4 and ICP27 genes (Figure 8), demonstrating that these HATs are recruited to IE viral promoters during HSV infection. Interestingly, CBP was preferentially associated with the ICP0 and ICP4 gene promoters whereas p300 was preferentially associated with the ICP27 promoter. Therefore, these two highly related HATs can be differentially recruited to IE gene promoters, despite the similarities in the *cis* regulatory elements at those promoters.

During infection of HeLa cells by RP5 (i.e., in the absence of the VP16 AD), recruitment of CBP and p300 to viral IE promoters was drastically reduced (Figure 8). No specific PCR signal was detected for the ICP0 and ICP27 promoters in samples immunoprecipitated with either anti-CBP or anti-p300, and only a weak signal was detected for the ICP4 promoter in the anti-CBP sample. These results reveal that the VP16 AD is required for the efficient recruitment of CBP and p300 to HSV-1 IE promoters during infection.

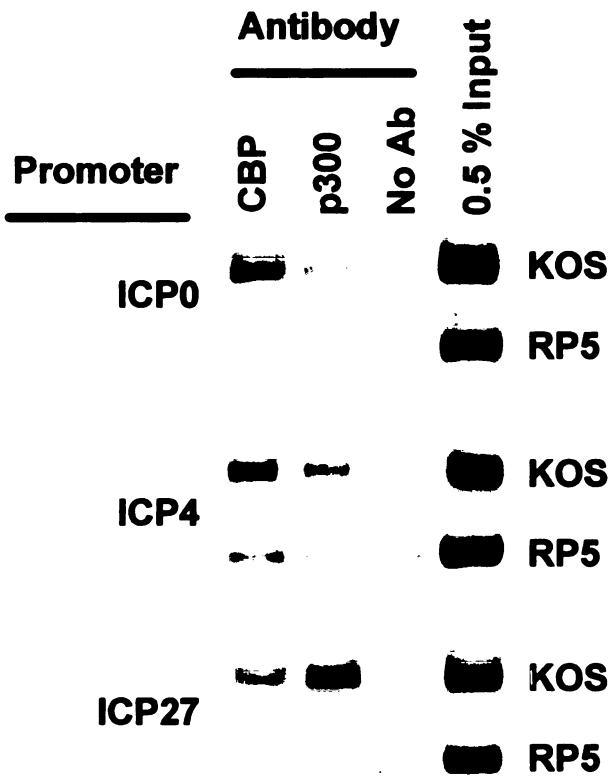


Figure 8. Recruitment of histone acetyl transferases CBP and p300 to IE promoters during HSV-1 infection. ChIP assays were performed using antibodies specific for CBP or p300 in lysates of cells infected with KOS or RP5 for 2 h. Immunoprecipitated samples were analyzed using PCR detecting viral IE gene promoters ICP0, ICP27, ICP4. Controls include precipitations performed without specific antisera (No Ab) and aliquots of samples prior to precipitation (0.5% input). Negative images of ethidium bromide-stained gels are shown.

We also tested for the presence of another class of coactivator complex, namely, the ATP-dependent chromatin remodeling complex SWI/SNF. BRM and BRG-1 are the ATPase subunits of two distinct human SWI/SNF complexes [reviewed by (212)]. ChIP assays of nuclear extracts from KOS-infected cells using antibodies against BRG-1 and BRM precipitated the promoters of the ICP0, ICP4 and ICP27 genes, indicating that the human SWI/SNF complexes are recruited to IE promoters during infection. Similar to the HAT complexes described above, BRG-1 and BRM showed different preferences for interacting with various IE promoters. The ICP0 and ICP4 promoter fragments were preferentially detected in the BRG-1 IP samples, whereas the ICP27 promoter fragment was preferentially detected in the BRM IP sample (Figure 9).

In the absence of the VP16 AD (i.e., in RP5 infection), the association of BRG-1 and BRM with the ICP0 promoter was lost. In contrast, BRG-1 (but not BRM) was still associated with the ICP4 promoter, and BRM (but not BRG-1) was still associated with the ICP27 promoter. Therefore, the ATP-dependent remodeling complexes are indeed recruited to viral DNA templates during lytic infection, and specific remodeling complexes preferentially associate with distinct IE gene promoters. Moreover, the VP16 AD is required or important for the association of these complexes with some promoters but not with others. This latter conclusion suggests that other activators present at IE promoters (for instance, Oct-1 or HCF-1) might contribute to the recruitment of BRG-1 or BRM.

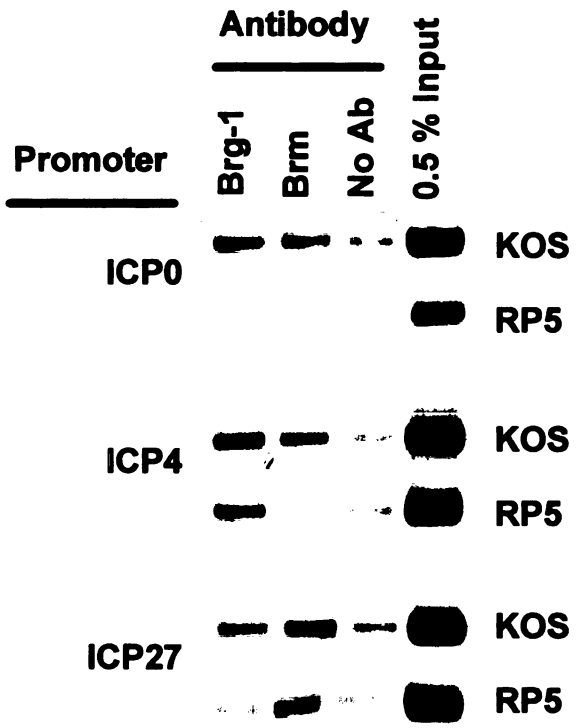


Figure 9. Recruitment of ATP-dependent chromatin remodeling complexes to IE promoters during HSV-1 infection. ChIP assays were performed using antibodies specific for Brg-1 or Brm in lysates of cells infected with KOS or RP5 for 2 h. Immunoprecipitated samples were analyzed using PCR detecting viral IE gene promoters ICP0, ICP27, ICP4. Controls include precipitations performed without specific antisera (No Ab) and aliquots of samples prior to precipitation (0.5% input). Negative images of ethidium bromide-stained gels are shown

The recruitment of HATs and SWI/SNF complexes to viral IE promoters was apparently the direct result of the VP16 AD and not a consequence of IE proteins themselves. In ChIP experiments performed on KOS-infected cells in the presence of cycloheximide, the ICP0 and ICP27 promoter fragments were present in the IP pellets obtained using antibodies recognizing CBP, p300, BRM, or BRG1 (data not shown), indicating that recruitment of these coactivators does not depend on IE protein synthesis.

2.3.4. ARC92 mediator subunit and IE gene expression.

The mediator has been proposed as the target for different mammalian activators including nuclear receptors, SREBP, p53 and viral activators E1A and VP16 [reviewed by (210)]. The interaction of mediator complex with the activation domain of VP16 has been studied *in vitro* and in heterologous systems using Gal4-VP16AD. The TRAP80 (MED17) subunit of the mediator complex interacts physically with VP16AD in binding reactions *in vitro* suggesting that is the direct target for VP16AD in the mediator complex (112). More recently, two independent studies identified another mediator subunit, ARC92 (MED25), as target of VP16AD in the mediator complex. The ARC92 interacts not only in binding assays but also in functional tests indicating that ARC92 is required for the activity of Gal4-VP16AD (201, 322).

These experiments indicate that the mediator complex is another target for the VP16AD and suggest that it might be involved in the activation of IE genes during

infection. To explore the role of the mediator complex and in particular the role of ARC92 in the activity of VP16 during infection we performed ChIP using antibodies against ARC92. ChIPs were performed under the same conditions used to detect transcriptional activators, general transcription factors and chromatin-modifying coactivators. HeLa cells were infected with KOS and RP5 viruses for 2 h. Chromatin was fixed and immunoprecipitated with anti-ARC92 and with the pre-immune serum as control.

Figure 10 shows that in KOS infections the ICP27 gene promoter is immunoprecipitated above background levels by anti-ARC92 serum. The immuprecipitation of the ICP27 gene promoter is not detected in RP5 infected cells indicating the VP16AD is required for the efficient recruitment of ARC92 to the ICP27 gene promoter. Specific immunoprecipitation of the other IE promoters ICP4 and ICP0 was not detected neither in KOS or RP5 infected cells. These results suggest that ARC92 mediator subunit is also part of the transcription factors recruited to the IE promoter ICP27 and again indicate that differential recruitment of cofactors is detected among IE promoters at two hours post-infection.

To further explore the role of ARC92 in the activation of IE genes, we tested whether a dominant negative form of ARC92 interferes with IE gene expression. The VP16 binding domain (VBD) on ARC92 resides between amino acids 402 and 590 and shows a dominant negative effect on Gal4-VP16AD activation of plasmid-based reporter gene (322). To test whether ARC92 VBD interferes with IE gene expression,

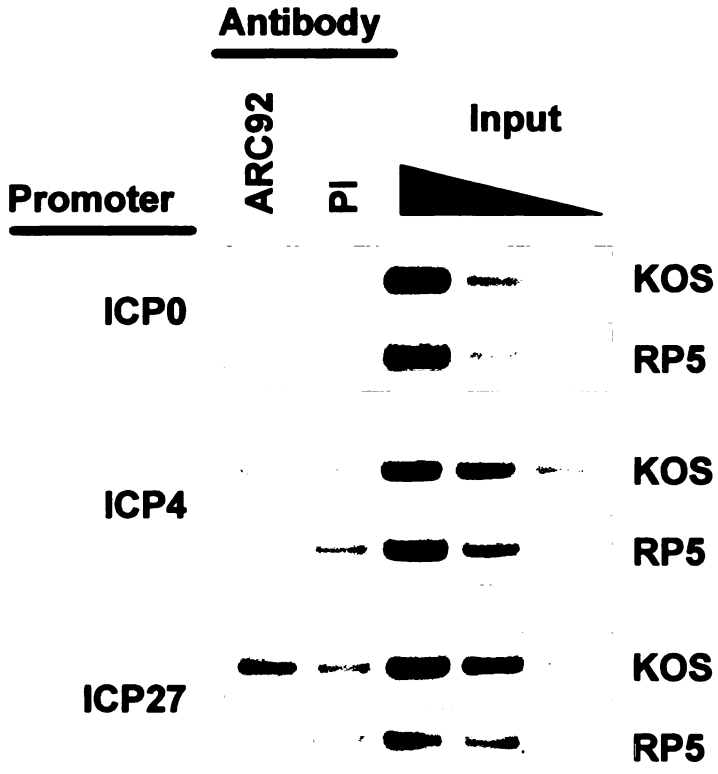


Figure 10. Recruitment of mediator subunit ARC92 to ICP27 promoter during HSV-1 infection. ChIP assays were performed using antibodies specific for ARC92 in lysates of cells infected with KOS or RP5 for 2 h. Immunoprecipitated samples were analyzed using PCR detecting viral IE gene promoters ICP0, ICP27, ICP4. Controls include precipitations performed with pre-immune serum (PI) and aliquots of samples prior to precipitation (0.5%, 0.1% and 0.02% input). Negative images of ethidium bromide-stained gels are shown

cells were transfected with a plasmid expressing ARC92 VBD and subsequently infected with HSV-1 for two hours. To select the cells that received the plasmid, the samples were co-transfected with pBABE plasmid encoding a puromycin resistant gene and grown on media containing puromycin 2 μg/ml. The selected cells from both samples (empty vector and ARC92 VBD) were collected and seeded to growth overnight on media without puromycin. Infections were performed in duplicate for each condition (empty vector or ARC92 VBD) with wild type HSV-1 viral strain KOS at MOI of 1 pfu/cell. Viral gene expression was quantified using quantitative RT-PCR (qRT-PCR). Relative cDNA levels were obtained using $2^{-\Delta\Delta Ct}$ analysis and 18S rRNA as endogenous control (see appendix B for details). Figure 11A shows a representative result for these experiments. The presence of the dominant negative form of ARC92 did not affect the steady state levels of RNA levels of ICP4 and ICP27 when compared to infections performed in parallel cells transfected with the empty vector. The expression of the ARC92 VBD peptide was confirmed in the same samples using immuno blot analysis of protein extracts. As expected, Figure 11B shows that a specific peptide is detected by antibodies directed against ARC92 only in samples transfected with vector expressing ARC92 VBD and not with the empty vector. The ARC92 VBD is a 188 amino acid peptide and thus a predicted molecular of approximately 19 kDa. These results show that the presence of ARC92 VBD does not affect the expression levels of IE gene under these conditions and thus they failed to add support to the hypothesis that the interaction of VP16 with ARC92 is required for the expression of IE genes. One caveat for these results is that we have not tried to replicate in our hands the dominant negative effect of ARC92 VBD observed on Gal4-VP16AD activity and thus no positive control was included.

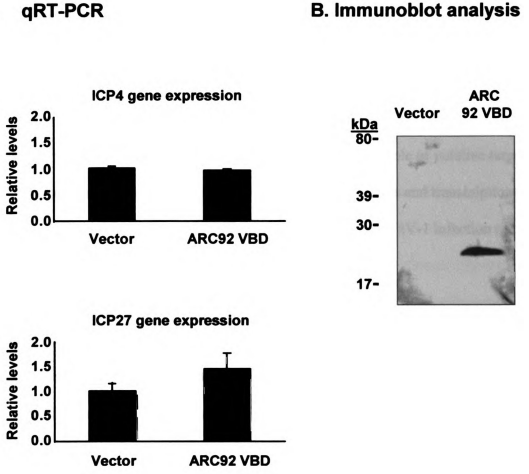


Figure 11. Effect of dominant negative form of ARC92 on IE gene expression. (A) Quantitative RT-PCR analysis of the steady-state mRNA levels of viral IE ICP24 and ICP27 genes in cells infected for 2 h with KOS in the presence or absence of plasmid expressing ARC 92 VBD. (B) Immunoblot analysis using antibodies against ARC92 of protein samples from the same infected cells used to analyze gene expression on panel A.

2.4. Discussion.

Although much has been learned about mechanisms of transcriptional activation by using VP16 and other activation domains in heterologous or *in vitro* experimental systems, the validity of the models arising from such studies is best tested in an appropriate biological context. Here we probe the physiological role of putative targets of VP16 by analyzing the recruitment of general transcription factors and transcriptional coactivator proteins to IE viral gene promoters by VP16 during HSV-1 infection of cultured mammalian cells.

We demonstrate that the recruitment of TBP and RNA Pol II (as representative GTFs) depends on the VP16 activation domain, indicating that at least part of the *in vivo* mechanism of transcriptional activation by VP16 is the establishment of a pre-initiation complex on target gene promoters. This conclusion is consistent with previous evidence that VP16 can interact directly *in vitro* with GTFs including TBP, TFIIB, TFIIA and TFIIH (84, 111, 140, 173, 284, 315) and can stimulate *in vitro* assembly of a TFIID/TFIIA/DNA complex (142). Our results do not exclude the possibility that later stages of transcription, such as promoter escape or elongation, might also be stimulated by VP16 (324).

Our results also indicate that two different types of chromatin-modifying complexes, HATs and ATP-dependent chromatin remodeling complexes, are recruited to viral IE gene promoters during HSV-1 infection. The recruitment of the HATs p300 and

CBP was fully dependent on the VP16 AD, whereas recruitment of the SWI/SNF proteins BRM and BRG-1 to some but not all IE promoters was affected by the VP16 truncation mutant. The ability of VP16 to recruit these HATs and remodeling enzymes during infection *in vivo* is consistent with results from assays using the Gal4-VP16 fusion protein in transfection or *in vitro* experiments (146, 195, 214, 296). The presence of BRM and BRG1 at some IE promoters during RP5 infection indicates that both VP16-dependent and VP16-independent mechanisms can recruit these proteins. In other circumstances, recruitment of SWI/SNF complexes has been shown to be independent of a given activation domain and yet dependent on the DNA-binding domain of a regulatory protein (44, 124, 126). We cannot yet distinguish whether the partial recruitment of BRG1 and BRM to IE promoters depends on the core domain of VP16 or on other activators that bind these promoters. Although hSWI/SNF has been implicated as a component of the RNA Pol II holoenzyme (48, 215), our results are not fully consistent with that model. In the absence of the VP16 AD, we observed BRG1 and BRM present at certain IE promoters despite the absence of RNA Pol II, indicating these ATPases can be recruited independent of the RNA Pol II holoenzyme as seen in *in vitro* systems (326).

We find it intriguing that the coactivators were differentially recruited to the various IE promoters. For example, the ICP0 and ICP4 promoters seemed to favor the presence of CBP, whereas the ICP27 promoter was prominently associated with p300. Likewise, BRG-1 was somewhat preferred at ICP0 and ICP4 promoters, whereas BRM was slightly more prevalent at the ICP27 promoter. Together, these observations suggest that BRM and p300 may cooperate specifically at certain promoters, whereas BRG-1 and

CBP function together at other promoters. The presence of ARC92 mediator subunit at the ICP27 promoter but not at the ICP0 and ICP4 promoters further illustrates the differential recruitment of transcriptional coactivators.

Curiously, these promoter preferences exist even though the important *cis* regulatory elements, including TAATGARAT, GA-rich, and Sp1-binding sites, are common to all of the IE promoters. The differential recruitment may reflect additional, undefined promoter elements that might distinguish the various promoters. Alternatively, the specific arrangements of the binding sites within the various IE promoters (and thus the quaternary structure of the various regulatory proteins) might be responsible for preferential coactivator recruitment. Whether the promoter-specific differences in coactivator recruitment have functional consequences for gene expression remains to be determined. Collectively, our results reveal that the assembly of the transcription machinery can be accomplished through multiple pathways, and that subtle differences in promoters might have significant effects on the recruitment of particular factors.

Other transcriptional regulators including Oct-1, HCF1-1, GABP and Sp1 also bind to the IE promoter regions (122, 229, 267, 303). We presume that the binding of these regulators to their cognate *cis* elements is not affected by the presence or absence of the VP16 AD, and in fact we show that Oct-1 is present at IE promoters during RP5 infection. However, the presence of these regulatory proteins is apparently not sufficient to recruit either the HATs or GTFs to the IE promoters, since the recruitment was ineffective during RP5 infection. This conclusion is somewhat surprising, given evidence

that CBP and p300 can interact *in vitro* with GABP and Sp1 (12, 273, 294). This reinforces the value of testing such interactions in an appropriate *in vivo* context.

The presence of coactivator proteins at IE promoters during infections, detected by ChIP assays, does not demonstrate that these factors are required for the expression of viral genes. Alternative approaches might be used to test whether these coactivators are required for the efficient expression of viral genes including RNA interference (RNAi), mutant cell lines and overexpression and dominant negative strategies. To that end, we developed quantitative reverse-transcriptase PCR (qRT-PCR) to analyze viral gene expression in cultured cells (see appendix B for details). To test the functional roles of these coactivators we explored RNAi approaches delivering the siRNA by transfections or by plasmids encoding the sequence producing siRNA within the cells. The results from these experiments did not show consistent and robust reduction of targeted coactivators (CBP, p300, Brg-1 and Brm) and so no clear answer has been obtained thus far on the effect on IE gene expression (data not shown).

The VP16AD binding domain of ARC92 (ARC92 VBD) is an effective dominant negative factor for the activity of Gal4-VP16AD in transfection experiments (322). We explored the role of the mediator subunit ARC92 on VP16 activity during infection in a collaborative effort with Dr. Anders Näär at Harvard Medical School. We used the same strategy developed by Näär and collaborators to study Gal4-VP16AD but now tested the dominant effect of ARC92 VBD on VP16 activity during HSV-1 infections. Several transfection conditions were used to test whether the presence of ARC92 VBD might

interfere with VP16 activity during infection. The negative results obtained with these experiments do not add support to the hypothesis tested. Different strategies might be used to further test the role of ARC92 and mediator on IE gene expression including ChIP assays using antibodies against other mediator subunits and RNAi strategies to knock down the expression of the ARC92 or other mediator subunits.

Chapter III

Association of histones with viral DNA[3]

3.1. Introduction.

The presence of CBP, p300, Brg1 and Brm coactivator proteins at IE gene promoters detected by ChIP assays suggests that the activation of IE genes during infection requires chromatin-modifying coactivators (see chapter II). These results are in agreement with the well documented recruitment of chromatin-modifying coactivators by the activation domain of VP16 in *in vitro* or heterologous systems (see chapter I for further details). However, they raise the question of why these chromatin modifying coactivators are recruited to an apparently non-nucleosomal DNA template during infection.

Several different alternatives might explain these seemly contradictory observations. Perhaps, the enzymatic activities of these chromatin-modifying coactivators might be involved in roles other than nucleosomal remodeling. Several histone acetyl transferases are known to acetylate substrates other than histones, such as activators p53, c-Myb, E2F, GATA-1, MyoD, the general transcription factors TFIIE and TFIIF and the high-mobility-group (HMG) chromatin-associated proteins [reviewed by (248, 268)]. This evidence indicates that some HATs might not be modifying only histones but also

[3] Part of this chapter was published in "Herrera FJ and Triezenberg SJ (2004). VP16-dependent association of chromatin-modifying coactivators and underrepresentation of histones at immediate-early gene promoters during herpes simplex virus infection. Journal of Virology 78(18): 9689-9696".

other proteins involved in transcriptional activation as part of potential cascades of covalent modification controlling transcriptional activation [reviewed by (75)].

Among the best-characterized chromatin remodeling complexes is the yeast SWI/SNF. This protein complex can relocate nucleosomes, alter DNAase accessibility patterns and alter the superhelicity of DNA *in vitro* [reviewed by (212)]. Although SWI/SNF complexes have been characterized by their ability to modify nucleosomal structure, naked DNA also stimulates SWI/SNF ATPase activity. Interestingly, SWI/SNF ATPase activity induces change in DNA topology in nucleosomal and naked DNA templates (78, 99). Thus, some of these SWI/SNF enzymatic activities in non-nucleosomal templates might be relevant for activating transcription on nucleosome-free HSV-1 viral DNA.

Another non-nucleosomal role for CBP and p300 during transcriptional activation might be their function as a structural component required for the formation of the pre-initiation complex or as a part of the holoenzyme (211). Thus CBP and p300 might not be recruited to the IE gene promoters to acetylate histones but as an integral component of the pre-initiation complex. In principle, hSWI/SNF might also be recruited to HSV-1 IE gene promoters as part of the RNA Pol II holoenzyme (48, 215). However, the results obtained with RP5 infections indicate that BRG-1 and BRM are recruited to HSV-1 IE gene promoters when RNA Pol II is not present at IE promoters, suggesting that BRG-1 and BRM are not recruited as part of the RNA Pol II holoenzyme but rather by specific interactions with activators present at IE gene promoters (see chapter II).

Alternatively, the presence of chromatin-modifying coactivators at viral gene promoters might suggest that incoming viral DNA associates with histones and thus the recruitment of these factors represents a mechanism used by the virus to remodel chromatin. This hypothesis contradicts prevalent models describing the HSV-1 DNA as non-nucleosomal during lytic infections. However, much of the evidence describing viral DNA as non-nucleosomal has been obtained for the newly synthesized DNA. Therefore we tested whether incoming viral DNA is associated with histones at two hours post-infection using ChIP assays. We used ChIP assays with antibodies that recognized acetylated histone H3 (K9, K14) and a C-terminal epitope of histone H3 for assessing total levels of histone H3 regardless of covalent modifications. We performed these ChIP assays using the same conditions used to detect activators, general transcription factors and coactivator proteins (see chapter II for further details). Defining the nucleosomal status of incoming viral DNA might help to understand the possible roles of chromatin-modifying coactivators in viral gene expression.

3.2. Materials and methods.

3.2.1. Cells and viruses.

HeLa and Vero cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Stocks of wildtype HSV-1 (strain KOS) and the

VP16 truncation mutant RP5 (276) were prepared in Vero cells and titered by plaque assays. For gene expression and ChIP assays, HeLa cells (approx. 3 x 10^7) were infected with KOS at a multiplicity of infection ranging from 0.1 to 5 pfu/cell. Infections with RP5 were performed with comparable amount of viral DNA to infected cells as in wild type infections, using multiplicity of infection ranging from 0.1 to 0.5 pfu/ml. The levels of viral DNA delivered during infection are reflected on the input titration used for the PCR analysis of IP samples. Typically input titration used 0.5%, 0.1% and 0.02% of input material used in each IP reaction.

3.2.2. Chemical crosslinking and immunoprecipitation assays.

To crosslink protein-DNA complexes, formaldehyde was added to the medium overlaying infected cells to a final concentration of 1% for 15 min. The crosslinking reactions were quenched by adding glycine to a final concentration of 125 mM. Cells were collected, resuspended in a hypotonic buffer, and dounce homogenized to release nuclei which were collected by centrifugation. Nuclear pellets were sonicated to obtain DNA fragments with an average length of 300-400 basepairs. Aliquots corresponding to 10% of the input material were reserved.

Prior to immunoprecipitation, samples were precleared using protein-G agarose beads (Upstate, Charlottesville VA). Immunoprecipitations were performed using specific antibodies at concentrations ranging from 10 to 50 μg/ml at 4°C overnight. Antigen-antibody complexes were precipitated using protein-G agarose beads. The beads

were washed extensively before protein-DNA complexes were eluted using 100 µl of 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS for 20 min at 65 °C. A second eluate, using 150 µl TE with 0.67% SDS, was added to the first. The combined eluates are referred to as the pellet sample. Crosslinks were reversed by adding NaCl to 200 mM and 10 µg of RNAase A and incubating at 65°C overnight. After ethanol precipitation, samples were digested with proteinase K (Boehringer) at 42°C for 2 hours and then extracted with phenol/chloroform. After another ethanol precipitation, DNA samples were resuspended in 75 µl TE pH 8.0.

ChIP assays were performed using antibodies or antisera directed against VP16 (283), Oct-1 (a gift from W. Herr, Cold Spring Harbor Laboratory), histone H3 acetylated at Lys9 and/or Lys14 (Upstate), a C-terminal epitope of histone H3 (ab1791, Abcam), and RNA polymerase II (8WG16, Covance). Control IPs using pre-immune sera exhibited essentially the same results as mock IPs using no antibody (data not shown).

Semi-quantitative polymerase chain reactions (PCRs) were performed to detect specific viral or cellular gene fragments in the immunoprecipitated samples. These fragments included the promoters of the HSV IE genes (ICP0, ICP27, and ICP4); the coding region of the ICP27 gene; the promoters of viral DE (TK) and L (VP16, glycoprotein C) genes; and the promoters of cellular genes U3 snRNA and IFN-β (See further details in appendix A). Parallel PCRs were routinely performed on serial dilutions of input samples (typically corresponding to 0.5%, 0.1%, and 0.02% of the total material) to confirm that observed signals were within linear range of the assay and were

comparable between different set of primers. Standard PCR conditions included 0.25 μM of each primer, 2.5 U *Taq* DNA polymerase (Invitrogen), 0.1 mM each deoxynucleotide triphosphate, 2 mM $MgCl_2$, 10% Enhancer solution (Invitrogen), with incubation at 95°C for 5 min followed by 30 to 35 cycles of 95°C for 30 sec, 65°C for 30 sec and 72°C for 1 min and ending with 5 min at 72°C. The Enhancer solution was omitted from reactions amplifying the ICP27 ORF, U3 snRNA promoter, and IFN-β promoter fragments. Annealing of primers for amplification of the IFN-β promoter and the GAPDH ORF was performed at 55 °C.

Quantitative real time PCR analysis (qPCR) was also used to quantify specific viral and cellular DNA fragments in the immunoprecipitated samples. qPCR were performed on a ABI Prism 7700 Sequence Detection System using SYBR Green Core Reagents (Applied Biosystems). Standard PCR conditions included 3 μL of template (IP sample or input dilution), 0.25 μM of each primer, 0.75 U Taq DNA polymerase (Applied Biosystems) and 2-3 mM $MgCl_2$ in 30 μL final volume. Parallel PCRs were routinely performed on serial dilutions of input samples (3%, 1%, 0.3%, 0.1%, 0.03%, 0.01% of the input material) to obtain standard curves for each PCR and to determine the percentage of input present in each IP. Each sample was measured in duplicate. Error bars represent range of the technical duplicates.

3.2.3. Sequential chromatin immunoprecipitation protocol.

A sequential chromatin immunoprecipitation protocol (Sequential-ChIP) was used to test co-occupancy of RNA Pol II and histone H3 at the same DNA fragment. The first immunoprecipitation and subsequent elution was performed using antibodies directed against RNA Pol II and the same conditions described on section 3.2.2. The eluted material (before reversing the cross-links) was subjected to a second immunoprecititation using antibodies directed against histone H3. After the second immunoprecipitation, the samples were treated following the same protocol described for standard ChIP (section 3.2.2). An aliquot of the eluted material from the first immunoprecipitation was reserved to test whether the first IP reaction was successful. Semi-quantitative PCR analyses of first and second IP were performed as in section 3.2.2. Parallel PCRs were used on serial dilutions of input samples (0.5%, 0.1%, and 0.02% of the total material) to confirm that the observed signals were within the linear range of the assay and were comparable between different set of primers.

3.3. Results.

3.3.1. Histone H3 is present on viral DNA but under-represented at IE gene promoters.

The presence of chromatin-modifying coactivators at viral promoters prompted us to test whether histones associate with viral DNA during early times of HSV-1 lytic infection. Initial experiments used ChIP assays to detect the presence of acetylated

histone H3 (K9, K14) on IE gene promoters. We tested first the presence of histones on viral DNA using this antibody because it has been extensively used in ChIP assays and because hyperacetylation of histone H3 (K9, K14) generally correlates with gene expression. Therefore, these modifications might be expected on IE promoters if these promoters are associated with histones during early times of infection. Approximately 6 x 10^7 HeLa cells were infected with KOS virus at a multiplicity of infection of 10 plaque forming units per cell. Cells were fixed at two hours post infection by adding formaldehyde directly to the medium at a final concentration of 1%. Chromatin was prepared and immunoprecipitations were performed using the same conditions described on Chapter II for activators, general transcription factors and coactivators.

Antibodies against acetylated histone H3 on K9, K14 (AcH3) were used along with antibodies against VP16 and Oct-1 as positive control for the ChIP assay. PCRs were performed to analyze the levels of IE gene promoters and the cellular U3 snRNA gene promoter in the IP samples. Figure 12 shows that antibodies directed against VP16 and Oct-1 immunoprecipitated IE gene promoters (ICP0, ICP27 and ICP4) as expected. Antibodies against Oct-1, but not antibodies against VP16, immunoprecipitated the U3 snRNA promoter as previously observed (see chapter II). In parallel IP samples, the IE gene promoters ICP0, ICP4 and ICP27 were not detected using antibodies against acetylated histone H3. The efficient precipitation of U3 snRNA gene promoter using anti-AcH3 serum indicates that the IP was successful. This result indicates that acetylated histone H3 on K9 and K14 is not detected on IE gene promoters at early times of infection.

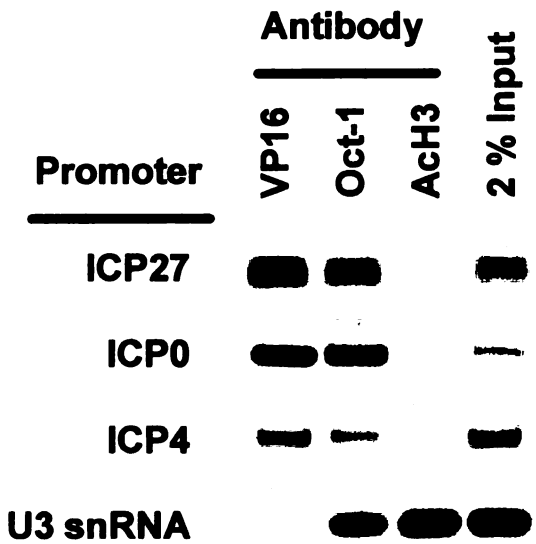


Figure 12. Acetylated histone H3 at K9, K14 (AcH3) is not detected at IE gene promoters during early times of infection. ChIP assays were performed using antibodies specific for VP16, Oct-1 and acetylated histone H3 at K9, K14 (AcH3), in lysates of cells infected for 2 h by KOS at MOI:10. Controls include precipitations performed without specific antisera (No Ab) and aliquot of sample prior to precipitation (2% input). Samples were analyzed using PCR detecting viral IE gene promoters (ICP0, ICP4, ICP27), and the promoter of the cellular U3snRNA gene. Negative images of ethidium bromide-stained gels are shown.

The absence of acetylated histone H3 (K9, K14) is in agreement with models describing viral DNA as non-nucleosomal, but does not necessarily demonstrate that histones are not associated with viral DNA. To more directly test whether histone H3 is associated with viral DNA regardless of covalent modifications, ChIP assays were performed on extracts of infected cells using an antiserum that recognizes a C-terminal epitope of histone H3. Approximately 6 x 10^7 HeLa cells were infected with KOS virus at a multiplicity of infection of 5 pfu/cells for 2h. After crosslinking, chromatin was prepared and immunoprecipitations were performed with antibodies directed against AcH3 and total H3. PCRs were performed to analyze the immunoprecipitated levels of viral gene promoters and selected cellular promoters as positive controls for the IP reactions. Figure 13 shows that the promoter regions of ICP27, ICP0 and ICP4 were not immunoprecipitated above control levels in AcH3 IP sample as observed previously. Similarly, no clear immunoprecipitation of IE gene promoters was detected using antibodies against H3. The cellular U3 snRNA gene promoter was detected in both samples as expected, confirming that both IPs were successful. The IFN-β promoter was detected in the anti-H3 sample but not effectively with AcH3 antibody indicating that the levels of histone H3 acetylation (K9, K14) at IFN-β are relatively low under these conditions. These results show that our ChIP conditions differentiate a hyperacetylated (U3 snRNA) from a hypoacetylated promoter (IFN- β). As further controls, we analyzed the promoter regions of a viral DE gene (TK) and two viral late genes (VP16 and gC). Unexpectedly, the TK, VP16 and gC gene promoters were immunoprecipitated with the anti-H3 and at some extent with the anti-AcH3 antisera, indicating that some regions of the viral DNA do associate with histones

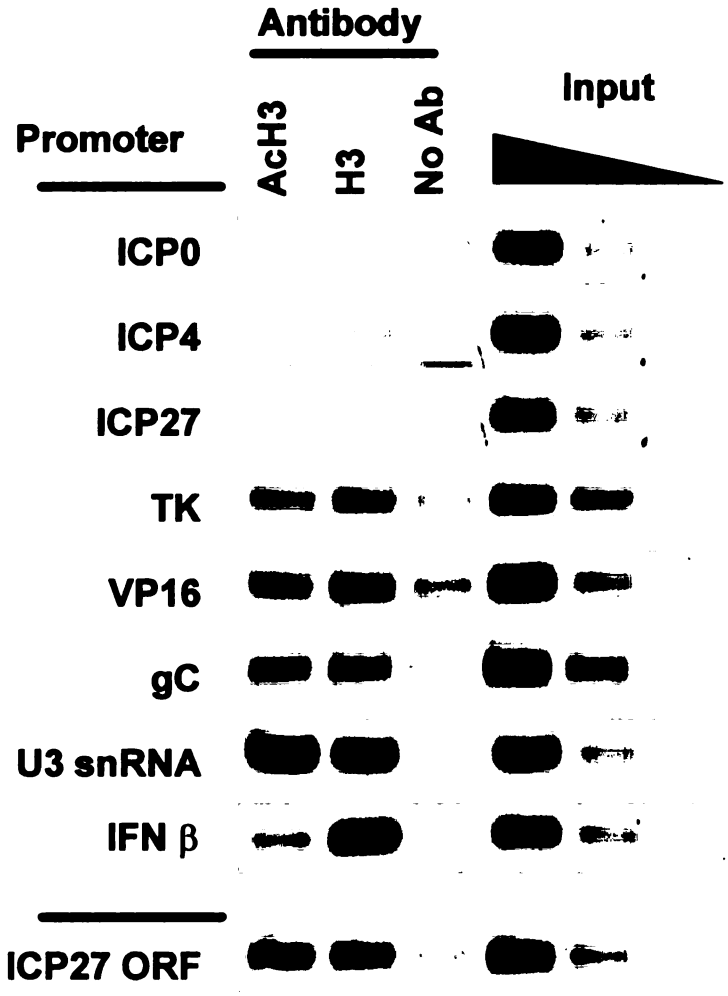


Figure 13. Histone H3 associates with HSV-1 DNA during infection and is under-represented at transcriptionally active IE gene promoters. ChIP assays were performed using antibodies specific for histone H3 acetylated at lysines 9 and 14 (AcH3) or for a carboxyl-terminal epitope of histone H3, in lysates of cells infected for 2 h by KOS at MOI:5. Controls include precipitations performed without specific antisera (No Ab) and aliquots of samples prior to precipitation (0.5%, 0.1% and 0.02% input). Samples were analyzed using PCR detecting viral IE gene promoters (ICP0, ICP4, ICP27), DE (TK), L (VP16, gC), the promoter of cellular genes U3snRNA and IFN-β and the coding region of IE gene ICP27 (ICP27 ORF). Negative images of ethidium bromide-stained gels are shown.

Moreover, the coding region of ICP27 was also immunoprecipitated in both IP samples, indicating that the dearth of histones at IE genes seems to be restricted to the promoter region of IE genes.

To test whether the association of histones with viral DNA was dependent on multiplicity of infection and thus on the amount of viral DNA delivered to the cell nucleus, we performed similar ChIP analysis using anti-AcH3 and anti-H3 in cells infected with KOS virus at multiplicity of infection of 0.1 pfu/cell. As shown in figure 14 the IE promoters were not immunoprecipitated above background levels in either IP sample, consistent with the previous experiments using cells infected at higher multiplicity of infections. The comparatively low levels of PCR amplification of the input samples is in agreement with reduced levels of DNA delivered by lower multiplicity of infection. The cellular U3 snRNA gene promoter was immunoprecipitated in both IP samples indicating that ChIP assays were successful. The promoter regions of TK and VP16 were inmmunoprecipitated with anti-H3 antisera indicating that the association of histones with DE and L gene promoters is not affected by low multiplicity of infections, although the acetylation levels of histone H3 seem to be reduced. This result shows that the differential detection of histone H3 between IE gene promoters and a DE and L gene promoter does not change at low multiplicities of infection and confirms that histones are associated with DE and L gene promoters.

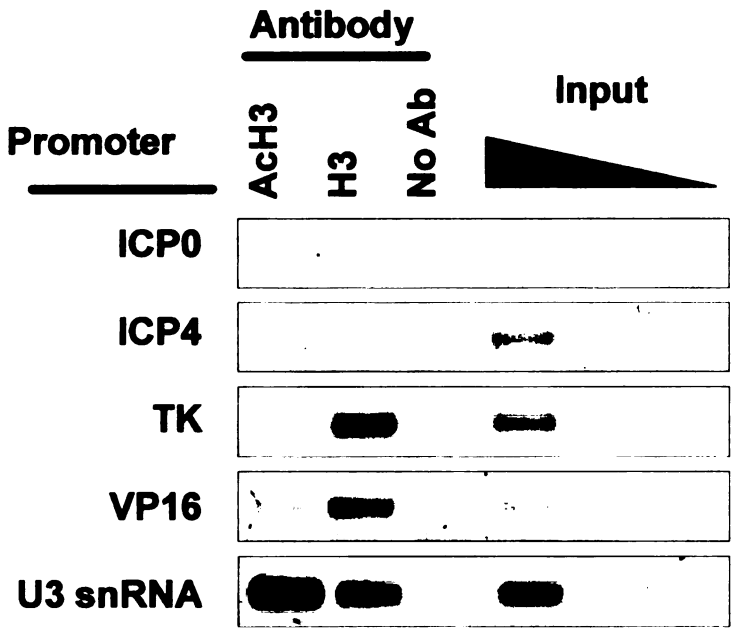


Figure 14. Histone H3 associates with viral promoters at low multiplicity of infections and is under-represented at IE gene promoters. ChIP assays were performed using antibodies specific for histone H3 acetylated at lysines 9 and 14 (AcH3) or for a carboxyl-terminal epitope of histone H3, in lysates of cells infected for 2 h by KOS at MOI: 0.1. Controls include precipitations performed without specific antisera (No Ab) and aliquot of sample prior to precipitation (0.5%, 0.1% and 0.02% input). Samples were analyzed using PCR detecting viral IE gene promoters (ICP0, ICP4), DE (TK), L (VP16), and the promoter of cellular U3snRNA gene. Negative images of ethidium bromide-stained gels are shown

These results indicate that histone H3 is under-represented at the actively transcribed IE gene promoters during early times of infection. To further test whether the promoters of actively transcribed IE genes are relatively deprived of histones, we performed sequential immunoprecipitations using antibodies directed against RNA Pol II followed by antibodies against histone H3. The first IP reaction result in the enrichment of viral promoters engaged on transcription (or at least with RNA Pol II at the promoter) and the second IP is aimed to detect the association of histones at those promoters. Figure 15A shows that the IE gene promoter ICP0 and ICP4 and cellular U3 snRNA gene are immunoprecipitated with antibodies against RNA Pol II as expected. The second immunoprecipitation using anti-H3 serum resulted in little or no enrichment of IE gene promoters over background levels (No Ab sample) (Figure 15B). In contrast, the U3 snRNA gene promoter was efficiently detected the second IP indicating that the sequential-ChIP was successful. This result indicates that the actively transcribed IE gene promoters are relatively deprived of histones compared to the cellular gene U3 snRNA and suggest that chromatin-modifying coactivators detected at IE gene promoters are associated with promoters containing relatively low levels of histones.

3.3.2. Histone H3 is detected in all classes of viral promoters in the absence of the VP16AD.

The results described in section 3.3.1 suggest that transcription activity is correlated with the under-representation of histones at viral IE gene promoters. To test

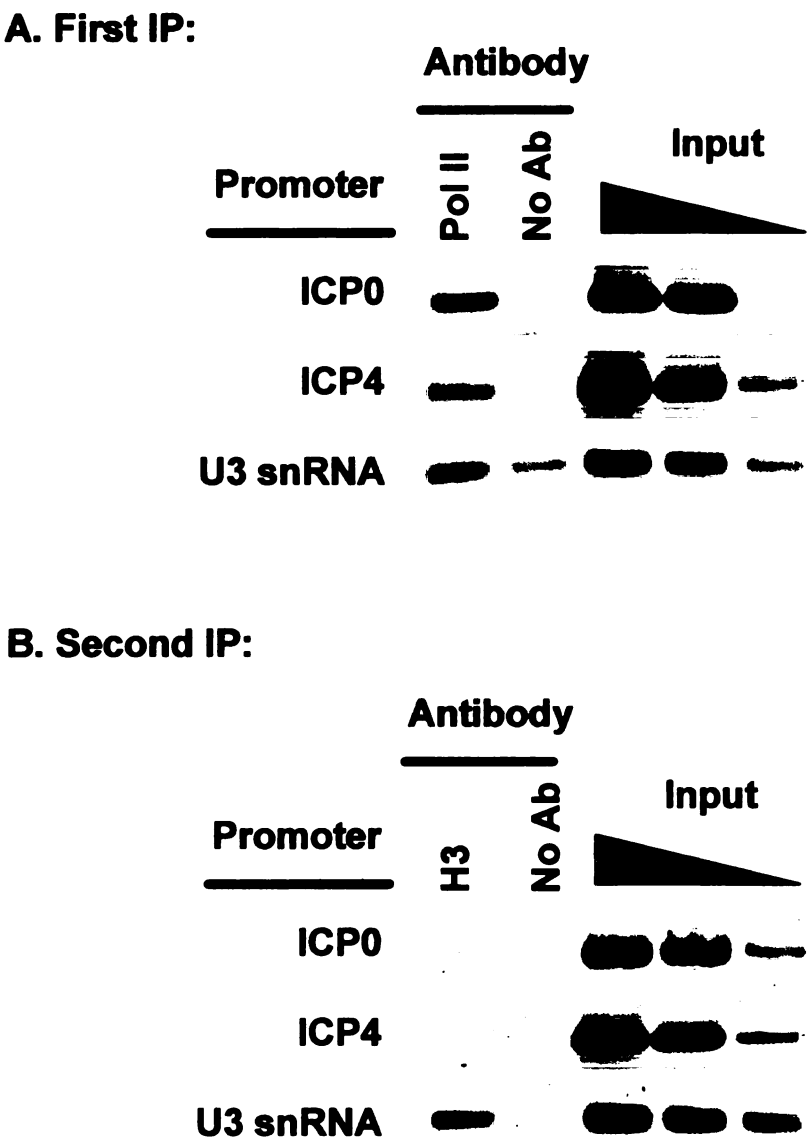


Figure 15. Histone H3 is not detected at IE gene promoters that are associated with RNA Pol II during infection by KOS virus. Double-ChIP assays were performed using antibodies specific for RNA Pol II (A, first IP) and for a carboxyl-terminal epitope of histone H3 (B, second IP), in lysates of cells infected for 2 h by KOS at MOI: 5. Immunoprecipitated samples from first IP and from the second IP were analyzed using PCR detecting viral IE (ICP0, ICP4) and the cellular U3 snRNA. Controls include precipitations performed without specific antisera (No Ab) and aliquots of samples prior to precipitation (0.5%, 0.1%, 0.02% input).

this hypothesis, we assayed the association of histone H3 at IE gene promoters during infection by the RP5 virus, when transcription of those genes is diminished. In marked contrast to the previous result, the anti-H3 antiserum efficiently immunoprecipitated the IE gene promoters in extracts from RP5-infected cells (Figure 16). These promoter fragments were not evident in IP using the anti-AcH3 antiserum. This result strengthens the correlation between transcription and absence of histones, and indicates the VP16 AD is responsible for either preventing deposition of histones or for the removal of histones from viral IE gene promoters. Moreover, in RP5 infections the TK, VP16 and gC gene promoters and the ICP27 ORF were also immunoprecipitated by the anti-H3 antiserum but not by the antiserum recognizing acetylated H3. The presence of the cellular gene promoters (U3 and IFN-β) in the IP pellets was the same for the two infections, confirming that the IP reactions were successful. We conclude that transcriptional activation of IE genes by VP16 has downstream effects on the acetylation status of histones associated with DE and L gene promoters.

To further examine the differences in histone H3 occupancy between KOS and RP5 infections, ChIP assays were analyzed using quantitative real time PCR. ChIP assays using anti-H3 antiserum were performed in cells infected with KOS and RP5 for 2 hours. Parallel immunoprecipitations using no antibodies were performed as control for background signals (No Ab sample). Aliquots of samples prior to precipitation (1%, 0.3%, 0.1%, 0.04% input) were used to obtain standard curves for the quantification of the IP samples.

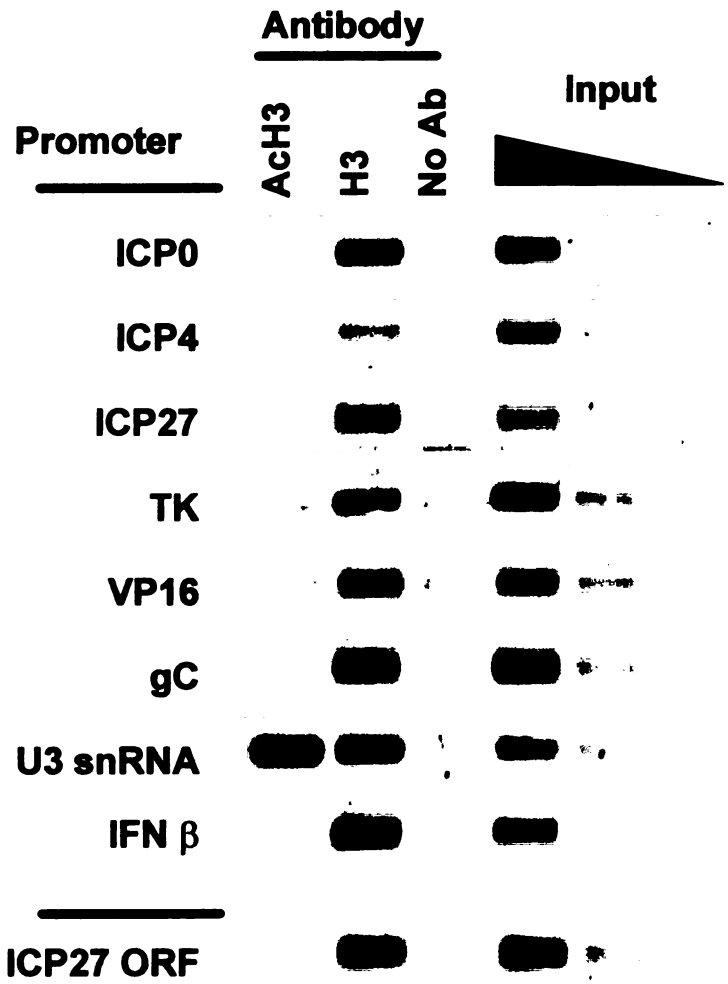


Figure 16. Histone H3 associates with all classes of viral promoters during infection of RP5 virus. ChIP assays were performed using antibodies specific for histone H3 acetylated at lysines 9 and 14 (AcH3) or for a carboxyl-terminal epitope of histone H3, in lysates of cells infected for 2 h by RP5 at MOI: 0.1. Immunoprecipitated samples were analyzed using PCR detecting viral IE (ICP0, ICP4, ICP27), DE (TK) or L (VP16, gC) gene promoters, the cellular U3 snRNA or IFN-β promoters, or the coding region (ORF) of ICP27. Controls include precipitations performed without specific antisera (No Ab) and aliquots of samples prior to precipitation (0.5%, 0.1%, 0.02% input).

To account for the background levels of immunoprecipitated materials the percentage input from No Ab samples was subtracted from the percentage input of IP samples (Normalized IP).

Figure 17 shows that during wild type infection histone H3 is under-represented at IE gene promoter ICP0 and ICP27 compared to the levels of histone H3 observed in RP5 infections. This result is consistent with the previous experiments using semi-quantitative PCR analysis. These results confirmed that histone H3 levels are dependent on the presence of the activation domain of VP16 and on the levels of transcription of the IE genes. In contrast, no clear difference is detected on histone H3 occupancy between wild type infections and RP5 infections at the DE gene promoter TK or L gene promoter gC. This result indicates that the absence of the activation domain of VP16 results primarily in differences on histone H3 occupancy at VP16-dependent genes (IE) but not on DE (TK) and L (gC) genes at early times of infection. This result also further strengthens the inverse correlation between histone H3 occupancy and levels of transcription at the IE gene promoters.

3.3.3. Histone H3 occupancy of viral promoters decreases at 4 hours post-infection.

The under-representation of histone H3 at IE gene promoters observed during infections by wild type KOS compared to infections by RP5 virus shows an inverse correlation between transcription activity and histone H3 occupancy at those

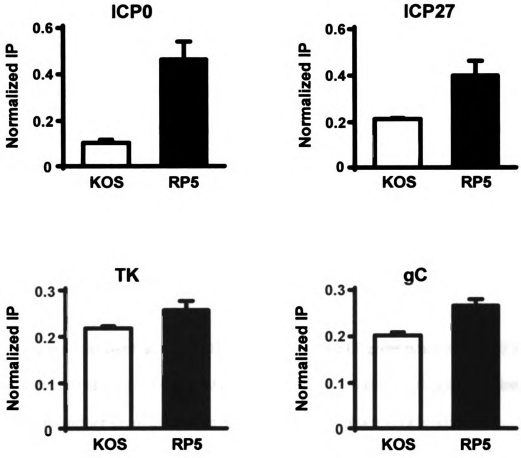


Figure 17. Histone H3 occupancy at IE gene promoter is dependent on VP16AD. ChIP assays were performed using antibodies specific for a carboxyl-terminal epitope of histone H3, in lysates of cells infected for 2 h by KOS at MOI: 5 and by RP5 at MOI: 0.01. Immunoprecipitated samples were analyzed using quantitative real time PCR detecting viral IE (ICP0, ICP27), DE (TK) or L (gC) gene promoters. Controls include precipitations performed without specific antisera (No Ab) and aliquots of samples prior to precipitation (1%, 0.3%, 0.1%, 0.04% input) used to obtain standard curves for the quantification of the IP samples. Normalized IP = (% input IP - % input No Ab).

IE gene promoters. This result suggests that histone occupancy at viral promoters might change as infection progresses and the levels of viral transcription increases. To test that hypothesis, ChIP assays using antibodies specific for a carboxyl-terminal epitope of histone H3 were performed in cells infected with wild type infections for 2 and 4 hours. As a control, parallel immunoprecipitations were performed with no antibodies (No Ab samples). The immunoprecipitated samples were analyzed using quantitative real time PCR. Input dilutions (1%, 0.3%, 0.1% and 0.04% input) were included for each PCR analysis to obtain standard curves for the quantification of the IP samples. To obtain the specific IP levels, the percentage of input detected in No Ab samples was subtracted from the percentage of input detected in IP samples (normalized IP).

Figure 18 shows that histone H3 occupancy at the IE genes promoters ICP0 and ICP27 decreased at 4 hours compared to the levels observed at 2 hours post-infection. These results further strengthen the inverse correlation between high levels of transcription and levels of histone H3 at the gene promoter. Interestingly, the levels of histones detected at the promoter of viral delayed early gene TK and leaky-late gene VP16 are also decreased at 4 hours compared to the levels observed at 2 hpi during wild type infections. Transcription from these two genes is not detected at 2 hours but is readily observed at 4 hours (see figure 7A). Thus the decreased in histone H3 signals correlates with the activation of TK and VP16 during the progression of the cascade of viral gene expression. This result indicates that the inverse correlation observed between histone H3 occupancy and transcription levels at IE gene promoters also is present at a DE and a leaky-late viral gene. The expression of the true-late genes such as gC requires

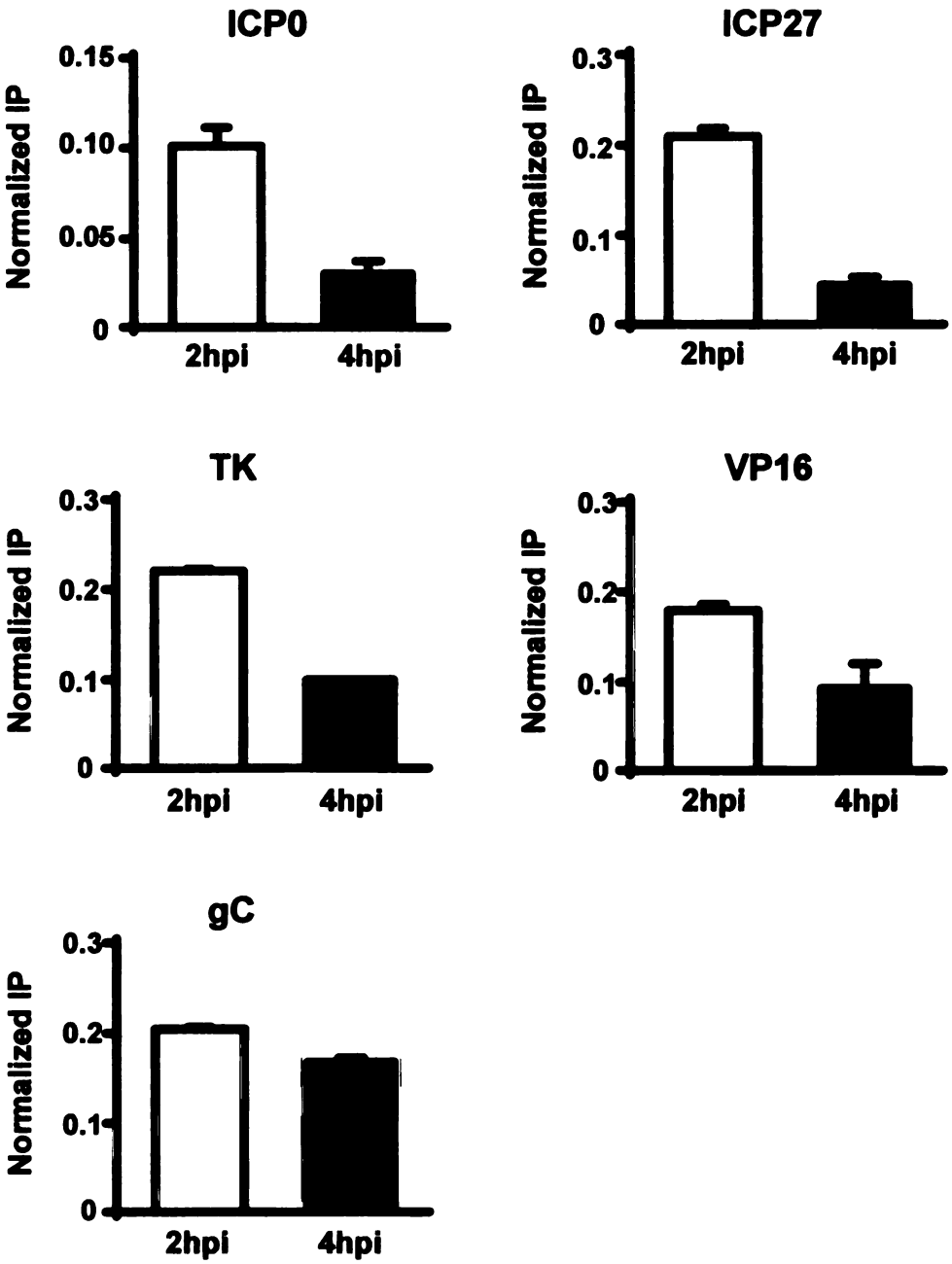


Figure 18. Histone H3 occupancy of viral promoters decreases at 4 hpi. ChIP assays were performed using antibodies specific for a carboxyl-terminal epitope of histone H3, in lysates of cells infected for 2 h or 4 h by KOS at MOI: 5. Immunoprecipitated samples were analyzed using quantitative real time PCR detecting viral IE (ICP0, ICP27), DE (TK), leaky-late (VP16) and true-late (gC) gene promoters. Controls include precipitations performed without specific antisera (No Ab) and aliquots of samples prior to precipitation (1%, 0.3%, 0.1%, 0.04% input) used to obtain standard curves for the quantification of the IP samples. Normalized IP = (% input IP - % input No Ab).

DNA replication and occurs later in infection than viral DE and leaky-late genes. The histone H3 occupancy at the gC gene promoter was not clearly affected when comparing 2 h and 4 h samples. This result indicates that as infection progresses from 2 hours to 4 hours histone H3 occupancy is primarily affected at the genes actively transcribed. It also suggests that histone depletion during transcriptional activation might occur at all classes of viral promoters.

3.4. Discussion

The recruitment of chromatin-modifying proteins to IE gene promoters might seem superfluous given previous evidence that HSV-1 DNA is not packaged in nucleosomes during lytic infection. In striking contrast to that model, our ChIP experiments detected a distinct association of HSV-1 DNA with histone H3, although this assay does not directly demonstrate that H3 is present in nucleosomal structures. ChIP assays using antibodies specific to other core histones (or variant histones), or other physical assays might be used to assess whether nucleosomes or some other histone based structures are present on viral DNA. Interestingly, in cells infected by wildtype virus, histone H3 was under-represented at IE gene promoters compared to DE (TK) and L (VP16 and gC) gene promoters at early times of infection.

The histone H3 levels also decreased at the DE (TK) and leaky-late (VP16) gene promoters at times when these genes are actively transcribed. Thus, the histone H3 (perhaps in nuclesosomes) is not only under-represented at the IE gene promoters but seems to also become under-represented at a DE and a leaky-late gene. Similarly, higher levels of histone H3 were detected at IE promoters during RP5 infection, when those genes are transcriptionally silent. These results indicate that the VP16 AD is required either to exclude histones from IE promoters or to remove them, perhaps through the action of the chromatin remodeling coactivators.

Consistent with our observations, recent reports demonstrated that nucleosomes are under-represented in the fully remodeled PHO5 promoter in yeast upon transcription induction (23, 239). Furthermore, the under-representation of nucleosomes at actively transcribed genes seems to be a general phenomenon in yeast (21, 165) (see chapter IV for further details). The effect of the VP16 AD on acetylation of histones associated with viral DNA is also noteworthy. Although acetylated H3 was associated with the ICP27 ORF and the DE and L gene promoters during infection by wildtype virus, that acetylation was absent during RP5 infection. This result suggests that the cascade of viral gene expression requires histone modifications that are directly or indirectly dependent on the VP16 activation domain. Given that robust expression of DE and L genes is observed later than at 2 hours, the H3 acetylation detected in wild type KOS infections at DE and L gene promoters might represent early events in the remodeling of these promoters.

The presence of CBP, p300, BRG-1 and BRM at IE promoters may reflect the mechanism by which histones are excluded from these promoters. These enzymes might also fulfill other functions for transcriptional activation in the absence of histones. For instance, CBP and p300 might be required for pre-initiation complex formation or as a part of the RNA Pol II holoenzyme (211). Another possibility is that CBP and p300 may acetylate and thus regulate the activities of other proteins such as activators, HMG proteins, coactivators and general transcription factors (248, 268). Although SWI/SNF complexes are best known for their ability to modify nucleosomal structure, naked DNA also stimulates SWI/SNF ATPase activity, and conversely SWI/SNF ATPase activity induces changes in DNA topology in nucleosomal and naked DNA templates (78, 99). Thus, SWI/SNF enzymatic activities on non-nucleosomal templates might be relevant for activating transcription on nucleosome-free HSV-1 viral DNA.

The relative levels of histone H3 occupancy detected at gene promoters by ChIP assays relies on the effective recognition of the histones by the specific antibodies used in the IP reactions. The changes in IP efficiency *per se* can be monitor by using internal controls when comparing two different IP samples. For instance, internal control used for ChIP assays using anti-AcH3 and anti-H3 antibodies included the promoter of the cellular genes U3 snRNA and IFN-β. However, changes in ChIP signal at a particular locus might also occur by epitope masking or by differences in crosslinking efficiencies between proteins and DNA at different loci or conditions. Our ChIP analyses do not distinguish whether epitope masking perhaps due to presence of large protein complexes (such us a RNA Pol II or coactivator complexes) or differences in crosslinking efficiency at

different loci are affecting IP efficiency within a particular IP sample. The use of polyclonal antibodies (such us anti-AcH3 and anti-H3 used in these experiments) might help to reduce potential problems in interpreting ChIP results due to epitope masking.

Chapter IV

Discussion and future work

4.1. Chromatin-modifying coactivators at viral IE gene promoters.

Large efforts have been made in the last decade toward understanding the mechanisms of chromatin remodeling during transcriptional activation in eukaryotes. We now know that nucleosomes are not just a static structure compacting DNA but instead they are highly modified during transcriptional activation. Some of this remodeling occurs by adding covalent modifications on particular histone residues and thus changing the structure of the nucleosome. Nucleosomes are also remodeled by changing the interactions between DNA and histones by ATP-dependent complexes. Chromatin-modifying coactivators are involved in transcriptional activation in organisms ranging from yeast to humans.

However, the role of chromatin-modifying coactivators during transcriptional activation of HSV-1 genes is largely unexplored. Current models describe that viral DNA during lytic infection does not become nucleosomal and therefore chromatin modifying coactivators might not be necessary for the activation of HSV-1 genes. Nonetheless, the activation domain of VP16 has been shown to interact with several chromatin-modifying coactivators *in vitro* or in heterologous systems (see chapter I for further details).

These observations led us to establish ChIP assays to test specifically whether chromatin-modifying coactivators were recruited to viral gene promoters during lytic infections. The results described on Chapter II indicate the presence of the HATs CBP and p300 and the ATPases subunits of human SWI/SNF complex Brg-1 and Brm. The presence of these coactivators at IE viral gene promoters led us further to test whether incoming viral DNA was associated with histones. The results described on Chapter III show that histones (at least histone H3) are associated with incoming HSV-1 DNA suggesting that chromatin remodeling might be required during activation of viral genes.

Several questions remain to be explored regarding the role of chromatin-modifying coactivators on viral gene expression, including whether chromatin-modifying coactivators are required for the expression of viral genes and whether other coactivators known to interact with activators present at IE promoters are also involved in the expression of IE genes.

4.1.1. Functional role of coactivators in viral gene expression.

The presence of CBP, p300, BRG-1 and BRM at IE promoters suggest that these coactivators participate in the expression of viral genes. However, whether these chromatin-modifying coactivators are required for the expression of IE genes during infection remain to be determined. Several approaches might be used to explore this question. One approach might be to use mutant cell lines defective for particular

coactivators. Mutant cells lines defective for the expression of p300, CBP, BRG-1 and BRM might be obtained from the corresponding knockout mice (33, 155, 242, 325). Since p300, CBP and Brg-1 knockout mice have an embryonic lethal phenotype, embryonic fibroblast cells might be used to test whether these coactivators are required for the activity of VP16 during infection. 3T3-derived cell lines expressing an inducible dominant negative mutant (tet-off system) form of hBRM and hBRG-1 have been obtained from T. Imbalzano lab (58). These cell lines might also be used to address the requirement of the ATPase activity of hBRM or BRG1 complexes for the expression of IE genes. Another alternative is to use an overexpression approach to test for functional interactions between VP16 and these coactivators. Increasing amounts of a plasmid encoding particular coactivators might be transfected into HeLa cells to test whether IE gene expression is affected on those cells. RNA interference might also be used to test whether these coactivators are required for the expression of IE gene. Initial attempts using plasmids expressing siRNA targeting CBP, p300, BRG-1 and BRM have shown variable and sometimes modest reductions of the levels of mRNAs encoding these coactivators (data not shown). Improved RNAi approaches including the use of siRNA pools and validated siRNA sequences to target human genes might be used to more effectively knockdown the expression of these coactivators.

4.1.2. Additional chromatin-modifying coactivators at IE gene promoters.

Other chromatin-modifying coactivators might also be involved in the expression of IE genes during infection. Possible candidates include coactivators known to interact with the activators present at IE promoters. The mammalian homolog of the yeast ADA/SAGA complex, known to interact with Gal4-VP16 in yeast, contains either hGCN5 or PCAF as a catalytic subunit (reviewed in Sterner and Berger, 2000). Furthermore, Gal4-VP16 interacts with GCN5 and PCAF *in vitro* and in heterologous systems (97, 195, 296). PCAF also interacts with CBP and p300, and thus the presence of these HATs might also contribute to the recruitment of PCAF to the IE gene promoters. HCF-1 interacts with the human Sin3 deacetylase and with Set1/Ash2 histone H3-K4 methyl transferase (314). Thus, antibodies against hGCN5 and PCAF, Sin3 and Set1 might be used in ChIP assays to test for their presence at IE promoters.

A recent report identified a family of chromatin modifying coactivator proteins associated with viral DNA at 6 hours post infection (280). This study used a proteomic approach to identify proteins interacting with a viral protein, ICP8, involved in viral DNA replication. Immunoprecipitation assays were used to pull down ICP8 and the interacting proteins were identified by mass spectrometry and immunoblot detection assays. Several chromatin-modifying coactivators co-immunoprecipitated with ICP8 in a manner dependent on DNA. These results strengthen the conclusion that chromatin-modifying coactivators associate with viral DNA during infection. However, this approach did not distinguish the region on which these chromatin-modifying proteins are bound to the viral DNA. The chromatin-modifying proteins identified include hSNF2 (ISWI complex), BRG-1 and BRM and several BRG-1 or BRM associated factors

(BAFs), histone deacetylase mSIN3a, histone deacetylase 2, nucleosome-associated protein-1-like (NAP-1-like) and REST corepressor (280). One possibility is that these coactivators might be associated with viral promoters. Thus, ChIP assays might be used to test for the presence of these proteins at IE and other viral promoters during infection. Since is not clear to which promoter these proteins might be recruited during infection, it might be interesting to use microarray technology to assess the occupancy of these factors using an unbiased approach in these ChIP assays (so-called "ChIP on chip" assays). These "ChIP on chip" assays might also be use to study the recruitment of other transcription factors in a global manner for all viral promoters during infection. A difficulty on establishing "ChIP on chip" assays is that HSV-1 promoter arrays are not currently available.

4.1.3. VP16AD subdomains.

ChIP results obtained with RP5-infected cells indicates that the activation domain of VP16 is required for the efficient recruitment of TBP, RNA Pol II, CBP, p300 and partially required for the recruitment of BRG-1 and BRM to IE promoters (see Chapter II for details). The VP16AD can be divided in to two sub-domains, namely VP16N (aa 410 to 456) and VP16C (aa 450 to 490), each capable of activating transcription when fused to a heterologous DNA binding domain. The different patterns of acidic amino acids surrounding the critical phenylalanines residues of VP16N and VP16C (54, 238, 272) and functional differences between these two subdomains (84, 140, 295) suggest that VP16N

and VP16C may utilize distinct mechanisms of action. Two recombinant viruses RP3 and RP4, lacking the C-terminal and N-terminal portion of VP16 AD respectively, (276) might be used in ChIP assays to determine which subdomain is required for the efficient recruitment of these transcription factors.

4.2. Is HSV-1 DNA non-nucleosomal during lytic infection?

The association of histones with HSV-1 DNA has been studied using different approaches including nuclease digestion assays of infected cells, electron and fluorescence microscopy and protein analysis of virion particles (see Chapter I for further details). The conclusion from these experiments is that most, if not all, viral DNA is non-nucleosomal during lytic infection. The viral DNA is not associated with histones in the virion particles and is thought to use the polyamines (spermidine and spermine) contained in the virion to counteract the negative charge of the DNA inside the capsid (82, 225). Upon entering the nucleus of the infected cells, prevalent models describe viral DNA as non-nucleosomal during lytic infection. Only during latent infections does HSV-1 DNA associates with histones, forming nucleosomal structures similar to that of cellular chromatin as probed by nuclease digestions assays (60).

However, the mechanisms that would maintain viral DNA free of histones after entering the nucleus are largely unknown. It has been suggested that the virion protein 22 (VP22) might help maintaining viral DNA in a non-nucleosomal state by inhibiting

histone deposition activities (288). VP22 is one of the most abundant protein components of the virion particle with approximately 2000 copies per virion (168). Together with other tegument proteins, VP122 is delivered to the newly infected cells upon infection where can be localized either in the cytoplasm or the nucleus of infected cells (67, 204, 227). VP22 interacts *in vitro* with two histone chaperones, template activator factor I α and β (TAF-Iα and TAF-Iβ), and inhibits the TAF-1 dependent histone deposition (288). Thus, the association of VP22 with viral DNA might serve as an inhibitor of histone deposition during early time of infection (288). This is clearly not the only VP22 function during infection. VP22 also associates with microtubules during infection and is capable of spreading to neighboring but uninfected cells, suggesting a role in modulating intracellular trafficking (66).

At later times during infection the decrease in histone synthesis (320) and the formation of the replication compartments that exclude cellular chromatin (233) might contribute to maintain newly synthesized viral DNA free of nucleosomes. The absence of nucleosomes on replicated viral DNA might help to package non-nucleosomal viral DNA into newly formed capsids.

Contrary to our expectations, chromatin immunoprecipitation results described in chapter III detected the association of histone H3 with incoming viral DNA at early times of infection. These results do not yet indicate that canonical nucleosomes are formed on viral DNA. Nonetheless, these observations contradict prevalent models describing viral DNA deprived of histones during lytic infection. A detailed analysis of previous

approaches used to explore specifically the association of histones with the incoming viral DNA might help to reconcile these seemly contradictory results. One study used micrococcal nuclease digestion to analyze viral DNA on infected Vero cells (166). The results of this study show that most incoming DNA was insensitive to nuclease digestion perhaps due to the presence of encapsidated DNA. As noted by the authors, the viral DNA that was digested yielded heterogeneous fragments and a portion with the size expected for nucleosomal DNA (166). In cell preparations from brainstems of acutely infected mice most of the viral DNA was nonnucleosomal, but again a minor fraction of viral DNA fragments of the size expected for nucleosomes was consistently detected using micrococcal digestions (205). In another study the nucleosome-like fragments were not detected among incoming viral DNA digested with staphylococcal nuclease and the TK coding region as a probe to detect viral DNA (167). Thus, in two reports using nuclease digestion sensitivity assays some fraction of the incoming viral DNA was observed with characteristics consistent with nucleosomal DNA.

As for later times in infection, dramatic changes in cellular chromatin are observed resulting in the perinuclear accumulation of cellular chromatin. Viral DNA replication occurs within this newly formed electron transparent regions in the replication compartments (233, 234). Electron microscopy studies have shown the accumulation of naked viral DNA in infected cells. Interestingly, the presence of non-nucleosomal DNA was dependent on viral DNA replication, thus the status of incoming viral DNA was not clear (206). The exclusion of cellular chromatin in the replication compartment has been also studied using fluorescence microscopy. Cells expressing a fusion protein of histone

H2B and the green fluorescence protein (GFP) were infected with HSV-1 and the nuclear changes were observed during late times of infection. The characteristic perinuclear distribution of cellular chromatin was again observed and no incorporation of H2B-GFP on replication compartments was detected (203).

A recent study explored once again the association of histones with incoming viral DNA using micrococcal nuclease digestion, with the addition of ChIP assays as a more direct method to test the association of histones with viral DNA (135). The nuclease digestion assay showed again the absence of the DNA ladder characteristic of regularly spaced nucleosomes at different times during lytic infections. However, a small fraction of mononucleosome size viral DNA was observed. The ChIP assays showed the association of acetylated H3 (K9, K14) with the ICP0, TK and VP16 promoters. The acetylation signal observed at 1 and 3 hpi is in overall agreement with the relative AcH3 levels observed at 2 hpi described in Chapter III. The levels of histone H3 were not monitored in this study, but the low levels of histone H3 acetylation detected at the ICP0 promoter by Kent et al. is in agreement with the under-representation of histones observed at this promoter (see chapter III). Interesting, the acetylation levels of histone H3 were drastically reduced at the TK and VP16 promoter at 6 hpi. This decrease might represent the deacetylation of histone H3 by HDACs or it might indicate that histone H3 levels are reduced at those promoters at 6 hpi. Similar results were observed with antibodies directed against methylated histone H3 (K4). The authors concluded that the HSV-1 genome is at least partially nucleosomal, although apparently not in a regular repeating structure, moreover the histone modifications observed at viral genes correlate

with histones modifications observed at actively transcribed genes in other biological systems (135).

4.3. Association of histones with incoming HSV-1 DNA.

One model consistent with all of the above observations would describe the association of histones with at least a fraction of the incoming viral DNA. Histones might be deposited on viral DNA by replication dependent or replication independent mechanisms using preexisting pools of canonical histones or any of the histone variants. The incoming viral DNA associated with histones might be actively engaged in the productive infection cycle and thus might require chromatin remodeling activities during transcription of the viral genes. The presence of histone and formation of chromatin on viral DNA might repress viral gene expression until the proper activation signal is present. The observed cascade of viral gene expression during lytic infections might be dictated in part by the repressive effect of nucleosomes on viral DNA. At later times in infection, the newly replicated viral DNA accumulates in regions that exclude cellular chromatin and would not become associated with histones. This nucleosome free viral DNA would then be packaged in capsids during virion assembly. The down-regulation of histone synthesis during infection (320) might also contribute to the accumulation of newly synthesized viral DNA deprived of nucleosomes.

This new model describing the incoming viral DNA as nucleosomal would predict that the expression of viral genes requires chromatin modifying coactivators. This is consistent with the association of HATs (CBP and p300) and ATP-dependent chromatin remodeling complexes (BRG-1 and BRM) with IE promoters (see chapter II). It would also help to explain why the viral activator VP16 evolved the capability to interact with chromatin-modifying coactivators and to remodel chromatin domains.

The association of histones with incoming viral DNA might result in a repressive environment for the transcription of viral genes. In different cell types, such as sensory neurons, the absence of the correct cofactors or differences in metabolic states might result in the inability of the virus to counteract the repressive effect of chromatin resulting in latent infections. During latency, the viral DNA is associated with histones and is transcriptionally silenced with the exception of the LAT genes [reviewed by (20)]. Using ChIP assays hyperacetylation of histone H3 (K9, K14) has been observed at the LAT promoter but not at the repressed ICP4 and DNA Pol viral gene promoters on latent HSV-1 genomes (151). These results suggest that chromatin remodeling might be involved during reactivation of latent viral DNA.

4.4. Does VP16 prevent deposition or promote disassembly of histones at viral IE gene promoters?

Chromatin immunoprecipitation analyses of histone H3 on viral DNA at early times of infection show that histone H3 is under-represented on IE gene promoters compared to DE (TK) and L (VP16 and gC) promoters. These lower levels seems to be restricted to the promoters since the ICP27 ORF is efficiently immunprecipitated with antibodies recognizing H3 (total H3 and AcH3 K9, K14). Since viral DNA is not associated with histones in the virion particle, the low levels of histones detected at the IE promoters might either result from the active disassembly of the nucleosomes upon activation of the IE gene or from preventing the deposition of histone at those promoters. The reduction of histone H3 levels between 2 and 4 hours post infection at the ICP0 and ICP27 gene promoter suggest that histone might be displace from the IE promoter. Time course experiments might be used to test more explicitly the dynamics of histone H3 occupancy at IE gene promoters during the first hours of infection. Alternatively, RP5 infected cells might be subsequently infected with HSV-2 to test whether the levels of histone H3 are reduced at the HSV-1 IE promoters by VP16 delivered by HSV-2.

In RP5 infections the expression of the IE genes is highly reduced (see Chapter II for further details). Under these conditions, the levels of histone H3 detected by ChIP assays at two hours post infections are higher that those observed in wild type (KOS) infections. This result indicates that the level of histones H3 at IE promoters inversely correlates with the levels of transcription of those genes. One hypothesis might predict that the high density of RNA Pol II at IE genes during wild type infections results in the under-representation of histone H3 at the IE gene promoters. ChIP assays performed with cells treated with transcriptional inhibitors might be used to distinguish whether

transcription *per se* is required for the low levels of histones detected at the IE gene promoters.

Alternatively, the under-representation of histones at IE promoters might be a result of a protein activity recruited directly or indirectly by VP16AD. This protein activity might either prevent the deposition of histone or induce the active disassembly of nucleosomes at the IE promoters. Precedents for both notions are found in the literature. van Leeuwen et al. proposed that the inhibitory activity of VP22 on TAF-I mediated histone deposition might contribute to maintain viral DNA deprived of histone (see further details on section 4.1). The model proposed by the authors assumes that VP22 binds non-specifically throughout the viral DNA preventing histone deposition. An alternative, and testable hypothesis, might propose that the under-representation of histones is the result of the localized activity of VP22 at IE promoters on the incoming viral DNA. Since VP22 binds to DNA without apparent sequence specificity, the targeting of VP22 to IE promoters might not involve direct *cis*-element recognition. The activation domain of VP16 interacts with VP22 and thus provides a specific mechanism for the recruitment of VP22 to IE promoters (65). It is interesting to note that the overexpression of TAF-Iα interferes with the progression of HSV-1 infection apparently by a general inhibition of viral gene expression (288). Perhaps, high levels of TAF-I overcome VP22 inhibition resulting on increased histone deposition on viral DNA and the silencing of the cascade of viral gene expression. ChIP assays might be used to address whether VP22 and TAF-I are recruited to IE promoters or other loci on viral

DNA. Overexpression of TAF-1 might be used to test whether histone deposition on viral DNA is increased by higher levels of TAF-1.

The disassembly of nucleosomes upon transcriptional activation at yeast promoter has been recently observed using ChIP assays. The first two of such studies used ChIP analysis for histones H3 and H2B at the endogenous Pho5 gene promoter. Upon activation, the nucleosomes were first hyperacetylated and then displaced or removed from the promoter (239). Another independent study used topological, sedimentation, nuclease digestion and ChIP analyses to show that nucleosomes are unfolded in the active Pho5 promoter (23). To distinguish between disassembly or sliding of the nucleosomes, the Pho5 promoter was excised from the chromosomal locus forming chromatin circles. Upon activation of the Pho5 promoter the number of nucleosomes was diminished in the chromatin circle indicating that nucleosomes are disassembled rather than moved to an adjacent location (24). This nucleosomal displacement is also observed in another yeast promoter (Pho8) and is reversible since the histone levels are restore to normal levels after transcription of the gene has been repressed (2). This disassembly of nucleosomes requires the histone chaperone Asf-1 and is essential for transcriptional activation (2).

More recently, genome-wide analysis of histone occupancy on *Saccharomyces cerevisiae* using "ChIP on Chip" assays determined that nucleosomal depletion is observed on promoters of actively transcribed genes (21, 165). During rapid mitotic growth, the amount of histones at gene promoters was inversely proportional with the level of transcription (165). Furthermore changes in global transcription program by heat

shock or a change in carbon source resulted in the increase in nucleosome occupancy at repressed gene promoters and decreased occupancy at the promoters that become active. Histone depletion was generally observed only at the promoter region, but was also observed at the coding region of heavily transcribed genes (165). Another study reported that upon activation of Gal1 promoter in yeast, nucleosomes were not only under-represented at the promoter region but also at the coding region. The reduced levels of histone H3 were not observed in the ORF of other three genes transcribed at lower levels than the galactose regulated reporter gene. The authors concluded that histones are displaced from actively transcribed promoters and suggested that histone depletion at ORF might be observed only at heavily transcribed genes (149). These recent reports collectively indicate that nucleosome depletion at gene promoters is a general phenomenon in yeast and suggest that it might occur during transcriptional activation in other eukaryotic organisms.

The cascade of events during transcriptional activation of a silenced locus has been observed in mammalian cells using light microscopy and a combination of fluorescent fusion proteins (116). Several interesting observations arose from these experiments including the replacement of canonical histone H3 by histone variant H3.3 during transcriptional activation of the silenced locus. The replacement of the histone H3 upon gene activation implies the active exchange of nucleosomes, or at least of nucleosome components, at an active gene promoter. This active exchange might be the result of the disassembly and subsequent reassembly of nucleosomes during transcriptional activation in a manner analogous to the Pho5 promoter. It will be

interesting to test whether the overall levels of histones are decreased at the promoter region during gene activation. Histone H3 replacement has been previously observed in *Drosophila* where actively transcribed regions including ribosomal DNA arrays become enriched with histone H3.3 (3). Furthermore, certain histone modifications associated with transcriptional activation, such as acetylation of Lys9 and Lys14 of H3, are over-represented in H3.3 compared to histone H3, strengthening the link between histone H3.3 and transcriptionally active genes (193). Collectively this evidence suggests that histones can be disassembled and reassembled at actively transcribed genes and that this assembly might result in the enrichment of nucleosomes with particular histone variants including histone H3.3.

Since many of the steps that control gene regulation are conserved among eukaryotes, some of these mechanisms involving balance between histone disassembly and histone deposition might be relevant for viral gene expression. It would be interesting to study the mechanisms involved in the deposition of histones on incoming viral DNA and whether any of the histone variants are associated with the viral DNA. The ChIP assays described in Chapter III do not discriminate between histone H3 variants and thus the question of which histone H3 is associated with viral DNA remains open. Recently developed antibodies (Abcam) that specifically recognized histone H3.3 might be used in ChIP experiments to directly test whether this histone variant is associated with viral DNA.

4.5. Are histones displaced from all classes of HSV-1 gene promoters during cascade of gene expression?

The disassembly of nucleosomes during transcriptional activation has been observed in yeast promoters (see section 4.3. for details) and thus might be an integral step in transcriptional activation in eukaryotes. The IE gene promoters show an inverse correlation between levels of histones and transcription activity. In that context, the activation of the DE and Late genes might require the disassembly of the nucleosomes present at those promoter regions. The differences observed in histone H3 occupancy between 2 and 4 hours post-infection at IE genes ICP0 and ICP27 as well as at the TK and VP16 gene promoter further suggest that histone disassembly might occur during viral gene activation. Time course experiments using ChIP assays detecting the levels of histones might be used to test whether the histone content decreases at all classes of viral promoters as the cascade of viral gene expression progresses.

The decreasing complexity in the architecture of viral promoters between IE, DE and L promoters might result in differences in the ability of these promoters to recruitment chromatin-modifying coactivators. It is tempting to speculate that the true-late gene promoters consisting only of core promoter elements might not be able to overcome the repressive effect of nucleosomes. It is well established that true-late genes require viral DNA replication in order to be transcribed [reviewed by (303)]. No mechanisms have been accepted for this seemly paradoxical phenomenon where DE and

L genes require the same IE proteins to be expressed but L genes remains silenced until DNA replication occurs.

One hypothesis consistent with the model described above (section 4.1.2.) might predict that IE, DE and leaky-late promoters recruit chromatin modifying coactivators and thereby overcome the repressive effect of nucleosomes. The different *cis* elements upstream of the IE, DE and leaky-late core promoters might be involved in the recruitment of these coactivators. True-late genes consisting only of core promoters might not be able to overcome the repressive effect of nucleosomes. Only after DNA replication might the core promoter become accessible for the binding of the general transcriptional machinery allowing transcription. The presence of newly synthesized template DNAs devoid of histones might help exposing the core promoter elements. Time course ChIP experiments might be used in combination with inhibitors of viral DNA replication to test the predictions arising from this model.

4.6. Chromatin and other animal viruses.

The genetic material of many viruses is targeted to the nucleus where is transcribed and replicated using cellular factors. Thus, the association of viral DNA with histones has been studied in several viruses including retroviruses and DNA viruses. The results of these studies reveal both common and distinct strategies to overcome the repressive environment that chromatin poses to viral gene expression. The role of

chromatin in the expression of genes of a retrovirus (HIV), double stranded circular DNA virus (SV40), double stranded linear DNA virus (adenovirus) and other herpesviruses (CMV, EBV and KSHV) will be discussed to illustrate these differences and similarities,.

4.6.1. Human immunodeficiency virus type-1.

The viral RNA of the human immunodeficiency virus type 1 (HIV-1) is reverse transcribed and integrated into the host genome. The viral DNA is packaged into chromatin and remains transcriptionally silent in the absence of stimuli [reviewed by (187)]. The reactivation of HIV-1 occurs from a nucleosomal template and thus the virus has evolved mechanisms to recruit chromatin-modifying coactivators to activate the transcription of viral genes. The long terminal repeat (LTR) region of viral DNA contains a core promoter and enhancer-like sequences that direct the viral gene expression during reactivation of the integrated viral DNA. The LTR core promoter and enhancer-like region contain DNA sequences recognized by several different cellular activators, including Sp1, NF-κB and nuclear receptors [reviewed by (187)]. Any of these cellular activators that bind to the LTR promoter might recruit chromatin-modifying coactivators during the reactivation of the viral genes. It has been shown that the transcriptional activation of the long terminal repeat (LTR) of HIV by thyroid hormone receptor (TR) requires chromatin disruption and histone acetylation (107). The Tat protein is the viral transactivator that activates LTR expression. Tat binds to a *cis*- element present in the nascent viral RNA (trans-activation response region, TAR) and helps to recruit an

elongation-competent RNA Pol II to the viral promoter boosting viral gene expression. Tat interacts with the positive elongation factor P-TEFb and with HATs including CBP, PCAF, GCN5 and TIP60 [reviewed by (27)]. Thus, Tat and any of the cellular activators bound to the LTR promoter might recruit HATs and chromatin-modifying coactivators to influence chromatin structure. It has been shown that activation of LTR by Tat results in the hyperacetylation of histone H3 and H4 and displacement of positioned nucleosomes at the promoter (183, 289, 290). Collectively this evidence shows that HIV-1 life cycle involves the association of viral DNA with chromatin and that the virus has evolved mechanisms to use the cellular machinery to remodel chromatin during transcription of viral genes.

4.6.2. Simian virus 40.

Simian virus 40 (SV40) is a double stranded circular non-enveloped DNA virus. The viral DNA is packaged into nucleosomes in the virion and remains associated with histones during lytic infection (review by (130) and references therein). SV40, a relatively small virus, contains two transcriptal units expressed in a temporal cascade. The early gene transcript due to differential splicing, encodes the large T antigen (LT), small T antigen (ST) and 17K T antigen (17KT) [reviewed by (251)]. These viral proteins induce cell cycle progression producing the proper environment for viral DNA replication. The SV40 genome contains a nucleosome-free region that includes the promoter directing early gene expression and the viral origin of replication (115, 256).

The absence of histones in these regions is thought to facilitate the binding of cellular transcription factors for the expression of the early genes. Viral early expression is then followed by viral DNA replication and late gene expression. The transcription start site of the late promoter is associated with nucleosomes and remains transcriptionally silent until viral DNA synthesis occurs [reviewed by (251)]. Thus, nucleosomes are present on SV40 throughout lytic cycle. The absence of nucleosomes at the promoter region and the dependence of late gene expression on viral DNA replication suggest that chromatin might be inhibitory for the expression of viral genes.

4.6.3. Adenovirus.

Adenovirus is a non-enveloped double stranded linear DNA virus. The viral DNA on the virion particle is associated with four viral encoded proteins polypeptide V, VII, mu and the terminal protein (TP). Among these core proteins, the basic viral protein VII is the most tightly bound to the viral DNA (reviewed by (130) and references therein). Micrococcal nuclease digestion assays have shown that during infection viral DNA is protected resulting in the formation of discrete nucleosomal-like fragments. However, the length of the protected DNA fragment does not seem to correspond to the size of the nucleosomal repeat of cellular chromatin (57, 199, 279). These results suggest that structures different than canonical nucleosomes are assembled on viral DNA during infection. Indeed, at least some fraction of the viral DNA remains associated with protein VII during lytic infection (42, 120). Thus, the association of protein VII with the viral

DNA observed in the virion particle is maintained throughout early times of infections and might be responsible for the nuclease protection of the viral DNA observed during infection. However, the absence of histones on viral DNA during infection has not been demonstrated (120).

The major viral activator of early gene expression, E1A, interacts with chromatin-modifying coactivators such as CBP/p300, TRRAP/GCN5 and ATP-dependent chromatin remodeling complexes (8, 76, 181). These interactions however are not necessarily an indication of chromatin remodeling on viral promoters, since E1A have been shown to activate cellular genes and disrupt normal cellular gene expression leading to cell cycle progression and tumorgenesis. The mechanisms used by E1A for disrupting the cellular gene expression also include displacement of HATs from their cellular functional targets and modulation of the catalytic activity of the targeted HATs [reviewed by (39)].

E1A has been shown to influence the chromatin structure of targeted cellular genes. The association of E1A with E2F responsive genes results in the elimination of histone H3 K-9 methylation and in the acetylation of the same residue on histone H3 (81). As for the viral gene expression, the activation of the early promoter by E1A in transfection assays requires the HAT activity of CBP/p300 and results in the hyperacetylation of histone H4 at the transfected early promoter (71). These results obtained with the adenovirus early promoter suggest that the activation of viral promoter during infection might require chromatin remodeling activities; however the actual role

of chromatin and chromatin modifying activities during adenovirus infection remains largely unknown (120).

4.6.4. Herpesviruses

Examples of well-studied herpesviruses where the association of histones with viral DNA and the role of chromatin during infections have been explored include cytomegalovirus (a beta-herpesvirus), Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus (both gamma-herpesviruses).

In the case of CMV, latency is established in monocytes and reactivation of the virus is observed during differentiation to macrophages (109, 198). The reactivation of viral gene expression induces changes in DNase I hypersensitive sites at the promoter region of the major immediate early protein (MIEP) (216). The CMV reactivation is also stimulated by inhibitors of histone deacetylase trichostatin A, further connecting changes in chromatin structure with activation of viral genes (208). In non-permissive cells the MIEP promoter is associated with heterochromatin protein 1 (HP1) suggesting the formation of heterochromatin-like structures on the silent viral genome (208). During reactivation, the MIEP promoter is associated with acetylated histone H3 and histone H4 indicating the presence of nucleosomes on CMV DNA (208, 278). As for chromatin-modifying activities that might be recruited to viral genes, the immediate early protein

IE86 has been shown to interact with HAT P/CAF and the viral activator pUL69 with the Spt6 subunit of the FACT complex (Facilitates Chromatin Transcription) (32, 309).

Epstein-Barr virus establishes latent infection in B lymphocytes. The latent viral DNA is organized in nucleosomes and maintained as an extrachromosomal episome (62, 258). During latent infections the viral activator EBNA2 activates the expression of viral proteins require for the immortalization of lymphocytes (50). The activation domain of EBNA2 can interact with chromatin-modifying coactivators including HATs CBP, p300 and P/CAF and the human SWI/SNF complex (296, 312). The activation of two viral promoters by EBNA2 results in the hyperacetylation of histones H3 and H4 (4). During the early stages of the lytic reactivation cycle the viral activator Zta activates the expression of several viral genes (134, 170). Zta interacts with CBP and stimulates its HAT activity in vitro (43). During reactivation of EBV infected cells CBP is recruited to viral promoters resulting in hyperacetylation of histone H3 and H4 (59).

Kaposi's sarcoma-associated herpes virus (KSHV) is maintained as a multicopy episome in latenly infected B-lymphocytes. During latency only a small group of viral genes are expressed and are thought to contribute to deregulate the cell cycle [reviewed by (118)]. The reactivation of the latent KSHV requires the viral transcriptional activator protein Rta (90, 180). Inhibitors of histone deacetylases trichostatin-A and sodium butyrate stimulate KSHV reactivation suggesting a role of chromatin in regulation of viral gene expression. Rta interacts physically and functionally with CBP and SWI/SNF and induces changes in chromatin structure on responsive genes during reactivation (93,

178). The promoter of the ORF50 coding for Rta contains a positioned nucleosome at the transcription start site that is remodel upon transcriptional activation observed during reactivation from latency (178).

Collectively this evidence indicates that viruses have evolved mechanisms to modulate chromatin structure during their life cycle. The presence of nucleosomes on viral DNA is generally associated with gene repression and thus viral activators are known to recruit chromatin-modifying coactivators to remodel the chromatin structure of promoters. Examples of chromatin-modifying coactivators that interact with viral activators are major HAT such as CBP and p300 and mammalian SWI/SNF complexes. Interesting, during lytic infections by different types of viruses including polyomaviruses (SV40), adenovirus, and HSV-1 late gene expression is coupled to viral DNA replication (reviewed by (130) and references therein) suggesting that changes in chromatin configurations during DNA replication might be another mechanism to overcome repressive effects of chromatin.

The presence of nucleosomes in viruses from the Herpesviruses family is well established during latency. The role of chromatin remodeling has been studied during reactivation of Beta- and Gamma-herpesviruses. For Alpha-herpesviruses (HSV-1) the role of chromatin remodeling during reactivation remain largely unexplored and only very recently the role of chromatin remodeling has been addressed on viral gene expression during lytic infection (105, 135).

Appendix A

Chromatin immunoprecipitation assays

A.1. Chromatin immunoprecipitation protocol.

The chromatin immunoprecipitation protocol used for studying association of proteins with viral DNA during herpes simplex virus infection is based on protocols described in (26) and in Current Protocols in Molecular Biology (Chapter 21).

ChIP assays typically used one p150 tissue culture dish with 2 x10^7 cells per immunoprecipitation sample. The protocol described below was typically used for three immunoprecipitation reactions.

Day 1:

1. Seed 3 p150 tissue culture dishes each with approximately 1 x 10^7 HeLa cells.

Day 2:

2. HSV-1 infection. Use multiplicity of infection of 5 pfu/cell.

    2.1. Wash cells with 10 ml DMEM w/o FBS and add inoculum in 4 mL of DMEM w/o FBS.

    2.2. Incubate 1 hour at 37 °C with periodic rocking of the plates.

    2.3 Aspirate inoculum. Wash cells with 10 mL of DMEM w/o FBS.

    2.4 Add 25 mL of DMEM 2% FBS. Incubate at 37 °C.

3. Cross link protein and DNA complexes with formaldehyde.

3.1. Add 0.69 mL formaldehyde 37% directly to the medium to obtain 1% final concentration. Incubate at room temperature for 15 minutes.

3.2. Quench cross link reactions by adding 3.67 mL of glycine 1 M to yield 125 mM final concentration.

4. Prepare chromatin samples.

4.1 Wash with cold PBS supplemented with 0.5 mM fresh PMSF (PBS/PMSF).

4.2 Add 10 ml of PBS/PMSF. Scrape cells. Transfer to a 50 ml conical tube.

4.3 Collect cells by centrifugation (1,000 rpm for 5 min).

4.4. Wash pellet with 10 mL PBS/PMSF. Centrifuge.

4.5. Resuspend cells in 3 ml swelling buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5 % IGEPAL CA-630, 0.5 mM PMSF, 100 μg/ml leupeptin and aprotinin). Incubate on ice for 20 min.

4.6. Disrupt cells using 20 strokes of dounce homogenize with B pestle.

4.7. Collect samples by centrifugation. 4,000 rpm for 10 min.

4.8. Resuspend pellet in 0.5 mL sonication buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1, 0.5 mM PMSF, 100 μg/ml leupeptin and aprotinin). Incubate on ice 10 min.

4.9. Sonicate samples on ice to obatain DNA fragments of 200-500 bp in average using sonicator. (Use microtip with 5 pulses of 30 seconds each. Cycle time 1 second. Out put control: 3).

4.10. Centrifuge samples at 14,000 rpm for 10 min at 4 °C. Save supernatant.

5. Immunoprecipitations.

5.1. Prepare 50% slurry of protein-G agarose (Upstate) in triton lysis buffer: (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% BSA. supplemented with 10 μg/ml salmon sperm DNA). Wash protein-G beads with triton lysis buffer 5 times. Centrifuge at 500 rpm for 5 min between washes.

5.2. Add 40 μL protein-G agarose beads (50% slurry) to supernatant from step 4.10. Incubate at 4 °C for 2 h on rotator. Centrifuge at 500 rpm for 5 min. Supernatants are referred as precleared samples.

5.3. Incubate 150-180 μL of the precleared sample (from step 5.2.) plus 0.9 mL triton lysis buffer plus antibody (10-50 μg/ml) overnight at 4 °C. Retain at least 20 μL of precleared sample to prepare 10% input samples.

Day 3:

5.4. Add 40 μL of protein-G beads. Rotate for 4-5 hours at 4 C.

5.5. Centrifuge at 500 rpm for 5 min. Save supernatant in case unbound fraction need to be analyzed.

5.6. Wash beads by adding 1 ml Triton wash buffer. Rotate 5 min at room temperature.

5.7. Repeat step 5.5 with Lysis Buffer 500 (0.1% sodium deoxycholate, 1 mM EDTA, 50 mM HEPES pH 7.5, 500 mM NaCl, 1%, Triton X-100) then LiCl/detergent (0.5% sodium deoxycholate, 1 mM EDTA, 10 mM EDTA, 250 mM LiCl, 0.5% IGEPAL CA-630, 10 mM Tris-HCl pH 8.0) and finally with Tris-EDTA pH 8.0.

6. Elution:

6.1. Add 100 µL elution buffer (10 mM EDTA, 1% SDS, 50 mM Tris-HCl pH 8.0) to the beads. 20 min at 65 °C. Transfer supernatant to a new tube.

6.2. Add 150 µL TE/0.67% SDS to the beads, invert several times and then centrifuge up to full speed. Combine this second elution (150 µl) with the first – step 6.1.-. (This combined material is referred to as the precipitate).

7. Reverse cross links.

7.1. Add 62.5 µL of 1M NaCl to yield 200 mM final concentration and 10 µg of RNAase A to reverse crosslinks. Incubate samples at least 6 hours at 65 °C.

7.2. In parallel prepare 10% input sample. Use 15-18 µL of precleared sample from step 5.2. plus TE/0.67% SDS to 250 µL. Usually this incubation is overnight.

Day 4:

8. Purification of precipitated DNA.

8.1. Precipitate samples with 2 volumes of ethanol.

8.2. Resuspend samples in 100 µL of TE pH 7.5 plus 5X proteinase K buffer and 20 µg of proteinase K. Incubate at 42 °C for 2 hours.

8.3. Extract with phenol/chloroform/isoamylalcohol (1 vol = 125 µL) *.

8.4. Precipitate with 2 volumes of ethanol and 1/10 volume 3M sodium acetate (pH 5.3).

8.5. Resuspend in 75 μL TE pH 8.0.

*. Alternatively DNA can be purified after proteinase K treatment using QIAquick PCR purification kit (QIAGEN cat# 28104). Elute DNA using 75 μL of TE pH 8.0.

PCR analysis.

Precipitated samples can be analyzed using semi-quantitative polymerase chain reaction (PCR) follow by ethidium bromide stained agarose gels or using quantitative real-time PCR (qPCR). PCR conditions used for analysis of ChIP samples are described below.

Semi-quantitative PCR.

PCR were performed to detect specific viral or cellular gene fragments in the immunoprecipitated samples. These fragments included the promoters of the HSV IE genes (ICP0, ICP27, and ICP4); the coding region of the ICP27 gene; the promoters of viral DE (TK) and L (VP16, glycoprotein C) genes; and the promoters of cellular genes U3 snRNA and IFN-β (Table 1). Parallel PCRs were routinely performed on serial dilutions of input samples (typically corresponding to 0.5%, 0.1%, and 0.02% of the total material) to confirm that observed signals were within linear range of the assay and were comparable between different set of primers. Standard PCR conditions included 0.25 μM of each primer, 2.5 U Taq DNA polymerase (Invitrogen), 0.1 mM each deoxynucleotide triphosphate, 2 mM $MgCl_2$, 10% Enhancer solution (Invitrogen), with incubation at 95°C

for 5 min followed by 30 to 35 cycles of 95°C for 30 sec, 65°C for 30 sec and 72°C for 1 min and ending with 5 min at 72°C. The Enhancer solution was omitted from reactions amplifying the ICP27 ORF, U3 snRNA promoter, and IFN-β promoter fragments. Annealing of primers for amplification of the IFN-β promoter and the GAPDH ORF was performed at 55 °C.

Quantitative real-time PCR.

qPCR was used to quantify specific viral and cellular gene fragments in the immunoprecipitated samples. qPCR were performed using SYBR Green PCR core reagents kit (Applied Biosystems) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The fragments analyzed included the promoters of the HSV IE genes ICP0 and ICP27; the coding region of IE genes; the promoter and coding regions of viral DE (TK) and L (VP16, gC) genes; and the promoter of cellular genes U3 snRNA and IFN-β (Table 2). Parallel PCRs were routinely performed on serial dilutions of input samples (3%, 1%, 0.3%, 0.1%, 0.03%, 0.01% of the input material) to obtain standard curves for each PCR. IP and input dilution samples were routinely analyzed in duplicate. Figure 19 shows representative standard curves for qPCR detecting IE gene promoters ICP0, ICP27, L gene promoter VP16 and the promoter of the cellular gene U3 snRNA. The standard curves were used to determine the percentage of input present in each IP sample for a given fragment of DNA. To account for the background immunoprecipitation, the % input of "No Ab" samples was subtracted from the % input of IP samples and reported as "normalized IP" signal. The intercept with y-axis of a particular standard curve might be used to compare the amounts of viral DNA when two

different infections are analyzed. Standard PCR conditions included 3 μL of template (IP sample or input dilution), 0.25 μM of each primer, 0.75 U Taq DNA polymerase (Applied Biosystems) and 2-3 mM $MgCl_2$ in 30 μL final volume.

Table 1. Oligonucleotides used in semi-quantitative PCR reactions analysis of ChIP samples. PCR primers were designed to detect viral immediate early (IE), delayed early (DE) and late (L) gene fragments. Selected cellular (C) gene fragments were also used. The orientation of the primer compare to the transcript unit is designed as (F) for forward and (R) for reverse direction. PCR end point are relative to the transcription start site (+1) of each gene.

Gene	Class	Region	Oligonucleotide sequence	PCR ends
ICP0	IE	P	(F) 5'-CCCTGGGGTTCCGGGTATGGTAATGAG (R) 5'-GGCTAACTTATACCCCACGCCTTTCCCC	-322 to -12
ICP4	IE	P	(F) 5'-GGGCTCGTATCTCATTACCGCCGAACC (R) 5'-GGCGTCCTCGGGCTCATATAGTCCCAG	-376 to -9
ICP27	IE	P	(F) 5'-GTGTAGCCTGGATCCCAACGACCCC (R) 5'-CGGCCGGGTGGTGGATGTCCTTATAC	-281 to -3
		ORF	(F) 5'-TGCATCCTTCGTGTTTGTCATTCTGG (R) 5'-GCCGTCAACTCGCAGACACGACTC	1099 to 1249
TK	DE	P	(F) 5'- GAGCGTCCGTTGGGCGACAA (R) 5'- TTCGAGGCCACACGCGTCAC	-297 to 1
VP16	L	P	(F) 5'-CAGCCCGCTCCGCTTCTCG (R) 5'-GCCGCCCCGTACCTCGTGAC	-272 to -47
gC	L	P	(F) 5'-GGCGTCGCCTGCACAGGAAG (R) 5'-CCCGTGTCCGGAATTT	-269 to -7
U3	C	P	(F) 5'-GCACCACACCAGGAGCAAAC (R) 5'-CGCTAGTTCCGATGCCATTAGG	-295 to -71
IFN-β	C	P	(F) 5'-CCTCACAGTTTGTAAATCTTTTTCC (R) 5'-TCGAAAGGTTGCAGTTAGAATG	-197 to 12
GAPDH	C	ORF	(F) 5'-AGGTCATCCCTGAGCTGAAC (R) 5'-GCAATGCCAGCCCCAGCGTC	731 to 980

Table 2. Oligonucleotides used in quantitative real-time PCR analysis of ChIP samples. PCR primers were designed to detect viral immediate early (IE), delayed early (DE) and late (L) gene fragments. Selected cellular (C) gene fragments were also used. The orientation of the primer compare to the transcript unit is designed as (F) for forward and (R) for reverse direction. PCR end point are relative to the transcription start site (+1) of each gene. (N.A.= not available)

Gene	Class	Region	Oligonucleotide sequence	Location
ICP0	IE	P	(F) 5'-GGCCGTGCATGCTAATGATA (R) 5'-CTTATACCCCACGCCTTTCC	-172 to -20
		ORF	(F) 5'-CTGTCGCCTTACGTGACCAA (R) 5'-CCATGTTTCCCGTCTGGTC	3022 to 3133
ICP4	IE	P	N.A.	N.A.
		ORF	(F) 5'-GAAGTTGTGGACTGGGAAGG (R) 5'-GTTGCCGTTTATTGCGTCTT	4125 to 4257
ICP27	IE	P	(F) 5'-TGGTGTCTGATTGGTCCTTG (R) 5'-CGGGTGGTGGATGTCCTTAT	-129 to -6
		ORF	(F) 5'-TGCATCCTTCGTGTTTGTCATTCTGG (R) 5'-GCCGTCAACTCGCAGACACGACTC	1099 to 1249
TK	DE	P	(F) 5'- GAGCGTCCGTTGGGCGACAA (R) 5'- TTCGAGGCCACACGCGTCAC	-297 to 1
		ORF	(F) 5'-TACCCGAGCCGATGACTTAC (R) 5'-AAGGCATGCCCATTGTTATC	354 to 506
VP16	L	P	(F)5'-CAGCCCGCTCCGCTTCTCG (R) 5'-GCCGCCCCGTACCTCGTGAC	-272 to -47
		ORF	(F) 5'-TGTTTGACTGCCTCTGTTGC (R) 5'-GTTAAGGTGCTCGCGAATGT	925 to 1079
gC	L	P	(F) 5'-TCGGGCGATTGATATATTTTT (R) 5'-TGTCCCCTTCCGGAATTTAT	-131 to -11
		ORF	(F) 5'-AGAGGAGGTCCTGACGAACA (R) 5'-GCCCGGTGACAGAATACAAC	663 to 806
U3	C	P	(F) 5'-GCACCACACCAGGAGCAAAC (R) 5'-CGCTAGTTCCGATGCCATTAGG	-295 to -71
IFN-β	C	P	(F) 5'-CCTCACAGTTTGTAAATCTTTTTCC (R) 5'-TCGAAAGGTTGCAGTTAGAATG	-197 to 12

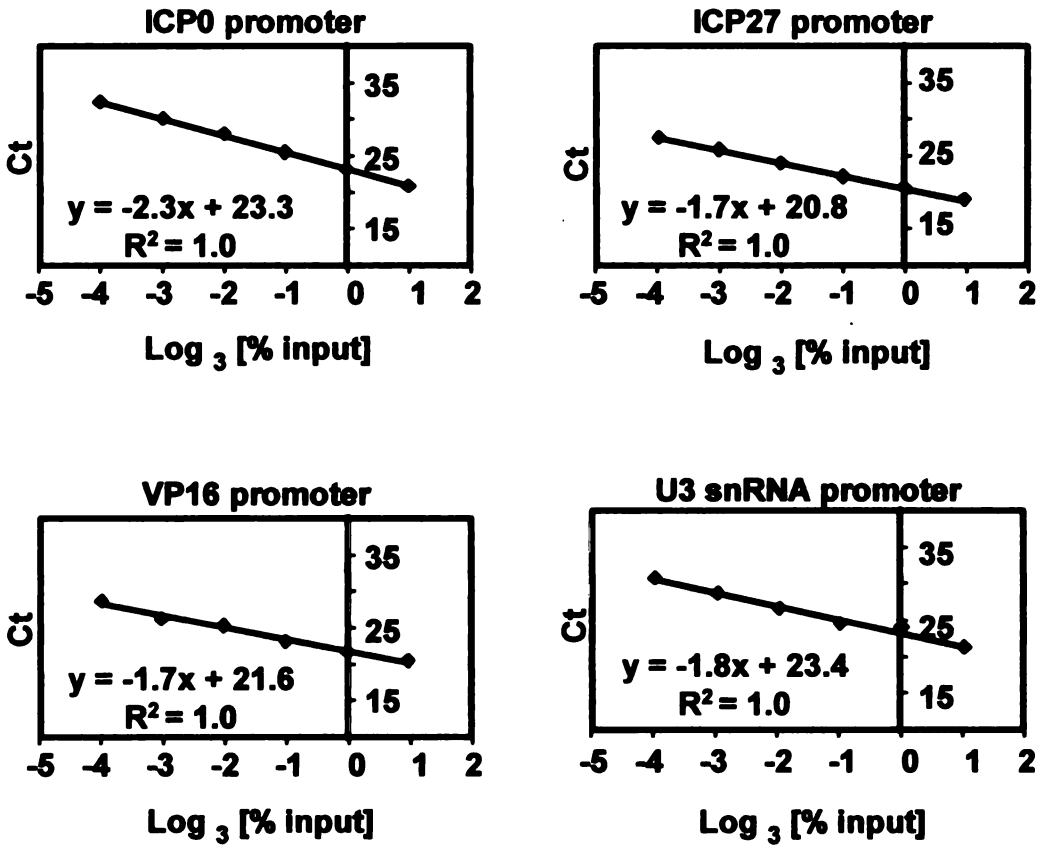


Figure 19. Standard curves for quantitative real-time PCR used for analysis of ChIP samples. 10% input material were prepared in parallel with immunoprecipitation samples and subsequently 3-fold diluted to obtain a range from 3% to 0.012% input.

Appendix B

Quantitative RT-PCR analysis for viral gene expression

B.1. qRT-PCR protocol.

Quantitative real-time PCR analysis (qRT-PCR) was established to measure viral gene expression during infection of cultured cells. Gene expression analyses were typically performed by infecting approximately 2 x10^6 HeLa cells at multiplicity of infection of 1-10 pfu per cell. Total RNA was isolated at 2 hours post-infection using TRI-Reagent following the instructions provided by the manufacturer (Molecular Research Center). cDNA was prepared using 1 μg total RNA and a randomly-primed reverse transcription system (Promega). cDNA samples were then analyzed using qRT-PCR analysis as described below.

cDNA samples from infected cells were analyzed with quantitative PCR using SYBR Green PCR core reagents kit (Applied Biosystems) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). PCR conditions included 0.25 μM each primer and 2-3 mM $MgCl_2$. Relative levels of viral gene expression were obtained using the $2^{-\Delta\Delta Ct}$ method (175). The two samples a and b, measuring gene "x" and using gene "y" as endogenous control, the equation used for obtaining ΔΔCt was [Ct(X)a-Ct(Y)a]-[Ct(X)b-Ct(Y)b]. PCR detecting 18S rRNA was used as an endogenous control to normalize gene expression. The use of 18S rRNA controls for potential differences in cDNA preparation between samples. Since the Tri-Reagent protocol allows the simultaneous isolation of DNA and RNA from the infected cells, viral gene expression

can be normalized to the amount of viral DNA delivered during each infection. This control is important especially when comparing gene expression levels between different viral strains that might have different infectivity. To compare the amount of DNA delivered between infections, total DNA was isolated from infected cells following the instructions provided by the manufacturer (Molecular Research Center) and used for qPCR analysis detecting a viral DNA fragment. Relative levels of viral DNA delivered between infections were calculated with the $2^{-\Delta\Delta Ct}$ method (175) and using 18S rDNA gene as endogenous control.

Table 3. Oligonucleotides used in quantitative real-time PCR analysis of cDNA samples. PCR primers were designed to detect viral immediate early (IE), delayed early (DE) and late (L) genes. The cellular (C) gene coding for 18S rRNA was used as endogenous control. The orientation of the primer compare to the transcript unit is designed as (F) for forward and (R) for reverse direction. PCR endpoints are relative to the transcription start site (+1) of each gene.

Gene	Class	Oligonucleotide sequence	PCR end points
ICP0	IE	(F) 5'-CTGTCGCCTTACGTGACCAA (R) 5'-CCATGTTTCCCGTCTGGTC	3022 to 3133
ICP4	IE	(F) 5'-GAAGTTGTGGACTGGGAAGG (R) 5'-GTTGCCGTTTATTGCGTCTT	4125 to 4257
ICP22	IE	(F) 5'-TTTGGGGAGTTTGACTGGAC (R) 5'-CAGACACTTGCGGTCTTCTG	1575 to 1712
ICP27	IE	(F) 5'-TGCATCCTTCGTGTTTGTCATTCTGG (R) 5'-GCCGTCAACTCGCAGACACGACTC	1099 to 1249
ICP47*	IE	(F) 5'-GTACGACCATCACCCGAGTC (R) 5'-GACGGCACGCCTTTTAAGTA	808 to 935
TK	DE	(F) 5'-TACCCGAGCCGATGACTTAC (R) 5'-AAGGCATGCCCATTGTTATC	354 to 506
VP16	L	(F) 5'-TGTTTGACTGCCTCTGTTGC (R) 5'-GTTAAGGTGCTCGCGAATGT	925 to 1079
gC	L	(F) 5'-AGAGGAGGTCCTGACGAACA (R) 5'-GCCCGGTGACAGAATACAAC	663 to 806
18S rRNA	C	(F) 5'-CCGCAGCTAGGAATAATGGA (R) 5'-CGGTCCAAGAATTTCACCTC	850 to 975

* The ICP47 primer overlaps with the US11 transcript. At early times of infections US11 is not expressed and thus this PCR detects ICP47 gene expression levels.

B.2. Assay validation.

B.2.1. Standard curves for qPCR primer pairs.

In order to validate qRT-PCR analysis for viral gene expression, each PCR primer pair was tested using standard curves prepared with two fold dilutions of DNA template. These standard curves were used to confirm that the assays yielded the expected linear relationship between logarithm of the template concentration and the Ct values for the range used in gene expression analysis during infection (data not shown). The slope of the standard curve was used to confirm that each cycle resulted in a doubling of template as expected when using the $2^{-\Delta\Delta Ct}$ method for relative quantification. The presence of a single product in each reaction was verified by analyzing the dissociation curves generated by the ABI Prism 7700.

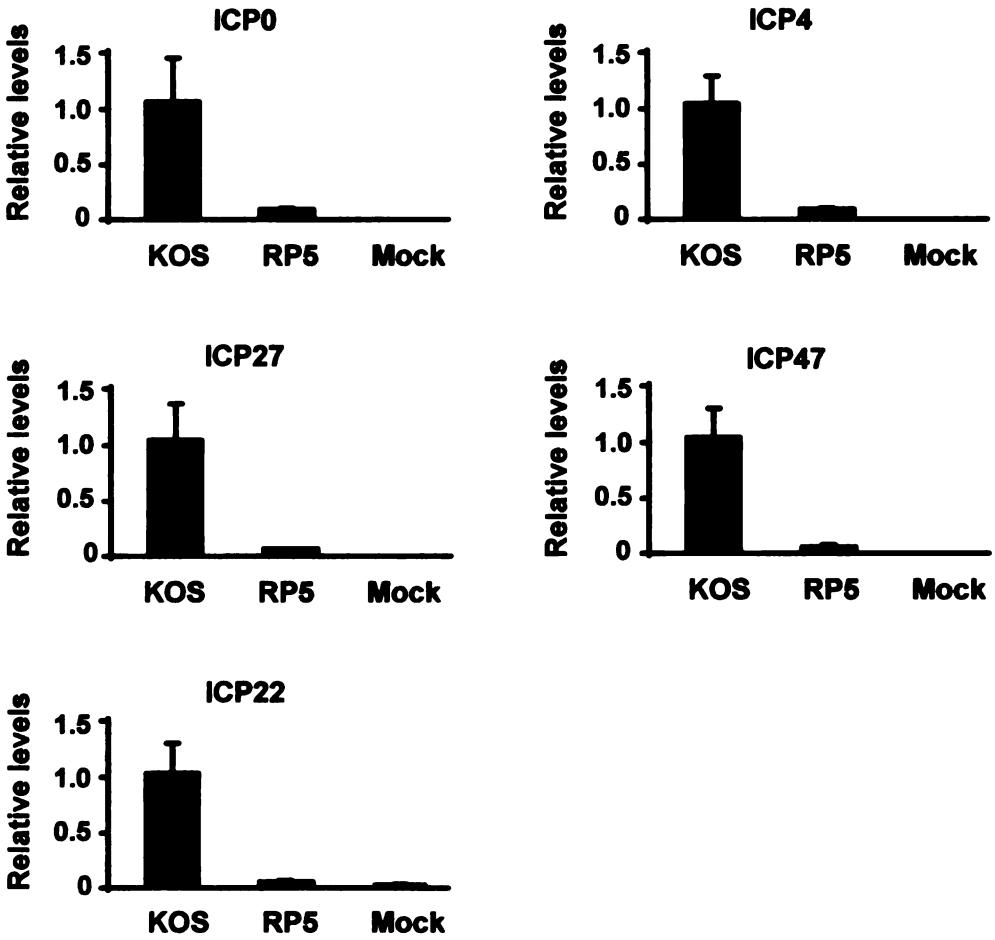
B.2.2. Analysis of viral gene expression of KOS, RP5 and mock infected cells.

The specificity of the qRT-PCR analysis was tested by comparing viral gene expression of KOS, RP5 and mock-infected cells. RP5 virus lacking the activation domain of VP16 is defective for IE gene expression (263, 276, 323). Mock infections were included to further test the specificity of the PCR reactions for viral gene expression. Infections were performed in triplicate at a multiplicity of infection of 1 plaque forming unit per cell for KOS. RP5 infections used multiplicity of infection of

0.01 plaque forming unit to deliver similar amount of viral DNA. Infections were performed by incubating the appropriate viral inculum in 200 μL DMEM without FBS in p60 tissue culture plates. After one hour, the inoculum was washed and 2 mL of DMEM with 2% FBS was added. After one additional hour of incubation at 37 °C, total DNA and RNA was isolated and cDNA was prepared as described on B2.1.

Figure 20 shows the result of qRT-PCR analysis of IE gene expression at 2 hours post-infection using the $2^{-\Delta\Delta Ct}$ method and normalized to the amount of DNA delivered in each infection. As expected, RP5 infections resulted in reduced expression of IE genes compared to viral gene expression in KOS infection (Figure 20A). Little or no signal is detected in mock-infected samples further showing the specificity of the qPCR for viral gene expression. To normalize for the amount of viral DNA delivered during KOS and RP5 infections, total DNA was analyzed using qPCR detecting the ICP27 promoter (as a probe for viral DNA) and 18S rDNA as an endogenous control. Figure 20B shows that RP5 infections did not deliver the same amount of viral DNA in these parallel infections. This observation reinforces the importance of analyzing the levels of viral DNA delivered when comparing gene expression levels between different viral strains. Using this analysis of the amount of viral DNA delivered during infection, the inoculum volumes can be corrected so subsequent infections using the same viral stocks will deliver comparable amounts of DNA.

A.



B.

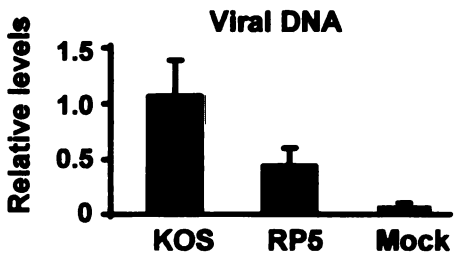


Figure 20. qRT-PCR analysis of viral gene expression of cells infected by KOS or RP5 viral strains. A) qRT-PCR analysis of viral gene expression in KOS, RP5 and mock-infected cells normalized to the amount of DNA delivered in each infection. B) Amount of viral DNA delivered in KOS, RP5 and mock infected cells. Errors bars represent the range of values obtained between biological triplicates, each measured in duplicate.

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