DIFFERENTIAL CELLULAR REQUIREMENTS FOR ICP4-MEDIATED ACTIVATION OF HSV-1 EARLY AND LATE GENES

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The herpes simplex virus type 1 immediate-early protein, ICP4, is the major transcriptional activator of viral early and late genes. ICP4 has been shown to bind DNA and interact with components of the general transcription machinery to activate or repress viral transcription, depending on promoter context. Microarray and northern blot analysis indicated that the abundance of the basal Pol II transcription machinery was significantly altered at late times of infection. Because viral early and late genes have very different promoter architectures, the cellular requirements for ICP4-mediated activation of early and late genes may differ. In testing this hypothesis using tk and gC as representative early and late promoters respectively, the general transcription factor TFIIA was found not to be required for ICP4 activation of the late gC promoter but was essential for activation of the early tk promoter. An intact INR element was required for TFIIA to be dispensable for ICP4 activation of the gC promoter. In the presence of TFIIA, ICP4 overcame the requirement for an intact INR on the gC promoter enhancing ICP4 activation of an INR-mutated gC promoter. When examining the binding properties of TFIIA, ICP4 and TBP/TFIID on early, late, and INR-mutated late promoters, ICP4 could only effectively substitute for TFIIA in stabilizing the binding of TFIID to the TATA box of the late promoter containing a functional INR. ICP4 required the additional activities of TFIIA to stabilize the binding of TFIID to the TATA box of an INR-mutated late promoter. Additionally, microarray and northern blot analysis indicated that TFIIA expression was reduced at late times

of infection. The decrease in TFIIA expression during infection, the ability of ICP4 to substitute for TFIIA in stabilizing TFIID binding to an INR-containing late promoter, and its dispensability for activation of late but not early genes suggests a mechanism for the shutoff of early gene expression while allowing the continued expression of late genes.

ACKNOWLEDGEMENTS

In loving memory of
Chet Zabierowski
(July 20, 1929 – October 8, 2003)

"This is for you, dad……."

Stevie Nicks
Landslide

TABLE OF CONTENTS

LIST OF FIGURES

1. INTRODUCTION

1.1. HERPESVIRIDAE FAMILY

Members of the Herpesviridae family consist of a group of DNA viruses composed of a 150 - 200 nm diameter virion containing at least four components: a lipid bilayer envelope studded with 10 to 12 different surface glycoprotein projections, an amorphous protein layer known as the tegument containing viral proteins necessary for lytic infection, a 100 nm icosahedral nucleocapsid which houses the viral genome, and a DNA core organized into a toroidal form with no associated packaging proteins (15). The viral genome consists of 120 - 200 kb of linear double stranded DNA encoding 60 – 120 genes. Members of this family replicate in the nucleus of a wide range of vertebrate hosts: including humans, horses, cattle, mice, pigs, chickens, turtles, lizards, fish, as well as in some invertebrates (167). Eight varieties have been isolated in humans that include herpes simplex virus 1 and 2 (HSV-1, HSV-2), varicella-zoster virus (VZV), human cytomegalovirus (HCMV or HHV-5), Epstein-Barr virus (EBV or HHV-4), human herpesvirus 6 and 7, and Kaposi's sarcoma herpesvirus (HHV-8).

Members of the herpesviridae family are further classified into three subfamilies: the alpha-, beta- and gamma-herpesviruses, based on tissue tropism, host range, duration of the reproductive life cycle and behavior under tissue culture conditions in the laboratory (reviewed in 168). Alpha-herpesviruses have a variable host range, a relatively short reproductive life

cycle, visible cytopathic effects (CPE), and are neurotropic, establishing latency primarily in the sensory nerve ganglia. Members of this subfamily include Herpes simplex virus type 1 and 2, the primary agents of recurrent facial and genital herpetic lesions, and Varicella-zoster virus (VZV), the causative agent of chicken pox and shingles. Members of the beta-herpesviruses are lymphotropic, establishing latency in secretory glands, lymphoreticular cells and kidneys. These have a more restricted host range, a longer reproductive cycle than alpha-herpesviruses, and infected cells frequently become enlarged (cytomegalo). Members of this subfamily include Human cytomegalovirus (HCMV or HHV-5), which causes infections in newborns and the immunocompromised, and Human herpesvirus 6 and 7 (HHV-6 and -7), which has been linked to mild early childhood diseases. Gamma-herpesviruses are also lymphotropic, specific for either T- or B-lymphocytes. Members of this subfamily include the causative agent of infectious mononucleosis, Epstein-Barr virus (EBV or HHV-4), and the recently described Kaposi's sarcoma herpesvirus (KSHV-8 or HHV-8), which has been linked to human cancers.

1.2. HSV PATHOGENESIS

Of the eight known human herpesviruses, herpes simplex virus type 1 (HSV-1) is the most extensively investigated. It is transmitted through close personal contact of susceptible oropharaynx or genital epithelia with infectious active virus. Under certain conditions, HSV can also infect the eyes resulting in ocular herpes or other facial regions resulting in herpes keratitis (161). It can also infect the central nervous system leading to herpes encephalitis (191). During primary productive infection HSV undergoes DNA replication and amplification in susceptible

surface cells of the oral or genital mucosa. Viral progeny are produced eventually leading to cell lysis and subsequent infection of neighboring cells. Skin lesions that result from inflammation and cell death at the site of infection are commonly termed as cold sores, fever blisters or genital herpes lesions. From the initial site of infection, viral progeny gain access to innervating sensory neurons and travel via retrograde transport through the axons to the neuronal cell bodies in the sensory ganglia, such as the trigeminal ganglion or dorsal root ganglia (33). In the neuronal nucleus, the virus either replicates or establishes latency (reviewed in 186). The spread of virus from these neurons to adjacent or synaptically-linked neurons results in transneuronal infection and may be one method that results in viral encephalitis (114, 192, 193, 215).

During latency, little to no viral gene expression occurs except for the latency-associated transcript (LAT) (30) the function of which is currently under debate. Since neurons lack MHC class II expression and MHC class I expression is low, CD8+ cytotoxic and CD4+ helper T cells do not target these cells (159). Thus viral genomes persist in these neurons in an inactive histone associated episomal state for the lifetime of the host, with little fear of elimination by the immune system. Upon conditions of stress, such as fever, UV light or physical or emotional stress, or immunosuppression, the virus can reactivate (reviewed in 186). Once reactivated, the virus undergoes de novo protein synthesis and replication. Viral progeny travel via anterograde transport back to the primary site of infection where it re-establishes a productive infection known as recurrent infection (reviewed in 198).

1.3. HSV PRODUCTIVE LIFE CYCLE

HSV infection begins with attachment of a virus particle to the surface of a permissive cell. This involves recognition of cell surface receptors by virion glycoproteins (reviewed in 183). The initial association occurs between heparan sulfate proteoglycans on the cell surface and the viral glycoprotein gC. This is followed by specific interaction of viral glycoprotein gD with one or more of several cell surface receptors. These include HVEM (herpesvirus entry mediators), a member of the TNF receptor family, and nectin-1 (HveC) and nectin-2 (HveB), receptors that are related to the immunoglobulin superfamily. Binding of gD to one of these receptors triggers viral fusion, which requires the action of other glycoproteins, gB and a heterodimeric complex of gH-gL (in addition to gD and the gD receptor). Following fusion of the viral envelope with the host cell plasma membrane, the glycoprotein-studded envelope remains at the surface of the plasma membrane, while the de-enveloped virus particle enters the cell and is targeted via the intracellular transport machinery to nuclear envelope pores (182). After reaching the nuclear pores the viral DNA is released into the nucleoplasm.

In the nucleus, the regulated and ordered gene expression of the HSV genome occurs. Each gene has its own promoter that utilizes the host RNA polymerase II transcription machinery to transcribe its genes at the expense of cellular gene expression (184). HSV genes are expressed in three major kinetic classes: immediate-early (IE, α), early (E, β) and late (L, γ) (reviewed in 199, 203). Immediate-early genes are the first viral genes expressed due to the potent transactivation potential of the virion-associated protein VP16. Viral mRNA is processed in the nucleus, transported and translated in the cytoplasm. IE genes mainly encode nuclear bound proteins that are involved in transcriptional and post-transcriptional regulation of the rest of the

viral genome. Once IE proteins are produced and localized in the nucleus, early genes are expressed. Early gene products are involved in DNA synthesis and viral replication. Viral replication takes place when sufficient quantities of early proteins are present. Viral replication was originally thought to occur via a rolling circle mechanism from circularized genomic DNA (reviewed in 14) however recent evidence indicates that genomic DNA does not circularize upon entry into the nucleus (98) suggesting that replication occurs from a linear DNA template. Once DNA replication is underway, late genes are expressed encoding proteins that make up the structural components of the virion such as glycoproteins, capsid components, and tegument proteins.

Empty procapsids are assembled in the nucleus and packaged with linear double-stranded viral DNA. Packaged nucleocapsids bud into the inner leaflet of the nuclear envelope. Fusion then occurs with the outer nuclear leaflet, releasing the nucleocapsid into the cytoplasm where it acquires tegument proteins. Nucleocaspid with tegument is doubly enveloped by host membranes containing Golgi modified glycoproteins. Once matured, virions exit the cell through fusion of the outer host membrane containing the enveloped viruses with the surface of the plasma membrane (reviewed in 134).

During infection many viral induced cellular changes occur to prime the cell for viral gene expression and replication. One rapidly occurring change upon initial infection is the shutoff of host protein synthesis. This is due to the functions of the virion host shutoff protein (*vhs*), a tegument protein encoded by the UL41 gene. Once released into the newly infected cell, *vhs* increases the rate of mRNA degradation in the cytoplasm contributing to the global suppression of cellular protein synthesis (reviewed in 179). *vhs* is an mRNA-specific RNase that disrupts preexisting polyribosomes, and triggers rapid degradation of all mRNAs. It exhibits a

high degree of specificity, as it apparently targets only mRNAs, while sparing other cytoplasmic RNA species. However, *vhs* is not selective to cellular over viral mRNAs, as both are affected. This results in a global increase in mRNA instability of even viral messages. The increased mRNA instability accelerates the kinetics of viral gene expression by promoting the transition between each successive phase of viral gene expression favoring the HSV life cycle.

1.4. HSV GENOME CONFIGURATION

The HSV genome is approximately 152 kb of linear double stranded DNA (108) with a base composition of 67% G + C (130), packaged in the form of a torus in the virion (67). It consists of a covalently linked long (L) and short (S) region, each containing unique sequences (Fig. 1) (reviewed in 129). The unique L (U_L) region is 108 kb and encodes at least 56 distinct genes that are involved in DNA replication, viral gene expression or that make up the structural components of the virion. The unique S (U_S) region is 13 kb and contains 12 open reading frames encoding glycoproteins. These unique sequences are each flanked by inverted repeats, the L (R_L) and S (R_S) repeat. The 9 kb L repeats (R_L) encode the immediate-early protein ICP0 and the latency-associated transcript, while the 6.6 kb short repeats (R_S) encode the immediate-early protein ICP4. Genes located in the inverted repeat regions are thus present in two copies. The repeats of the L component are designated *ab* and *b'a'* and those of the S component are designated *a'c'* and *ca*. The structure of the *a* sequence is highly conserved but the number of *a* sequences at the junction between the L and S region and at the L terminus is variable from strain to strain. Depending on the number of *a* sequences at the junction of the two unique

regions and at the L terminus, the genome sizes of various isolates of a particular virus can vary up to 10 kb (197). The long and short regions can invert relative to one another causing the genome to exist as a mixture of four isomers in equimolar proportions (42). The *a* sequences at the ends of the linear molecule are important in both concatemerization and in packaging the DNA in the virion. There are three origins of replication. The ori_L is located in the middle of the U_L region, while the ori_S is located in the R_S and is present in two copies.

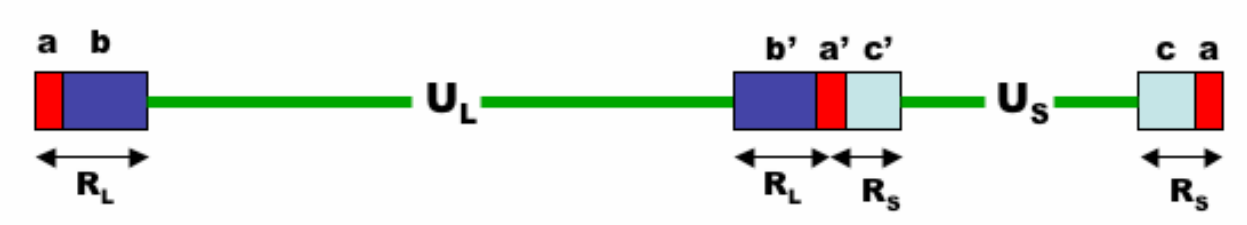


Figure 1. Schematic representation of the DNA sequence arrangement of the HSV genome.

The HSV genome is divided into unique long (U_L) and unique short (U_S) regions, composed of 108 kb and 13 kb of DNA, respectively. These unique sequences are separated by inverted repeats R_L and R_S (depicted as arrows) that are 9 kb and 6.6 kb, respectively. The R_L is composed of a and b sequences and the R_S is composed of a and c sequences. A varying number of a sequences are found at the terminus of U_L and the junction of U_L and U_S. Only one copy of a sequence is found at the U_S terminus. Each a', b' and c' represents an inverted sequence of a, b, and c sequences.

The HSV genome encodes at least 80 open reading frames that are distributed throughout the genome on both strands without many intervening sequences (reviewed in 129). The structure of HSV genes closely resembles that of cellular genes in that each viral gene contains a promoter, a 3' UTR, an open reading frame and a polyadenylation signal (8). However, one of the major distinctions between HSV and cellular genes is the lack of introns in the majority of HSV genes. Of the more than 80 viral transcripts expressed during infection, only four contain introns, with three of these four being immediate-early transcripts (155). The lack of introns serves to benefit the virus in mechanisms that will be discussed further below.

1.5. CASCADE OF HSV GENE EXPRESSION

During the lytic cycle of HSV-1 infection, synthesis of viral gene products occurs in three temporally regulated phases, immediate-early, early, and late (also termed α or IE, β or E, and γ or L respectively) (Fig. 2) (reviewed in 158, 199, 203). The pattern of viral protein synthesis is determined by the pattern of viral gene transcription and the level of viral mRNA accumulated in infected cells (102). Viral gene products regulate the cascade of gene expression through positive or negative regulation of their own genes or of genes in different kinetic classes. Expression of individual genes is regulated mainly at the level of transcription initiation (199, 200). Each gene contains its own promoter. Each promoter contains a TATA box that is recognized by the cellular TATA binding protein (TBP), allowing transcription of all viral genes by the host RNA Pol II transcription machinery (6, 36). Although HSV promoter structures contain many similarities to cellular RNA Pol II promoters, each class of genes differs with respect to the timing of expression and promoter architecture. Two major factors controlling transcription of HSV genes are the presence of both viral and cellular specific promoter elements associated with each of the three gene classes, and the trans-acting viral and cellular transcription factors that recognize these elements. The mechanism of sequential viral gene expression has been suggested to be due to class specific differences in promoter architecture. These are critical in determining the ability to nucleate the assembly of stable preinitiation complexes at various phases of infection mediating class-specific transcription kinetics. Additionally, DNA replication also has a significant influence on viral gene expression (reviewed in 199, 203). The

onset of DNA replication significantly reduces early gene expression while it is required for the initiation of late gene expression.

Expression of all three classes of genes ultimately leads to virus production, eventual death of the infected cell, virion release and spread to neighboring cells. In permissive cells, this entire process takes approximately 18 to 20 hours (Fig. 2) (93).

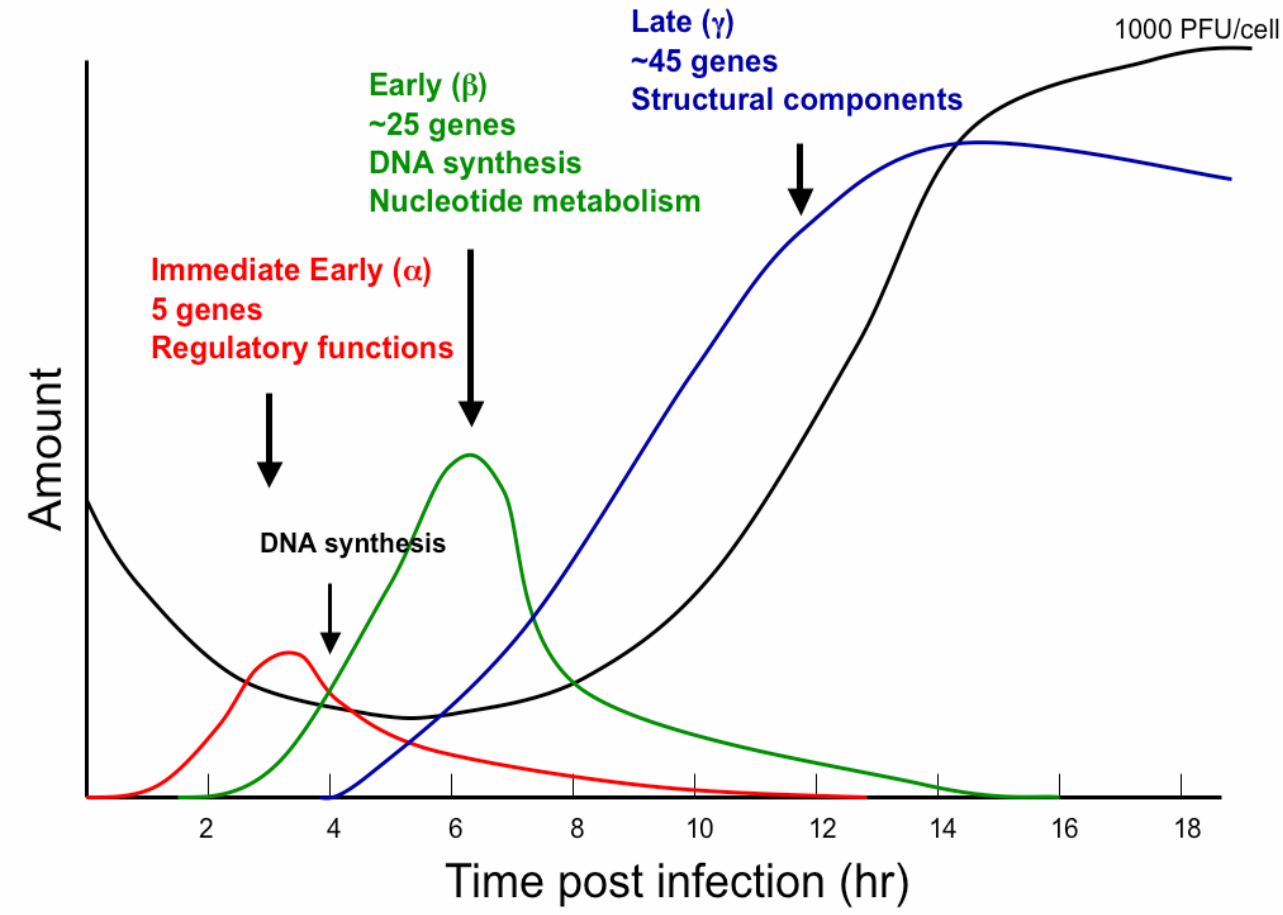


Figure 2. Cascade of HSV gene expression and viral progeny production in permissive cells.

The expression of immediate-early genes encoding viral transcriptional and post-transcriptional regulatory proteins, peaks expression at approximately 3 hour post infection and subsequently slowly decreases. Early gene expression encoding genes involved in nucleotide metabolism and DNA synthesis, progressively increases at 4 hours post infection, peaks at 6 hours and decreases thereafter. DNA replication can be detected at approximately 4 hours post infection. Late gene expression encoding structural components of the virus, steadily increases at 6 hours post infection and peaks at approximately 13 hours post infection. At around 8 hours post infection infectious viral progeny can be identified and the amount of infectious virus increases exponentially to 16 hours post infection when 1000 plaque-forming units per cell can be detected.

1.5.1. Regulation of immediate-early gene expression

Within the first hour of infection, immediate-early genes are transcribed, constituting the first phase of viral gene expression (Fig. 2) (93). Immediate early promoters are the most complex, with binding sites for both viral and cellular specific factors located upstream of the TATA box (Fig. 3). Expression of IE genes is mainly regulated at the level of transcription initiation through TAATGARAAT motifs found exclusively on IE promoters upstream of the TATA box. TAATGARAAT motifs are recognized specifically by the viral tegument-associated protein, VP16 (reviewed in 7, 89, 207). Upon release into newly infected cells, VP16 translocates to the nucleus where it associates with host cellular factors Oct1 and HCF. This viral and cellular protein complex then binds to TAATGARAAT motifs to trigger activation of IE genes. The activation domain of VP16 interacts directly with the general transcription factors TFIID, TFIIB and TFIIA to recruit and stabilize their interactions with IE promoters leading to the subsequent recruitment of the remaining general transcription factors to these promoter (reviewed in 65). In addition to VP16 binding sites, these promoters contain cellular cis-acting elements located upstream of the TATA box, such as CAAT box elements and Sp1 binding sites (101). These serve as binding sites for cellular specific factors that also interact with one or more of the general transcription factors. These factors synergize with the activities of VP16 to recruit and stabilize the cellular transcription machinery on IE promoters that in turn activate transcription. It is due to these VP16 specific binding sites that IE genes are rapidly expressed upon initial infection without any prior viral protein synthesis. Five immediate early products are produced:

ICP0, ICP4, ICP22, ICP27 and ICP47 (28, 93, 153). These proteins play critical regulatory roles in the progression of the lytic cycle. In the absence of competent immediate early proteins, particularly ICP4 and ICP27, minimal early proteins are produced, DNA replication is halted, and the lytic cycle does not progress (44, 51, 128, 169).

Peak synthesis of IE gene products occurs 3 hours post infection (hpi) and their expression significantly decreases thereafter (93). One mechanism for the attenuation of IE genes is through the actions of the immediate-early protein ICP4, which is largely responsible for the attenuation of its own expression (71, 166). ICP4 in conjunction with the general transcription factors TBP and TFIIB binds directly to high affinity ICP4 DNA binding sites located near the start site of transcription (80, 113, 181). Binding of ICP4 in a tri-partite complex to these high affinity sites is thought to block RNA Pol II from initiating transcription thus decreasing its expression. In the absence of ICP4, IE genes are over-expressed and E genes are under-expressed (31, 44, 51, 156, 176).

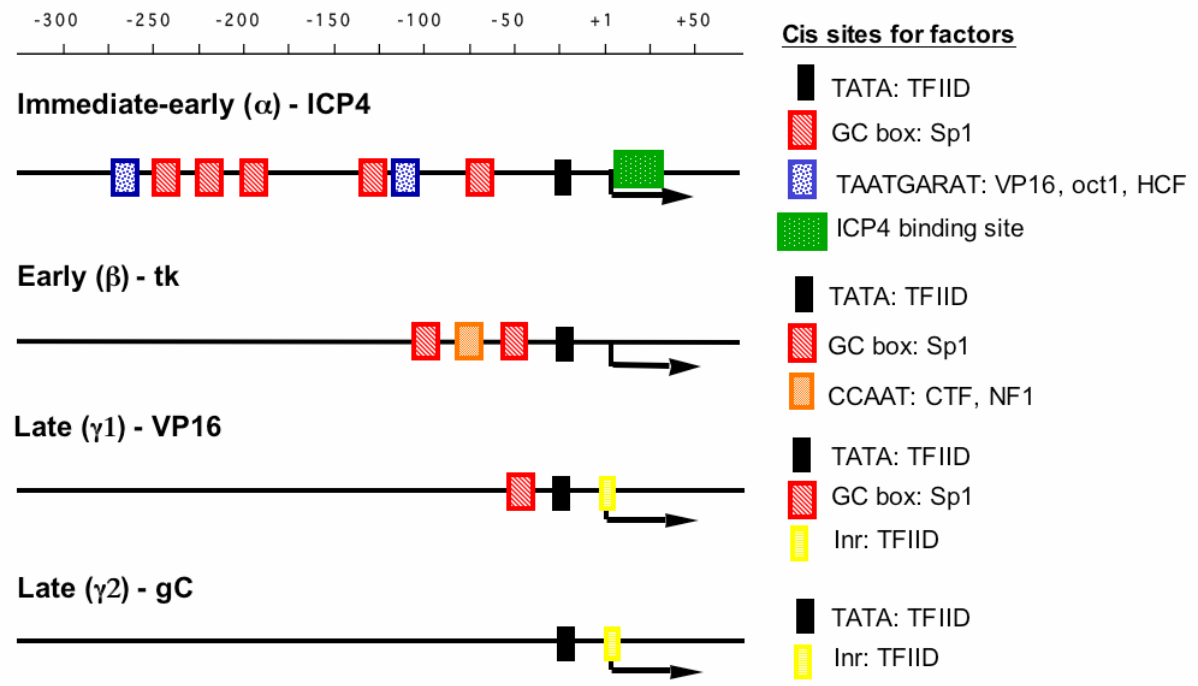


Figure 3. Representation of immediate-early, early, leaky-late and true late HSV promoters.

The general arrangement and composition of cis-acting regulatory elements for the indicated promoters are shown. One element common to all HSV promoters is a TATA box that is the binding site for the general transcription factor TFIID resulting in their expression by the host cellular transcription machinery. Aside from a TATA box the number and arrangement of cis-acting elements varies among each class of promoters.

1.5.2. Regulation of early gene expression

Early (E) gene products are involved in DNA synthesis and viral replication. Expression of these genes are strictly dependent on the presence of competent IE proteins particularly the immediate-early protein ICP4, which serves as the major transcriptional activator of early and late gene expression (45, 58, 72, 74, 142). While early promoters still retain many of the same cellular cis-acting sequences as those found on immediate-early promoters, these promoters are devoid of viral cis-acting sequences, particularly the TAATGARAT motifs present on immediate early promoters (Fig. 3). Early genes are not expressed immediately upon viral entry due to the lack of VP16 specific elements. However, E promoters like IE promoters contain a TATA box and still retain many of the same binding sites for upstream cellular factors. Despite the reduction in the number of cis-acting sequences in comparison to immediate-early promoters, these sites play an important role in the activation of early genes. Common cis-acting sequences found on early promoters are Sp1 and CAAT-box sites, that can be found on early promoters in multiple copies (29, 132). Although there are no ICP4 specific sites on early promoters, ICP4 is required for efficient expression of early genes. Through non-specific DNA binding, ICP4 can highly activate transcription of early promoters (96, 202). In the absence of either ICP4 or cellular activators, early genes are not efficiently activated or expressed. The cellular transcription factor Sp1 has been shown to play an important role in early gene transcription (101). The functions of

Sp1 and other cellular transcription factors work in concert with ICP4 to recruit and stabilize the cellular transcription machinery on early gene promoters.

Expression of early genes precedes viral DNA synthesis and reaches maximum levels between 4 and 6 hours post infection (Fig. 2) (93). With the onset of replication, early gene expression rapidly declines and is selectively repressed after viral replication. Inhibitors that block replication, allow for the continued high level expression of early genes (117), suggesting that the shutoff of early gene expression is somehow linked to viral DNA replication (reviewed in 199, 203). However, the exact mechanisms for early gene attenuation are not well understood. One possible mechanism is through modulation of the activities of transcription factors during infection that are required for early gene expression, as has been shown to occur with the transcription factor Sp1. Under normal conditions, Sp1 is a common cellular transcription factor that binds specifically to Sp1 sites and participates in the recruitment of the transcription machinery via interactions with co-activators of transcription, such as the 'Mediator' complex and the TBP-associated factor 110 (TAF110) of TFIID (68, 103, 139, 190). However, approximately 6 hours into HSV infection, Sp1 is quantitatively phosphorylated, coinciding with the time early gene expression declines (109). Although phosphorylation does not alter the abundance or the DNA binding ability of Sp1, it correlates with a decrease in the activation of the viral early gene, thymidine kinase. Additionally, Sp1 harvested from infected cells is less active than Sp1 from uninfected cells suggesting that phosphorylation of Sp1 interferes with its ability to activate transcription. Thus the phosphorylation of Sp1 during HSV infection may be one of the mechanisms that contribute to the reduced levels of IE and E gene expression late in infection.

Another mechanism of early gene attenuation that will be discussed in further detail in Chapter 3 and 4 is through alterations in the expression of components of the general transcription machinery. We show that the general transcription factor TFIIA, is required for ICP4-mediated activation of a representative early gene, yet during infection the expression of TFIIA decreases, suggesting that late in infection TFIIA is no longer available to participate in early gene expression and may also contribute to attenuation of early genes.

1.5.3. Regulation of late gene expression

The late or γ genes are last set of genes expressed, peaking at 13 hpi (Fig. 2) (93). Late genes products form the structural components of the virion such as glycoproteins, capsid, and tegument proteins necessary for virion formation. Late gene expression is highly dependent on viral DNA replication as well as the activities of the immediate-early gene products, particularly ICP4 (51, 202). Late genes can be classified as either leaky-late (γ1) or true-late (γ2) depending on their requirement for DNA replication (reviewed in 199, 203). Leaky-late genes are those that have a loose requirement for replication and whose products can be detected late in the lytic cycle, during the decline of early gene expression, but present prior to the onset of DNA replication (216). Moreover, these genes are expressed even in the presence of DNA replication inhibitors suggesting some other mechanism of regulation other than the requirement for replication. True late genes, however, are strictly dependent on viral DNA replication and are halted for gene expression in the presence of DNA replication inhibitors. Several mechanisms have been suggested to explain the dependence on viral replication for late-gene expression. These include differences in promoter strength and architecture, the presence of transcription

inhibitors that are titrated out following replication, conformational changes of the genome after viral replication, and differences in polyadenylation sites.

Examination of late gene promoters reveals that in contrast to immediate-early and early promoters which contain numerous upstream cis-activating sequences, most late promoters are deficient for sequences upstream of the TATA box (Fig. 3) (reviewed in 200, 203). While leaky-late promoters still contain one or more cis-acting site for cellular transcription factors that may work to activate transcription from these promoters prior to replication, most leaky-late and all true late promoters lack any influential upstream activating elements. In general the sequences upstream of the TATA box do not play a critical role in late gene expression as they can be mutated or deleted without detrimental effect on the ability of ICP4 to activate transcription from these promoters. Rather for most leaky-late and all true late promoters, the sequences located downstream of the TATA box, such as the initiator (INR) and the downstream activating sequence (DAS), are critical for activation (82, 83, 185, 205). True late promoters generally consist of a TATA box and an INR. The INR is a common feature of many cellular promoters with a loose consensus sequence, YYA+1N(T/A) YY (where Y is pyrimidine and N is any nucleotide) (178). It can direct the specific initiation of transcription by itself or synergistically with other promoter elements (177). We have shown that the INR is essential for ICP4-mediated activation of a representative true-late promoter, glycoprotein C (79, 110). The nucleotide contacts important for INR function are important for optimal induction by ICP4. Although the gC promoter contains a consensus DAS motif in addition to a TATA box and INR, only mutations in the INR severely compromise the ability of ICP4 to activate transcription of this promoter. Furthermore, *in vivo* analysis of wild-type and INR-mutated forms of the gC promoter showed that INR function is critical for high levels of gC mRNA accumulation late in infection

(110). These results suggest that the INR and TATA box are the only cis-acting elements that are absolutely essential for ICP4 activation of the late gC gene during viral infection.

1.6. PARTICIPATION OF VIRAL PROTEINS IN TRANSCRIPTION

The two virion-associated proteins, VP16 and *vhs*, that participate in viral gene expression very early during infection, have been discussed above. Four of the five IE proteins also play critical roles in the regulation of HSV gene expression (22, 44). ICP4, ICP0, ICP27 and ICP22 are all nuclear phosphoproteins that possess transcriptional and post-transcriptional regulatory activities (1). The activities of these proteins prepare the cell for and participate in the efficient cascade of viral gene expression, DNA replication, and the production of progeny virus (22, 44, 128).

1.6.1. ICP27

ICP27 is a 63 kDa nuclear phosphoprotein that is an essential regulatory protein in all experimental systems. It is a multifunctional protein that has been shown to regulate viral and cellular mRNA processing events, as well as modulate the activities of ICP4 and ICP0 (133, 162). ICP27 is found to co-localize with ICP4 within HSV replication compartments in infected and transfected cells. ICP27 is required for the transcription of a subset of viral early and has been implicated in the switch from early to late gene expression during viral replication (128). ICP27 facilitates DNA synthesis by stimulating the expression of early genes, which in turn allows for efficient DNA replication and late gene expression. ICP27 is essential for productive infection due to its multitude of regulatory effects both at the transcriptional and post-transcriptional levels. Evidence for these include effects on polyadenylation site selection, 3’

RNA processing, and splicing of mRNA transcripts (reviewed in 170). Transcription and pre-mRNA processing are coordinated events and ICP27 has been shown to interact with the RNA Pol II holoenzyme (217) and bind to many of the proteins involved in splicing. By binding to splicing proteins, ICP27 prevents their participation in the splicing process. Because only three of the four HSV transcripts that are spliced are IE genes, these genes are transcribed maximally before splicing is inhibited. In addition to interfering with splicing, ICP27 has been shown to stabilize labile 3' ends of mRNA, induce nuclear retention of cellular intron-containing transcripts while shuttling viral intronless transcripts out of the nucleus, and regulate distribution of host small nuclear ribonucleoproteins (snRNPs).

1.6.2. ICP0

Although not essential for growth in tissue culture, ICP0 deficient HSV is impaired for growth at low multiplicities of infection. ICP0 is a 110 kDa nuclear phosphoprotein that plays a key role during lytic infections, as well as in the establishment of and reactivation from latency (reviewed in 57, 84). ICP0, known as a promiscuous transactivator, will activate a broad range of viral and cellular promoters in transient assays and no promoter specific sequence is required for its activity. ICP0 has been found to elevate levels of viral gene expression and growth in tissue culture and in the trigeminal ganglia of mice. It has been shown to stimulate expression of all three temporal classes of viral genes (22) by increasing the rates of transcription. Although it can activate transcription alone, ICP0 in conjunction with ICP4 will synergistically activate transcription to a level that exceeds activation with either protein alone.

Early in infection, ICP0 localizes to the nucleus where it co-localizes with pre-existing nuclear sub-structures known as ND10 (or promyelocytic leukemia (PML) nuclear domain bodies) and within hours disrupts these domains by inducing the degradation of a number of ND10 components such as PML and Sp100 (59). ICP0 has also been found to co-localize at the centromeres of condensed chromosome disrupting these structures. The ring finger domain of ICP0, which is a ubiquitin E3 ligase, is required for ND10 and centromere disruption (16). ICP0 is thought to activate transcription indirectly through the induced degradation of specific cellular targets found within ND10s, such as PML and Sp1, that may repress viral transcription (reviewed in 57).

1.6.3. ICP22

ICP22 is a 68 kDa nuclear protein that is not essential for viral growth in many cell types yet acts to promote efficient expression of late genes in a cell type dependent manner (171). ICP22 in conjunction with UL13 have been shown to localize to replication compartments within infected cells and both are responsible for the altered phosphorylation of the carboxy-terminal domain of RNA polymerase II (121). The significance of this modification to RNA Pol II is not clear since ICP22-deficient virus will replicate in some cells types without observable altered Pol II phosphorylation (121). However, this modification is suggested to contribute to the shift in transcription from host to viral genes (121, 163, 164). Additionally, ICP22 interacts with several cellular proteins including cell cycle regulated proteins (17). ICP22 and the UL13 protein kinase mediate the stabilization of cdc2 while its cyclin partner, cyclin B, is simultaneously degraded (2). Cdc2 binds the viral protein UL42 and this complex recruits the cellular factor

topoisomerase IIa (3) to newly synthesized viral DNA. This is thought to untangle concatemeric DNA which is suggested to ensure the optimal expression of a subset of late genes such as UL11, UL38 and UL41, post DNA-synthesis (4).

1.6.4. ICP4

The immediate-early protein ICP4 has both positive and negative regulatory effect on viral gene expression. It is the major transcriptional activator of early and late genes (45, 58, 72, 74, 142) making it essential for productive lytic infection (51, 156).

1.6.4.1. Location

ICP4 can be detected in numerous discrete foci in the inner rim of the nucleus within 1 hour post infection. Many of these ICP4 containing foci are observed to associate with ND10 structures containing ICP0 during the early stages of infection (61). A proportion of these foci, containing one or more parental viral genomes, have been shown to develop into viral replication compartments later in infection. Positioning of ICP4-containing nucleoprotein complexes to ND-10 bodies is thought to potentate viral replication. It is these foci where not only replication but also transcription originates. These ICP4 concentrated areas predominate throughout lytic infection where ICP4 and other co-localizing viral transcriptional modulators, such as ICP27 and ICP22 (60) exert their effect on viral gene expression.

1.6.4.2. Structure

The ICP4 encoding gene, IE3, is located within the inverted repeats that flank the unique short region, resulting in two copies of the IE3 locus within the HSV genome (138). The ICP4 polypeptide is a 1,298 amino acid protein with a corresponding molecular weight of 175 kDa on SDS-polyacrylamide gels (37). However, in its natural state it exists as a highly phosphorylated 350 kDa homodimer in the nucleus of infected cells that has the ability to dimerize on DNA (37, 135, 137). It appears as three electrophoretic species on SDS-PAGE (153) and as seven species on two-dimensional gels (1). The fastest-migrating form seen on SDS gels accumulates in the cytoplasm, while the two slower-mobility species localize in the nucleus. The observed differences in mobility are, in part, due to differences in phosphorylation patterns (63, 153, 204, 208). ICP4 is also subjected to other post-translational modifications including adenylation, guanylation and adenosine diphosphate (ADP) ribosylation (12, 13). Of all these modifications, only phosphorylation has thus far demonstrated to affect the activity of ICP4 (10, 137, 149). ICP4 has been characterized as an elongated molecule as determined by gel filtration with a Stokes radius of approximately 90 Å (176). It can be divided into several discrete regions, which are conserved among the ICP4 orthologs of related alpha-herpesviruses, including the pseudorabies virus IE180 (27), the varicella-zoster virus ORF62 protein (131) and the equine herpesvirus immediate-early protein. These conserved regions of ICP4 include an N-terminal transactivation domain, a DNA binding and a nuclear localization domain and a C-terminal transactivation domain (Fig. 4) (47, 151, 172).

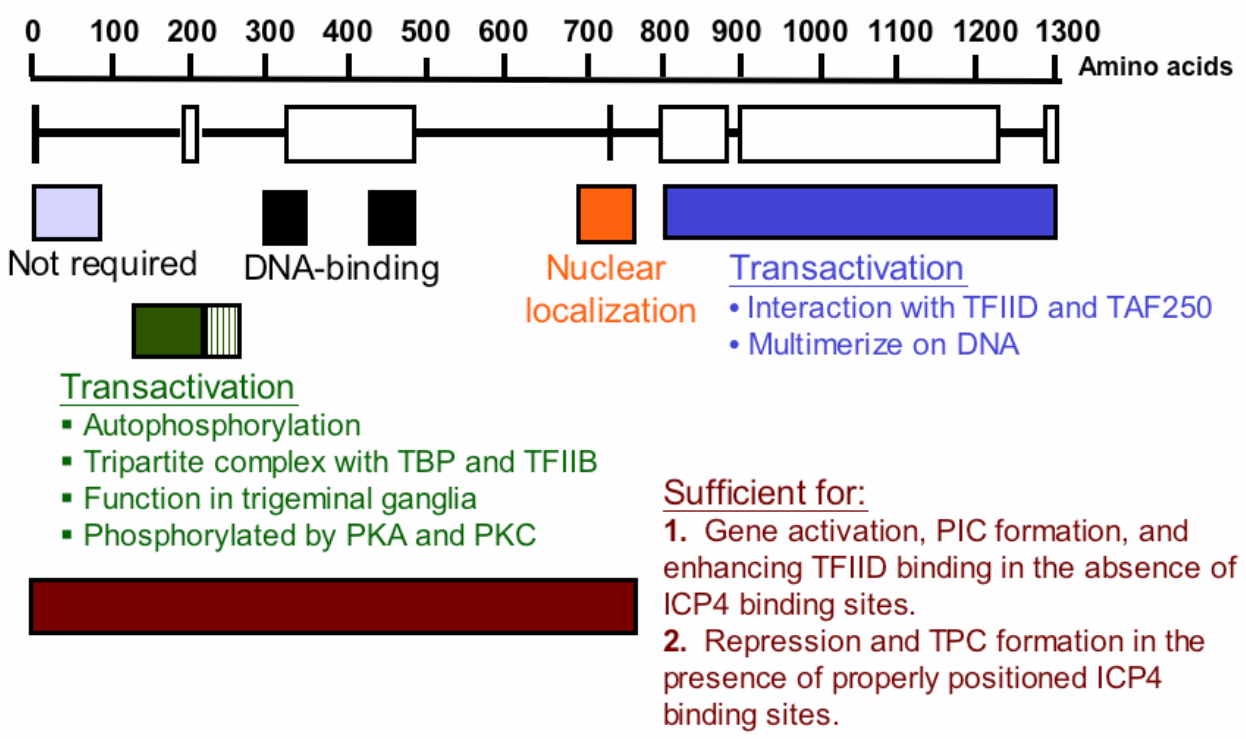


Figure 4. Primary structure of ICP4.

A comparison of ICP4 and its VZV ortholog ORF62 is indicated directly below the primary amino acid sequence with the white boxes specify regions of conservation. Colored boxes directly below their location within the primary structure indicate functional regions and physical properties of ICP4.

N-TERMINAL TRANSACTIVATION DOMAIN

The N-terminal transactivation domain of ICP4 lies between amino acids 142 - 210 and contains many of the features of ICP4 as an activator and repressor of transcription and also contributes to its regulation by ICP27 (Fig. 4). The first 774 amino acids of ICP4 containing the N-terminal activation domain, the DNA-binding domain, and nuclear localization signal possess most of the properties associated with the full-length wild-type ICP4 (47). This ICP4 molecule, named n208, retains the ability to form tripartite complexes with TBP and TFIIB on promoters containing ICP4 specific DNA binding sites and can repress transcription initiation from these promoters (80, 181). The N-terminal region is essential for this effect. Although this polypeptide can promote preinitiation complex formation on TATA boxes and activate

transcription of some early and leaky-late genes in transient transfections and *in vitro* reconstituted transcription systems, in the context of lytic infection this molecule lacks the complete transactivation potential of wild-type ICP4 (47). Viruses that express this 774 amino acid polypeptide are defective in activation of all early and leaky-late promoters impairing viral DNA synthesis which in turn, compromises the expression of late genes.

SERINE TRACT

The N-terminal activation domain of ICP4 contains a stretch of 24 residues, termed the serine-rich region. This peculiar stretch of serines is characteristic of all herpesvirus family ICP4 homologs, such as HSV-2, VZV, pseudorabies virus, Marek's disease virus, equine herpesvirus 1, and bovine herpesvirus 1 (27, 131). The serine-rich region of HSV-1 ICP4 contains consensus sites for phosphorylation by cellular protein kinase A (PKA), protein kinase C (PKC) and casein kinase II (208, 209). Although phosphorylation is not essential for activator function, the serine-rich region is a major determinant for phosphorylation of ICP4 in that this region itself or the phosphorylation of this region increases the phosphorylation of the rest of ICP4.

Viral mutants in which the serine-rich region of ICP4 has been deleted exhibit reduced viral yields and delayed E and L gene expression in tissue culture. Yet this serine tract can be deleted without detrimental consequence to lytic growth in tissue cultures. However, deletion of this serine rich region results in a virus that is completely impaired for viral growth in the trigeminal ganglia (10). In the trigeminal ganglia these viruses express only low levels of IE and E proteins and do not appear to replicate DNA. A block for replication in the trigeminal ganglia of infected mice suggest that the polyserine tract of ICP4 provides an activity required for viral DNA synthesis and full lytic gene expression. Thus the conservation of this serine tract among

neurotropic herpesviruses may indicate that it imparts to ICP4 and its homologs an activity required for function in neurons, despite its dispensability in cell culture.

DNA BINDING DOMAIN

ICP4 is a sequence-specific DNA-binding protein. The DNA binding domain of ICP4 is localized within residues 263 to 487 with a predicted helix-turn-helix motif structure common among many DNA-binding proteins (Fig. 4) (151, 175). This region is highly conserved between ICP4 and its homolog in VZV. The DNA-binding activity of ICP4 is essential to its function as a repressor and transactivator (47, 151, 175), since most mutations that disrupt DNA–binding also abolish or reduce both transactivation and autoregulation. The specific DNA binding-activity of ICP4 requires the direct binding of ICP4 in conjunction with TBP and TFIIB to high affinity ICP4-specific DNA binding sites located near the start site of transcription. The ICP4 consensus binding site is a fairly degenerate consensus sequence composed of RTCGTCNNYNYSG, where R is purine, Y is pyrimidine, S is C or G, and N is any base (49, 62). These ICP4-specific binding sites are orientation specific and location specific in that these sites must be located within the vicinity of the transcription start site in the proper orientation (113, 165, 166). When positioned at or near the transcription start of such promoters as the LAT, ORF-P and ICP4 promoters, these cis-elements allow ICP4 to function as a repressor. This allows ICP4 to autoregulate its own transcription (80, 113, 136, 166).

The DNA binding activity of ICP4 is also required for transcriptional activation. Yet certain mutants unable to bind to ICP4 consensus binding sites are still competent for activation (96, 152, 174). Although low affinity ICP4 binding sites have been identified on some early and late promoters, ICP4 will still activate transcription in the absence of these sites. In fact, ICP4

does not require any single or collection of ICP4 specific binding sites for activation and it will activate promoters *in vitro* without a consensus binding sites (29, 54, 56, 85, 180). This suggests that nonspecific DNA-binding will suffice for ICP4 activation (79, 96, 180).

NUCLEAR LOCALIZATION

The nuclear localization signal of ICP4 is located between amino acids 723 and 732 (Fig. 4) (47, 150). The NLS of ICP4 contains similarities to the SV40 large T antigen regulatory protein and the polyoma virus large-T regulatory protein. The large T antigen signal is recognized and bound by the importin 58/97 protein complex that targets nuclear proteins to the nuclear pore complex (75, 76, 95, 104). Due to the similarities between ICP4 and the SV40 large T antigen, it is likely that ICP4 is transported into the nucleus in an analogous manner.

C-TERMINAL DOMAIN

With the exception of the DNA binding domain, the bulk of amino acid conservation between HSV ICP4 and other ICP4 homologs resides in the C-terminal one-third of ICP4 (Fig. 4). The last 500 amino acids of the carboxy-terminal domain contain the greatest degree of amino acid similarity among all ICP4 homologs. Although ICP4 mutants that lack the carboxyl terminal 500 amino acids still function in DNA binding, repression and activation, viruses containing this truncation do not grow due to deficiencies in late gene expression. C-terminal ICP4 mutants do however express some early genes, suggesting a role for the N-terminal transactivation region in early and leaky-late gene expression. The amino acid similarity in within this C-terminal domain of ICP4 and ICP4 homologs must thus specify functions that are necessary for late gene

expression. The C-terminal 524 amino acids of ICP4 has been shown to interact with TAF250 of TFIID and this interaction correlates with its importance in the activation of late genes (25).

1.6.4.3. Repression and activation

ICP4 negatively regulates its own promoter, the LAT promoter and the promoter for ORF-P. Binding of ICP4 to its cognitive binding site in a tripartite complex with TFIIB and TBP is thought to block progression of the preinitiation complex assembly. The regions responsible for ICP4 repressor activity reside within the first 774 amino acids (46, 47). The ability of ICP4 to interact with TFIIB and TBP through its N-terminal activation domain is also detrimental to its function as a repressor (80). In the context of infection, in the absence of the repressive effects of ICP4, IE genes are inefficiently shutoff and continued to be expressed at high levels (44, 51, 128, 169).

As the major transcriptional activator of early and late genes, ICP4 functions to increase the rate of transcription by increasing the rate of preinitation complex formation (78, 79). The regions required for activator function reside within both the N- and C- terminal transactivation domains, however, these two regions activate transcription to different extents by different mechanisms (43, 45, 150, 175). The N-terminal domain is important for activation of early and leaky-late genes while interactions with TFIID through the C-terminal domain of ICP4 are important for activation of late genes.

ICP4 can activate transcription with a relatively simple set of RNA polymerase II general transcription factors in *in vitro* reconstituted transcription systems. This has been shown to be true particularly for ICP4 activation of a representative late gene promoter, gC (79). Most true late promoters are composed of a TATA box and an INR. The presence of an INR in addition to

a TATA box enhances the ability of ICP4 to activate transcription and the same nucleotide contacts that are important for classical INR function are also important for ICP4-mediated activation (79, 110). The TAFs of the general transcription factor, TFIID play an essential role in ICP4-mediated activation of late genes (79). TFIID is a multi-subunit complex that is composed of the TATA binding protein (TBP) and TBP-associated factors (TAFs) (reviewed in 5). TBP alone is unable to support ICP4-activation (79). ICP4, like other cellular transcriptional activators, requires the co-activator functions of the TAFs of TFIID in addition to TBP to activate transcription (196). ICP4 interacts with TAF250 of TFIID through its C-terminal transactivation domain (25). TAF250, an integral component of TFIID, contributes to the interaction with the INR. A complex consisting of TBP-TAF150-TAF250 is sufficient for INR activity (26, 195). ICP4 increases the rate of preinitiation complex formation on late promoters through recruitment and stabilization of TFIID to the TATA box and INR through interactions with TAF250 (25, 78). Thus, ICP4 assists TFIID to function more efficiently through the INR present on most late genes.

1.7. TRANSCRIPTION INITIATION BY THE RNA POL II COMPLEX

1.7.1. Promoter structure and elements

The accurate initiation of transcription is a key stage in the regulation of gene expression for mammalian protein-coding genes. The regions that regulate transcription can be separated into two categories: core promoter elements and regulatory elements. Regulatory elements are gene-

specific sequences that are located both up and downstream of core promoter elements and serve as binding sites for enhancers and repressors of transcription. Core promoter elements can consist of a TATA box, which is an A/T rich sequence located 25 to 30 bp upstream of the transcription start site, an initiator element (INR) that encompasses the start site, a downstream promoter element (DPE) localized approximately 30 base pairs downstream of the start site and a TFIIB response element (BRE). For promoters that lack a TATA consensus, the INR can direct accurate transcription initiation by RNA polymerase II by itself or in conjunction with other promoter elements (55, 120, 177). The presence of an INR in addition to a TATA box can serve to enhance the strength of that promoter, as is the case for most HSV late promoters (reviewed in 199, 203).

1.7.2. RNA Pol II machinery

Transcription initiation is an orchestrated process that requires the concerted functions of multiple transcription factors (reviewed in 116, 124, 146, 160). The general transcription machinery is composed of a ubiquitous group of factors that include RNA polymerase II and six general transcription factors (GTFs), TFIIA, -B, -D, -E, -F and –H that were originally identified biochemically as factors required for the accurate transcription initiation by RNA Pol II in *in vitro* reconstituted transcription systems. With the exception of TFIIB, which is a single polypeptide of 35 kDa, all other GTFs as well as Pol II, are multi-subunit complexes of 2 to 14 different proteins that exist as stable multi-protein complexes in solution. Although RNA Pol II is a large multi-subunit enzyme, composed of 12 different proteins with a molecular mass of approximately 0.5 MDa that shares five of its subunits with the two other eukaryotic RNA

polymerases (Pol I and III), Pol II alone is unable to recognize promoters and accurately initiate cellular transcription. Initiation of transcription by Pol II requires the cooperative assembly of GTFs and Pol II into pre-initiation complexes (PIC) on core promoter elements. This involves recognition of GTFs to various core promoter elements, which include the TATA box, the INR, TFIIB-response element (BRE) and downstream promoter elements (DPE) that are found in various combinations in different genes. Association of the general transcription factors with these core promoter DNA elements then allows the specific recruitment of Pol II to the core promoter of all class II genes.

The first step in PIC formation involves the binding of TFIID to the core promoter. TFIID is a multi-subunit complex composed of the TBP and 14 distinct TBP-associated factors (TAFs) that are highly conserved from yeast to Drosophila to humans (5). Binding of TFIID to the TATA box, via TBP, is the initial step of PIC assembly and is critical for the rate and efficiency of this process. The TBP subunit of TFIID binds to the minor groove of the TATA box inducing a dramatical bend in promoter DNA, while the TAFs interact with additional downstream DNA sequences up to +35. TFIID can also recognize TATA-less promoters to allow functional PIC formation.

In purified reconstituted systems, TBP can function in the absence of TAFs to promote basal levels of transcription from TATA-containing core promoters. However, TBP alone is insufficient to regulate transcription by upstream activators. Effective response to transcriptional activators requires the presence of TAFs in TFIID and many activators have been shown to interact with TAFs to mediate activation. TAFs also contribute to promoter selectivity by interacting with other basal transcription factors, other TAFs, and specific DNA sequences, such as the INR and downstream promoter element (DPE) or gene-specific core promoter elements.

Human TAF150 is involved in INR recognition and a complex consisting of TBP, TAF250 and TAF150 is sufficient for INR activity in the presence or absence of a TATA box (24, 26, 105-107, 120). Drosophila TAF60 recognizes the DPE and allows for TFIID to function through this core promoter element in the absence of a TATA box (reviewed in 20).

The binding of TFIID to the core promoter is stabilized by TFIIA and TFIIB. TFIIA is a three-subunit complex that stabilizes TBP binding to the TATA box by contacting TBP and DNA upstream of the TATA box. TFIIB also stabilizes the TBP-TATA interaction through contacts with both TBP and BRE DNA sequence that is 5' to the TATA element. TFIIB is responsible for properly positioning RNA Pol II on the start site and specifying the start site of transcription. While TFIIB is essential for basal transcription directed by either TBP or TFIID, TFIIA is dispensable for TBP-directed basal transcription in purified systems *in vitro*. However, it is essential for TFIID-directed basal and activated transcription. In Chapters 4 and 5 we show that although required for ICP4-activation of early genes, TFIIA is downregulated during HSV infection and is dispensable for ICP4 activation of late genes.

Assembly of a TFIID-TFIIA-TFIIB complex on DNA allows for the recruitment of TFIIF in association with RNA polymerase II. TFIIF is a heterotetrameric factor consisting of RAP30 and RAP74 subunits that prevents random initiation by RNA Pol II by inhibiting and reversing Pol II's binding to non-promoter sites. RNA Pol II is the enzyme that catalyzes DNA-dependent synthesis of mRNA. The crystal structure of RNA Pol II has recently been derived (38) and described as a "jaw" like structure that clamps down onto DNA (206). The largest subunit of RNA Pol II contains the essential carboxy-terminal domain (CTD) that is specific to only RNA Pol II. During initiation, the unphosphorylated form of Pol II is recruited to the initiation

complex. The CTD plays key roles in the regulation of transcription initiation and coordination of co-transcriptional mRNA processing events.

Although a complex of TFIIA, -B, -D, -F, and RNA Pol II is sufficient to initiate transcription from supercoiled DNA plasmids and pre-melted promoter template *in vitro*, the additional activities of TFIIE and TFIIH are required for efficient transcription initiation on linear DNA. TFIIE, a heterotetramer of 34 and 56 kDa subunits, joins the complex and recruits TFIIH and regulates TFIIH activities. TFIIH is a large complex GTFs, consisting of 9 subunits with a molecular weight comparable to Pol II. This 9-subunit complex with a ring-like architecture is the only GTF with enzymatic activities, including two ATP-dependent DNA helicases (XPB and XPD) that are involved in promoter melting at the transcription start site and in Pol II promoter clearance. TFIIH also contains a kinase that phosphorylates the CTD of the largest subunit of Pol II, a step that is thought to also facilitate promoter clearance by disrupting interactions of the CTD with components of the PIC. Phosphorylation of the CTD promotes the transition of RNA Pol II from a preinitiation complex to a stable elongation complex.

This ordered PIC assembly has been challenged with the discovery that the un-phosphorylated form of Pol II can be found in preassembled complexes in the form of a holoenzyme (reviewed in 9, 112). These complexes vary in composition but generally include TFIIF, -E, -H, Pol II and components of the 'Mediator' complex that can be recruited in a single step to DNA bound TFIID/TBP-TFIIA-TFIIB complex. After open complex formation and promoter clearance, the CTD-phosphorylated Pol II in association with TFIIF elongates, while TFIIA, -D, -E, and -H remain bound to the core promoter and TFIIB is released. Second rounds of transcription initiation may not require *de novo* recruitment of TFIIA, -D, -E, or -H but may just need re-incorporation of TFIIB, TFIIF and a dephosphorylated form of Pol II.

2. RATIONALE

HSV is known to cause immense changes in host cellular gene expression. In an effort to collectively determine differences in the individual expression patterns of a large number of cellular genes during infection, microarray analysis was conducted using Incyte DNA gene chip arrays containing approximately 30,000 representative cellular genes. Scatter plot representation of microarray analysis of wild-type (KOS at an MOI of 10) infected versus uninfected HEL cells showed that cellular gene expression was progressively dysregulated as a function of wild-type HSV infection (Fig. 5). At 1 hour post infection, cellular gene expression was minimally affected as most genes (indicated as dots) could be plotted on a central axis of one (Fig. 5). By 4 hpi, changes in cellular gene expression were more pronounced as many genes were displaced from the central axis of 1, indicating that many of these genes were moderately up or down regulated at this time of infection (Fig. 5). Genes that were upregulated could be located below the central axis of 1, whereas genes that were downregulated could be located above the central axis of 1. By 8 hour post infection, cellular gene expression was highly dysregulated as evidenced by the greater distribution of cellular genes away from the central axis of 1 (Fig. 5). Expression of cellular genes at this time of infection appeared to fall into three main categories, those that were downregulated during infection, those that were unaffected and those that were upregulated during infection, with the largest group of genes being those that were repressed. This was not unexpected since many cellular genes have been shown to be downregulated during

infection (88, 184, 201). Most importantly however, this microarray analysis showed that the expression of the small number of general transcription factor components present on these chips were also dysregulated during infection, suggesting that the expression of many more components of the general transcription machinery responsible for the transcription of all viral genes may also be perturbed.

Changes in the mRNA abundances of components of the cellular transcription machinery may lead to variations in the overall composition of the basal transcription machinery available for the transcription cellular genes. Alterations in the transcription machinery may contribute to the repression of cellular gene expression since the normal factors that make up the transcription machinery may be altered or not available for cellular activators to interact with or function through. However since immediate-early and early genes also rely on cellular as well as viral activators that function though the cellular transcription machinery, any changes in the composition of this machinery would also effect viral immediate-early and early gene expression. In contrast, late promoters do not contain binding sites for upstream activators but are maximally expressed late in infection suggesting that late gene expression may not require the normal set of cellular transcription factors necessary for immediate-early and early gene expression. Changes in the composition of the transcription machinery may in fact promote the shift from early to late gene expression.

ICP4 plays a major role in efficient expression of the early and late genes. If at late times of infection the make up of the cellular transcriptional machinery is greatly perturbed, but late genes are still maximally expressed, it follows that ICP4 can still function with changes in the transcription machinery to promote high levels of late viral transcripts. ICP4 is a large complex protein, with many unrecognized functions. Interactions with the general transcriptional

machinery may exist at various times of infection that allow optimal usage of the transcriptional machinery regardless of differences in the composition of the transcription machinery. Based on this, we hypothesize that as infection proceeds the mRNA abundance of the Pol II transcription factors and their subunit composition change as a function, but that the major HSV transcription regulatory protein, ICP4 may function with these changes and has differential requirements of the general transcription factors based on the viral promoter context.

Through cellular and biochemical methods, the goals of this study are to address the following: I) Identify changes in the expression patterns of the components that make up the cellular transcription machinery. Changes in the mRNA abundances of all the components that comprise the basal cellular transcription machinery will be analyzed at early and late times of wild-type infection via microarray and northern blot analysis. II) Determine whether there are differential requirements for any of the general transcription factors for ICP4-mediated activation of different promoters. Utilizing chromatographically enriched forms of transcription factors, ICP4 will be tested for the ability to activate transcription from representative viral early and late promoters using an *in vitro* reconstituted transcription system. III) Characterize and analyze the transcription factors of interest with ICP4 through protein-protein and protein-DNA interactions on early and late promoters. DNase I footprinting analysis will be conducted on representative early and late promoters to delineate differences in requirements of the transcription factors of interest to stabilize protein-DNA interactions in the presence and absence of ICP4.

Because HSV can be easily transmitted and is able to cause a variety of infections, some of which can cause severe and life-threatening diseases it is important to understand the mechanisms of pathogenesis for therapeutic approaches. If we can establish exactly what contacts between viral (i.e. ICP4) and cellular proteins affect transcription we can use the

information to develop therapeutics that block these interactions preventing viral gene expression of products that are cytotoxic to cells, essential for progeny production or both. These studies can provide a better understanding of how viral genes are regulated and how viral genes are efficiently expressed late in infection. Not only are these studies important for determining how viral genes are regulated, but they may also reveal novel functions of the cellular transcription machinery that would aid in understanding the mechanisms governing cellular gene expression.

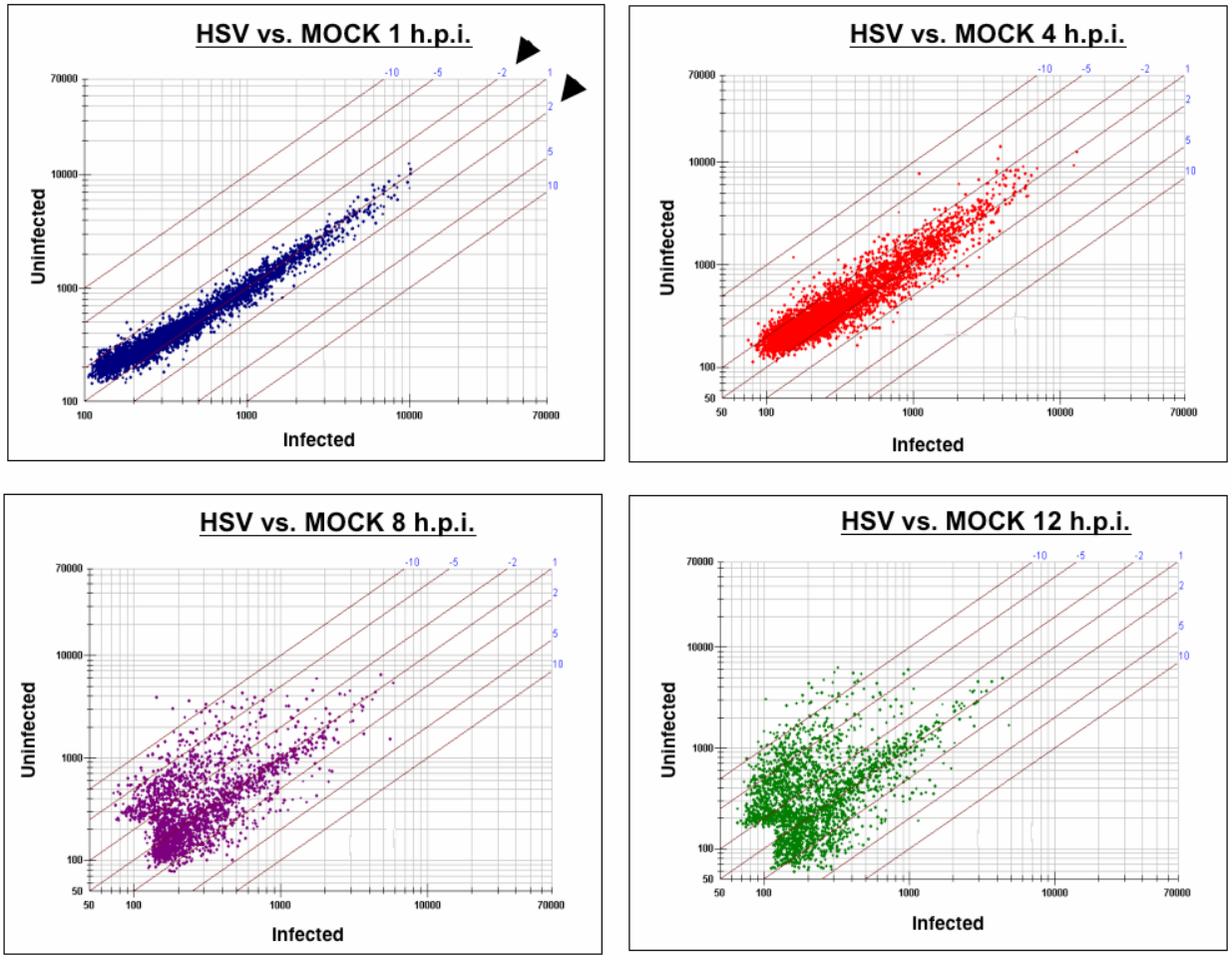


Figure 5. Incyte microarray analysis of changes in the expression of cellular genes as a function of wild-type HSV-1 infection

Scatter plot representation of changes in the expression patters of 30,000 cellular genes during the progression of wild-type KOS infected versus uninfected (MOCK) HEL cells analyzed through Incyte microarray DNA chips. Complements of William Hobbs.

3. CHANGES IN THE EXPRESSION OF COMPONENTS OF THE GENERAL TRANSCRIPTION MACHINERY AS A FUNCTION OF HSV INFECTION

3.1. INTRODUCTION

Infection with herpes simplex type 1 virus profoundly alters cellular homeostasis through a myriad of viral induced modifications. Many of these changes lead to a strong suppression of cellular gene expression with a concomitant increase in viral gene expression (88, 184, 201). Studies have shown that by 6 hours post infection, cellular gene expression is repressed to less than 40% compared to uninfected cell levels with a continuous decline thereafter (127, 184, 187). Within the first hours of HSV infection, the virion-associated protein *vhs* halts translation of cellular mRNA by disrupting preexisting polyribosomes and induces the degradation of preexisting and newly transcribed mRNA through an RNase-like activity (reviewed in 179). The immediate-early protein ICP27, blocks cellular splicing mechanisms and induces nuclear retention of intron-containing cellular messages while it increases transport of viral intronless mRNAs out of the nucleus (reviewed in 170). ICP0 causes dispersal of nuclear ND10 domains early during infection by inducing the degradation of ND10 proteins, including PML and Sp100, that is suggested would otherwise silence viral transcription (57, 84). The combined action of these viral immediate-early gene products and virion components contribute to dramatic alterations of host gene transcription for the benefit of viral gene transcription.

HSV-1 relies on the activities of the host cellular transcription machinery for efficient expression of all its genes and many viral induced cellular changes contribute to the redirection of the cellular transcription machinery from cellular to viral genes (164, 184). Initiation of transcription by the host RNA polymerase II transcription machinery is a highly regulated process that requires the cooperative assembly of Pol II and six general transcription factors (GTFs), TFIIA, -B, -D, -E, -F and –H into pre-initiation complexes (PIC) on core promoters (86, 124, 145). With the exception of TFIIB, which is a single polypeptide of 35 kDa, all the other GTFs as well as Pol II are multi-subunit complexes of 2 to 14 different proteins that exist as stable multi-protein complexes in solution. Recognition of these general transcription factors to core promoter DNA elements allows the specific recruitment of Pol II to the core promoter of all class II genes. HSV encodes at least two viral regulatory proteins, VP16 and ICP4, that directly interact with the general transcription factors and moderate preinitiation complex (PIC) formation on viral promoters. The virion-associated transcriptional regulatory protein, VP16, interacts with a number of the general transcription factors (GTFs) including TBP, TFIIB, TAF31 of TFIID, the gamma subunit of TFIIA, and the 62 kDa subunit of TFIIH (65). These interactions strongly stimulate preinitiation complex formation on immediate-early promoters triggering the expression of these genes rapidly upon infection. In addition, other cellular activators that also interact with the basal transcription machinery synergize with the effects of the powerful transactivation potential of VP16 to recruit and induce stabilized PIC formation on immediate-early gene promoters. ICP4, an immediate-early gene product that is the major transcriptional activator of early and late genes, interacts with the general transcription machinery to both repress immediate-early transcription and to activate early and late genes. For repression, ICP4 participates in a tripartite complex formation with TBP and TFIIB on ICP4

specific binding sites of immediate-early promoters blocking recruitment of RNA Pol II (80, 113, 165, 181). For the activation of early and late genes, ICP4 interacts with TAF250 of the general transcription factor TFIID through its C-terminal transactivation domain resulting in recruitment and stabilization of TFIID to viral core promoters (25). This serves to increase the rate of preinitiation complex formation on viral early and late promoters (78).

Due to drastic alterations in cellular gene expression, the question remained whether these viral induced changes also had any effect on the expression of components of the cellular transcription machinery. It seemed feasible that such drastic changes in cellular gene expression also resulted in changes in the expression levels of some of the general transcription factors that are required for viral as well as cellular mRNA synthesis. Changes in the expression of the basal transcription machinery components may contribute to suppression of host gene expression since the normal composition of these factors responsible may not be available for cellular transcription. However, repression of cellular gene expression occurs fairly early on during infection, thus it is likely that other factors such as *vhs*, ICP27, and ICP0 play greater roles in the suppression of cellular gene expression. Because the cellular transcription machinery also transcribes all three classes of HSV genes, changes in the transcription machinery would most likely have a great impact on viral gene expression. Late in infection, early gene expression is attenuated suggesting that changes in the transcription machinery may contribute to the shutoff of early gene expression. Yet late in infection, late gene expression appears to proceed at high levels suggesting that if there are any changes in the transcription machinery viral transcriptional regulatory mechanism, namely ICP4, can operate with these changes to transcribe viral genes. Perhaps changes in the transcription machinery during infection may be yet another mechanism for augmenting the progression from one viral kinetic class of genes to the next.

Microarray analysis was used to examine changes in the mRNA abundances of all the components that make up the general transcription machinery during HSV infection. Because the complete transcriptional machinery is a large complex composed of 43 distinct proteins with a total mass of over 2.2-MDa, microarray was the choice method of analysis. Microarray cDNA chips containing all the components of the general transcription factors were constructed in-house and changes in the mRNA abundances of these factors were examined at early (4 hpi) and late (8 hpi) times post wild-type infection. Microarray analysis indicated that components of the general transcription factors were changed as a function of infection and some of these changes were confirmed through Northern blot analysis. Together this provides evidence that expression of components of the general transcription machinery are altered during infection, suggesting that late in infection the composition of the transcription machinery may be distinct.

3.2. MATERIALS AND METHODS

3.2.1. Microarray analysis

Either 2 μg of total RNA or 3 μg of poly (A)+ RNA harvested from uninfected and KOS (multiplicity of infection of 10) infected HEL cells at 4 and 8 hpi were used for hybridization to chips arrays made in-house containing PCR amplified cDNA fragments representing the mRNAs encoding components of the general transcription machinery. Microarray chip construction, hybridization and data analysis was conducted as previously described (53).

3.2.2. Northern blot analysis

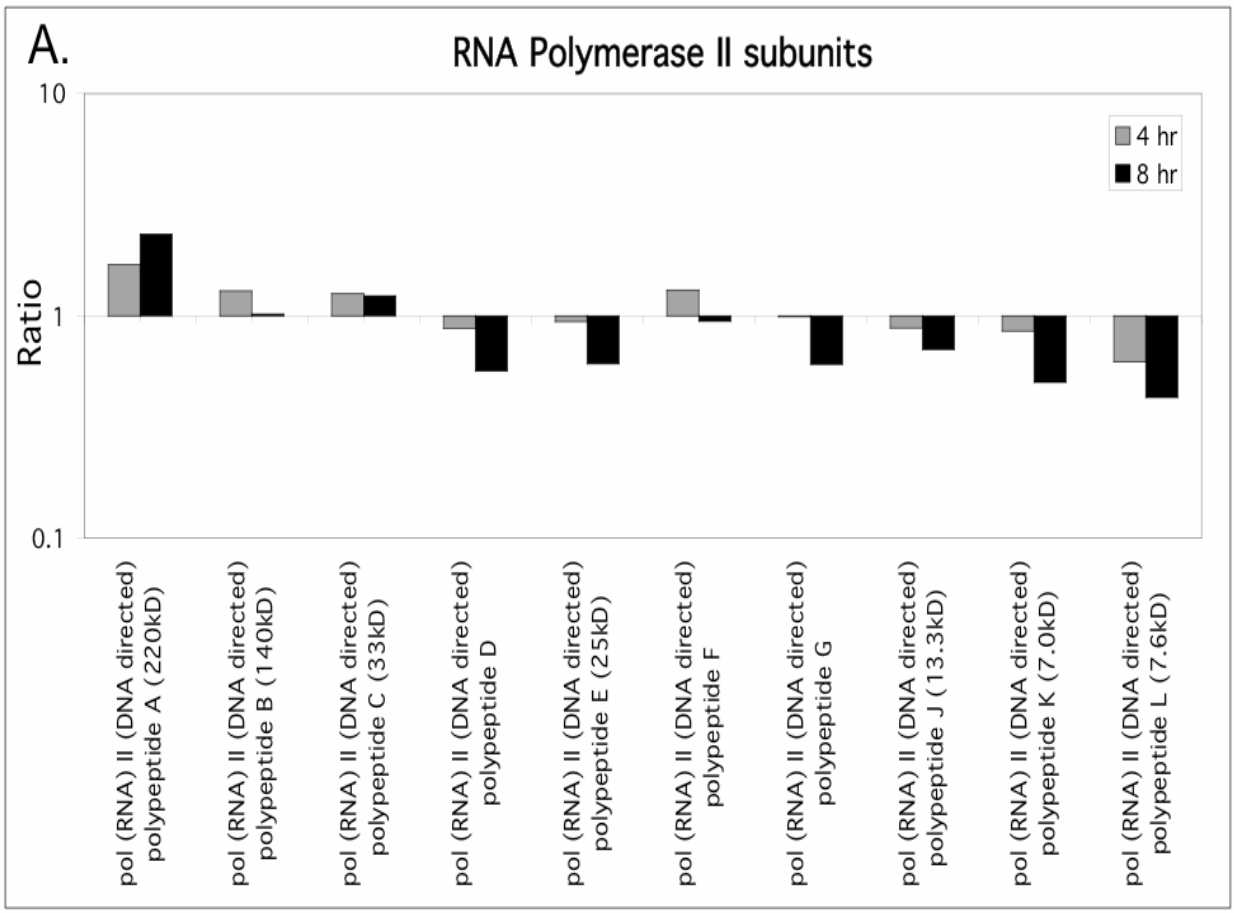
Poly A+ RNA (2 μg) harvested from uninfected and KOS (MOI of 10) infected HEL cells at 4 and 8 hpi was resolved by denaturing formaldehyde-agarose gel electrophoresis, transferred to nitrocellulose membranes and probed, washed and exposed as previously described. ^{32}P-labeled probes for Northern analysis, labeled by nick translation using [α-^{32}P]dCTP and [α-^{32}P]dGTP, were generated from PCR amplified products from plasmids that were used for microarray chip construction. TFIIA probes were generated from pQE-TFIIAαβ and pQE-TFIIAγ by nick translation using [α-^{32}P]dCTP and [α-^{32}P]dGTP.

3.3. RESULTS

Changes in the mRNA abundances of components of the general transcription machinery observed from at least three independent microarray hybridization experiments were averaged and graphed as fold change in log ratio. The ratios are a comparison of uninfected (MOCK) versus wild-type KOS (MOI of 10) infected HEL cells harvested at 4 and 8 hours post infection. Genes that were not affected during infection at the indicated time point, remain at a ratio of one. Genes that were downregulated fall below the ratio of one and those that were upregulated rise above the ratio of one.

3.3.1. Changes in the expression of components of the RNA Pol II enzyme and the general transcription factors.

RNA polymerase II is a large multi-subunit enzyme, composed of 12 different proteins. Microarray analysis of all the subunits of RNA polymerase II showed that while the largest subunit of RNA Pol II (220 kDa) was upregulated during infection, most of the smaller subunits of Pol II were downregulated during wild-type infection (Fig. 6A). Northern blot analysis confirmed an approximate 2-fold increase in expression of the largest subunit of RNA Pol II by 4 hpi and validated the decreased expression of two of the smallest subunits of polymerase II, 7.6 kDa and the 7.0 kDa, by 8 hpi (Fig. 6B).



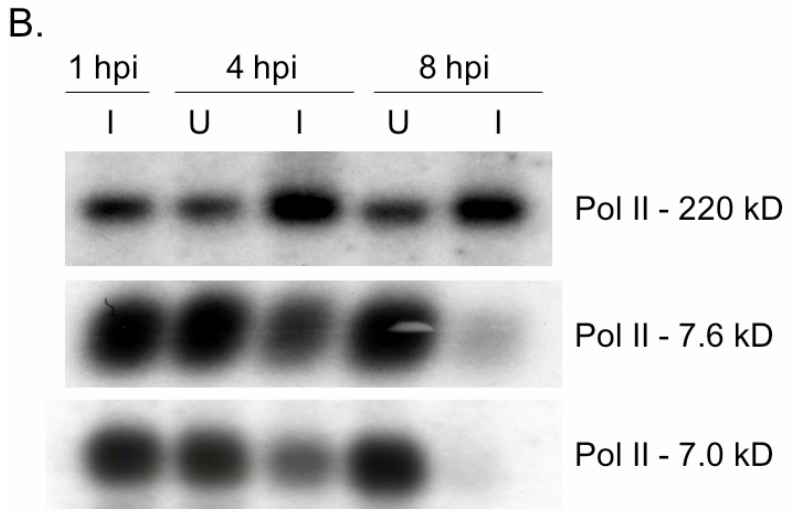
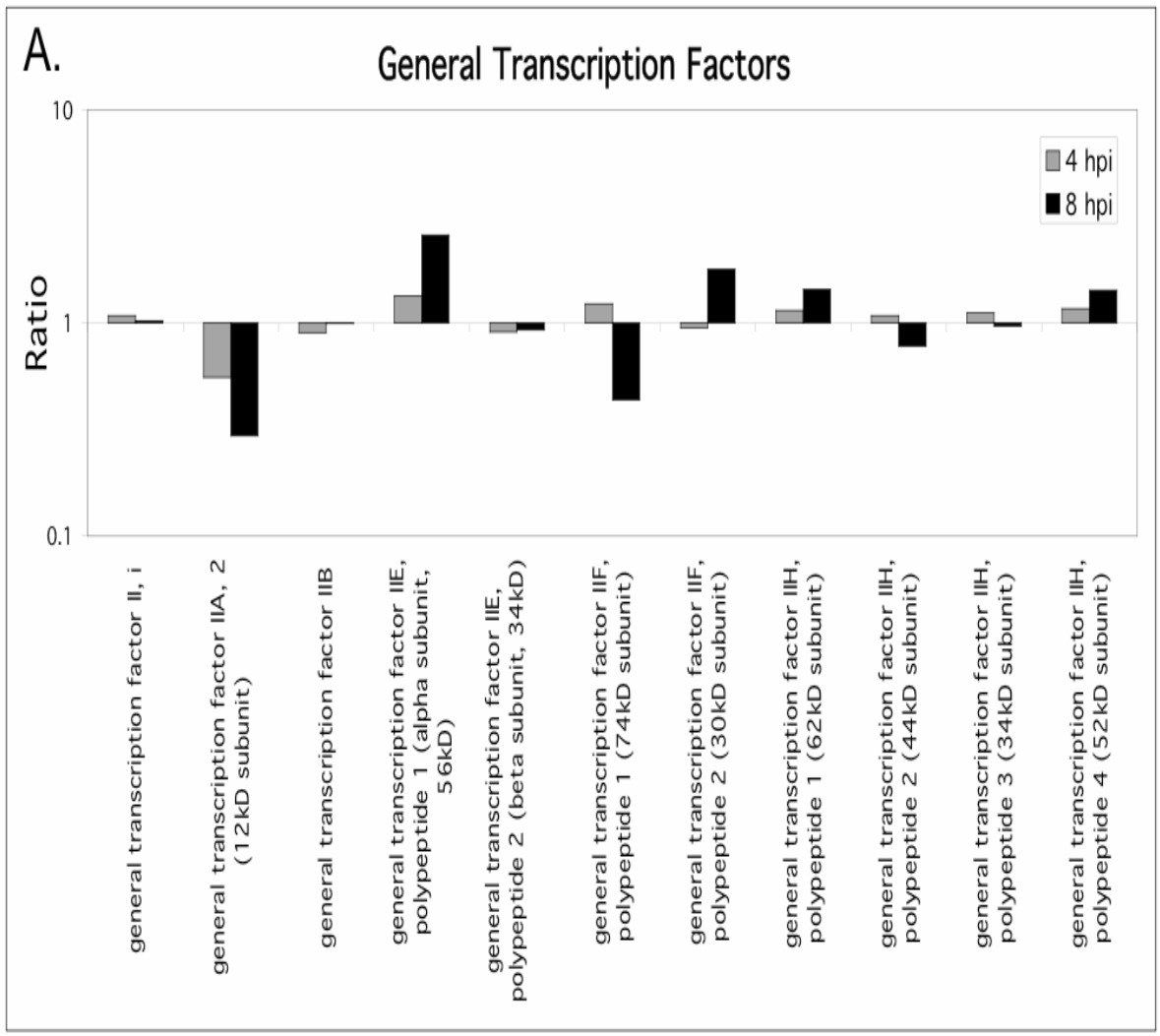
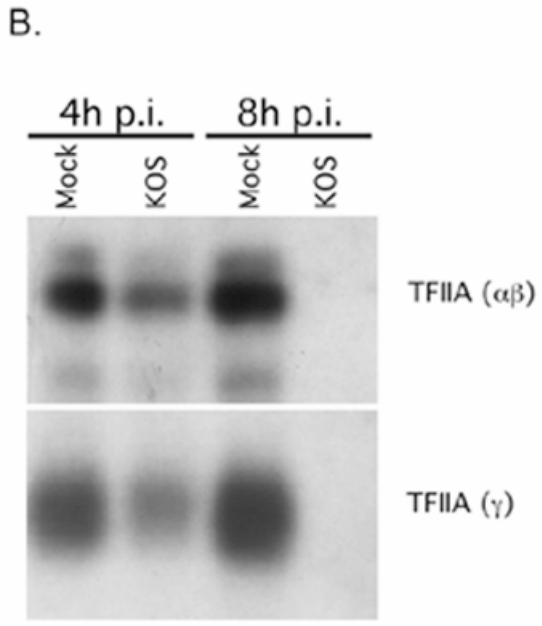


Figure 6. Microarray and northern blot analysis of changes in the expression of subunits of the RNA polymerase II enzyme during wild-type HSV infection.

A. Bar graph representation of the mRNA abundances of all twelve subunits of RNA Pol II at early (4 hpi) and late (8 hpi) times post wild-type (KOS at MOI = 10) infection of HEL cells. An average of at least three microarray experiments were calculated and graphed as log ratio changes in wild-type versus uninfected HEL cells. **B.** Northern blot analysis of poly A+ mRNA harvested at 1, 4, and 8 hour post wild-type infected (I) and uninfected (U) HEL cells probed for the indicated subunits of RNA Pol II.

Microarray analysis of components of the general transcription factors showed that the expression of the smallest subunit of TFIIA (12 kDa subunit) was sharply downregulated during infection (Fig. 7A). There was also a decrease in the expression of the RAP74 subunit of TFIIF and a slight decrease in the 44 kDa subunit of TFIIH by 8 hpi (Fig. 7A). In contrast the 56 kDa subunit of TFIIE, and to a much lesser extent the RAP30 subunit of TFIIF, were upregulated by 8 hpi (Fig. 7A). Northern blot analysis confirmed the downregulation of the smallest subunit of TFIIA, the γ–subunit, as well as of the other two subunits of TFIIA, α and β, such that by 8 hpi, the mRNA abundance was undetectable by northern blot analysis (Fig. 7B). Northern analysis also confirmed the decreased expression of the 44 kDa subunit of TFIIH (Fig. 7B).


A.
General Transcription Factors
10
1
0.1
Ratio
4 hpi
8 hpi
general transcription factor II, i
general transcription factor IIA, 2 (12kD subunit)
general transcription factor IIB
general transcription factor IIE, polypeptide 1 (alpha subunit, 56kD)
general transcription factor IIE, polypeptide 2 (beta subunit, 34kD)
general transcription factor IIF, polypeptide 1 (74kD subunit)
general transcription factor IIF, polypeptide 2 (30kD subunit)
general transcription factor IIH, polypeptide 1 (62kD subunit)
general transcription factor IIH, polypeptide 2 (44kD subunit)
general transcription factor IIH, polypeptide 3 (34kD subunit)
general transcription factor IIH, polypeptide 4 (52kD subunit)


B.
4h p.i.
8h p.i.
Mock
KOS
Mock
KOS
TFIIA (αβ)
TFIIA (γ)

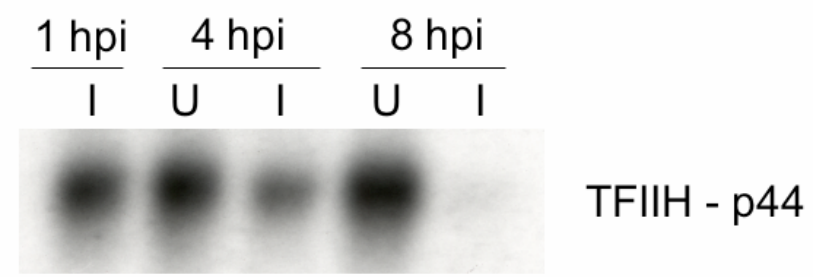
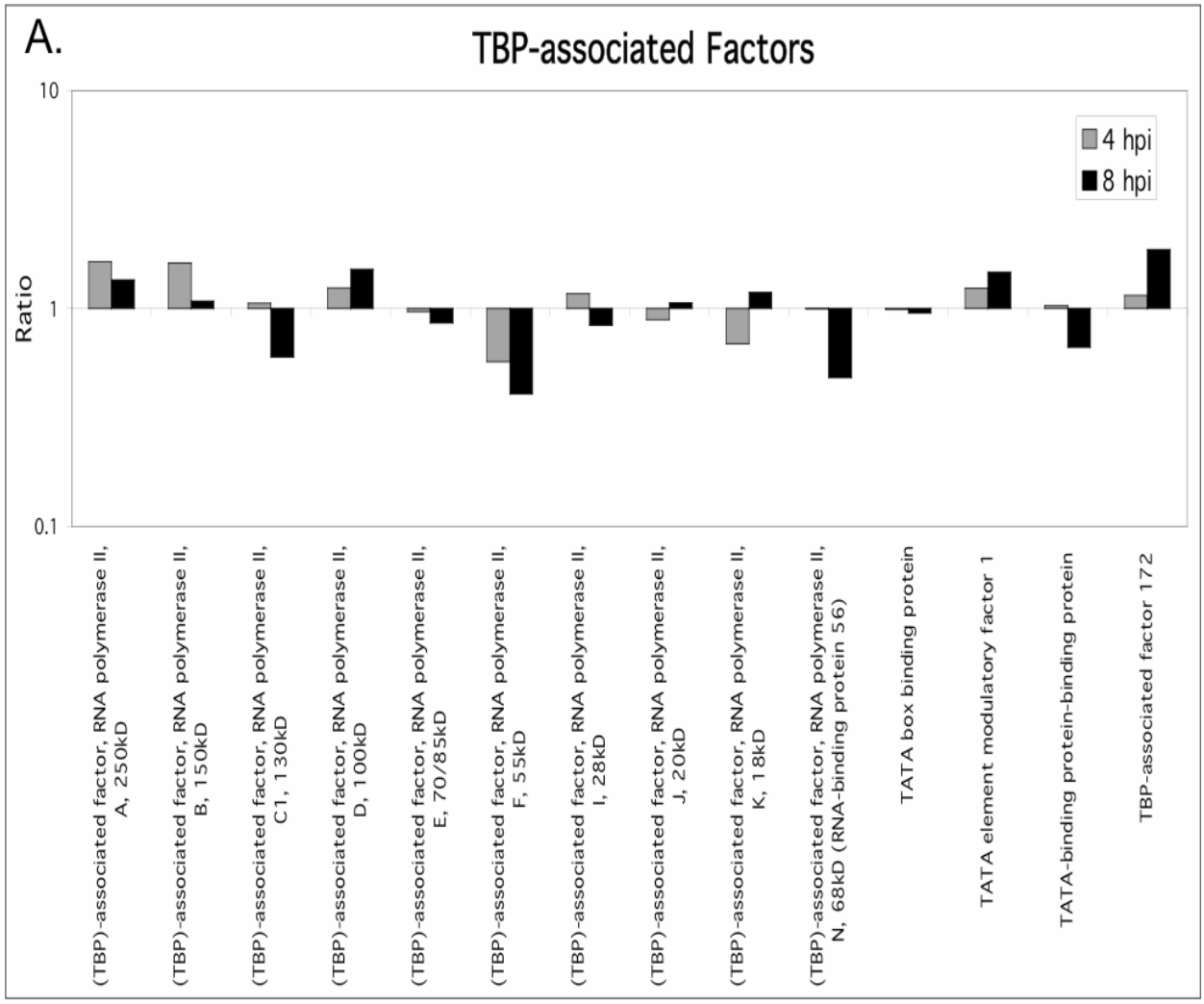


Figure 7. Microarry and northern blot analysis of changes in the expression of components of the general transcription factors during HSV infection.

A. Microarray changes in the mRNA abundances of subunits of the general transcription factor during wild-type infection. **B.** Northern blot analysis of poly A+ mRNA harvested at various times post wild-type infected (I) and uninfected (U) HEL cells that were probed for the indicated general transcription factor subunits.

When examining the TBP-associated factors of the general transcription factor TFIID through microarray, many factors appeared to be diffferentially expressed as a function of infection (Fig. 8A). The expression of TAF55 suggested to sharply decrease as a function of infection, was also shown to be dramatically downregulated during infection through northern analysis (Fig. 8B).



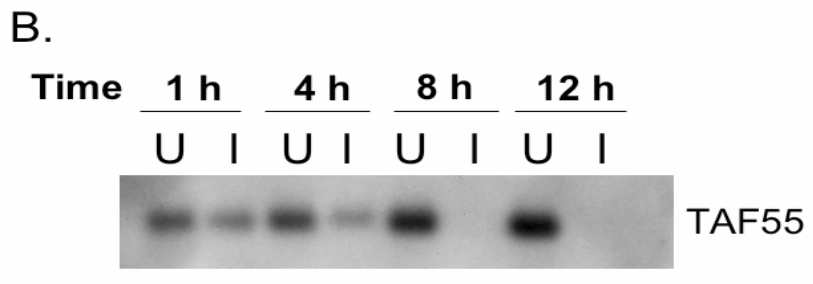


Figure 8. Microarry and northern blot analysis of changes in the mRNA abundances of the TBP-associated factors of the general transcription factor TFIID during HSV infection.

A. Changes in the mRNA abundances of subunits of the TBP-associated factors of TFIID. **B.** Northern blot analysis of poly A+ mRNA harvested at various times post wild-type infected (I) and uninfected (U) HEL cells probed for TAF55.

3.4. DISCUSSION

Using microarray and northern blot analysis, changes in the expression of components of the general transcription machinery were analyzed at early (4 hpi) and late (8 hpi) times post wild-type infection. Many changes in the mRNA abundances of these components were observed through microarray analysis with some being confirmed through northern blot analysis. Only a small number of these components were observed to be upregulated during infection while a larger number were observed to be downregulated during infection and these changes were particularly evident at later times of infection. These results suggest that changes in the expression of these components may result in alterations in the composition of the general transcription machinery available for transcription particularly at late times of infection.

3.4.1. Impact on cellular and viral gene expression

The general transcription machinery is essential to the expression of both cellular and viral genes. Alterations in the composition of the cellular transcription machinery may contribute to the global decline of host genome transcription (99). However, shutdown of host transcription is likely due to many viral induced mechanisms that exist earlier on during infection. Yet it is likely that alterations in the cellular transcription machinery can also help keep host genome transcription down. Because alterations in the composition of the cellular transcription factors occur later on in infection, these changes may have a greater impact on viral gene expression than cellular gene expression. At 4 hours post infection during the peak expression of viral early genes, the observed changes in the abundance of the general transcription machinery are only commencing suggesting that the early gene expression is most likely not affected prior to this

time. Since early promoters have cis-acting sequences that are common to cellular promoters, such as Sp1 and CCAAT box sites, viral early gene expression and cellular gene expression share similar requirements of the cellular transcription machinery. Alteration in the transcription machinery at this early time of infection would not be highly beneficial for early gene expression. ICP4 is also required for efficient early gene expression, yet the exact cellular requirements of ICP4-mediated activation of early genes are not well understood. For early gene activation, ICP4 synergizes with the activities of cellular activators, such as Sp1 to activate early genes. ICP4 and cellular activators most likely require the normal composition of the cellular transcription machinery for efficient early gene expression. The disruption or alteration of the normal composition would have severe consequences on early gene expression. The inability of cellular activators to function with altered transcription factors may in turn compromise ICP4's ability to activate transcription of early genes. With cellular activators unable to co-opt altered general transcription factors or operate in the absence of these transcription factors, these activators lose the ability to transactivate viral early genes and ICP4, although highly abundant at the time, is unable to activate transcription of early genes without the activities of these activators. Thus changes in the expression of the transcription machinery beyond 4 hpi, coincident with the time early genes are turned off may contribute to shutdown of viral early gene expression.

Late in infection (8 hpi), the abundance of the general transcription factors is grossly changed in comparison to earlier times of infection (4 hpi), suggesting that if viral gene expression would be affected, late gene expression would be impacted the most. However viral late gene expression proceeds efficiently and at high levels suggesting that if the normal composition of the cellular transcription machinery is altered, viral mechanisms exist to circumvent the normal

requirements. Late promoters are quite simplistic lacking many of the characteristics of viral early and cellular promoters. Late promoters, unlike any cellular promoters, are devoid of any influential upstream cis-activating sequences. Although these promoters still contain a TATA box, the only other element in addition to the TATA box that has been shown to be required for late gene expression is an INR (79, 110). In *in vitro* reconstituted transcription systems, ICP4 alone will activate late gene transcription to high levels with a simple set of general transcription factors in the presence of TFIID on late promoters that contain an INR element (79). Due to this and the fact that late promoters do not require cellular transactivators other than ICP4 for expression, late gene transcription may have differential requirements for the basal transcription machinery for expression. Since ICP4 alone is sufficient to activate late genes at high levels, ICP4 may cooperate in an optimal manner with a different set of factors late in infection to efficiently activate transcription of late genes by replacing or substituting for functions inherent to these factors.

3.4.2. Changes in the cellular transcription machinery

Infection with HSV caused the decreased expression of many components of the general transcription machinery more than it increased. A peculiar observation was the increased expression of the largest subunit of the RNA polymerase II enzyme. In contrast, the smaller subunits of pol II were observed to decrease during infection. The largest subunit is unique in that it contains a carboxyl-terminal domain that is absent from RNA pol I and III and plays an essential role in RNA pol II activity (reviewed 39, 144). Deletion of the CTD is lethal in mouse, Drosophila, and yeast. Phosphorylation of the CTD is important in the initiation and elongation

of transcription. Alternative states of phosphorylation are associated with different steps in the transcription cycle. The unphosphorylated CTD (IIa) is the form that is recruited to and assembled into the preinitiation complex. Extensive phosphorylation of the CTD (IIo) corresponds with the transition of RNA pol II from transcription initiation to elongation and is necessary for the progression of RNA synthesis. Many important transcription accessory factors are associated with the both forms of the CTD. During transcription initiation, the Mediator complex is associated with the CTD while splicing factors are associated during elongation. Interestingly during HSV infection, a form that deviates from the hypo- or hyper- phosphorylated form can be detected, termed the intermediate form (IIi) (164). This form runs intermediate to the unphosphorylated and the hyperphosphoylated forms of the CTD on SDS-polyacrylamide gels and is the predominant form late in infection. Two viral proteins, ICP22 and UL13 are responsible for this aberrantly phosphorylated form of the CTD (121). While the significance of this modification is not well understood it has been suggested that these changes may be responsible for the redirection of the transcription machinery from cellular to viral gene. In addition to redirecting the transcription machinery to viral genes, the aberrant phosphorylation of the CTD, the increased expression of this subunit, and the decreased expression of the smaller subunits of Pol II during infection may have a greater impact on viral gene expression. Transcription with the RNA pol II enzyme harvested from infected cells has not been assessed on viral early and late genes. It would be worthwhile to examine the properties of infected RNA Pol II in the presence of the other GTFs on the transcription of early and late genes and its ability to support ICP4-mediated activation. It is possible that late in infection cellular activators are not able to function with this altered RNA Pol II enzyme resulting in the shutdown of not only cellular gene expression but also viral early gene expression that require the actions of these

cellular activators. Late genes have no such requirements for cellular activators and it is possible that ICP4 can function with the infected form of RNA Pol II to efficiently activate late genes. This may be one mechanism that promotes the shutoff of early genes while allowing late gene expression to proceed.

The TBP-associated factor 55 (TAF55) of the general transcription factor TFIID was also suggested to be downregulated during infection though both microarry and northern blot analysis. TAF55 has recently been described to interact with TAF250 of TFIID and to inhibit its acetyltransferase (AT) activity (70). Reconstituted transcription showed that addition of exogenous TAF55 decreased TAF250-dependent transcription while having no effect on TAF250-independent transcription. TAF250-dependent transcription was decreased due to TAF55's ability to inhibit TAF250's AT activity. Given that late gene expression relies on TAF250 for INR function (48), late promoters could be considered TAF250-dependent. Future studies should be aimed at determining if the decreased expression of TAF55 during infection affects TAF250-dependent viral promoter activity. The presence of TAF55 could be hypothesized to block TAF250-promoter dependent late gene expression at early times of infection by blocking TAF250 AT activity. Late in infection the decreased abundance of TAF55 may lift the block in TAF250 activity resulting in an increase in the expression TAF250-dependent late promoters. This may be one of the mechanisms to prevent expression of late genes at early times of infection.

All three subunits of the general transcription factor TFIIA were suggested to be downregulated during wild-type infection through microarray and northern blot analysis. In the subsequent chapters we correlate the decreased expression of TFIIA with the attenuation of early gene expression. We show that TFIIA is required for efficient ICP4-activation of a viral early

promoter but that late in infection, TFIIA is downregulated and is not required for efficient ICP4-activation of an INR containing late promoter. We also show that TFIIA is not required for ICP4-activation of an INR-containing late promoter because ICP4 can substitute for TFIIA in stabilizing the binding of TFIID to the TATA box in the presence of a functional INR. Because TFIIA is required for ICP4-mediated activation of early genes, but is not required for ICP4-mediated activation of INR-containing late genes, the decreased expression of TFIIA may serve as yet another mechanism to shutoff early gene expression, while having no effect on late gene expression.

4. DIFFERENTIAL CELLULAR REQUIREMENTS FOR THE ACTIVATION OF HSV-1 EARLY (TK) AND LATE (GC) PROMOTERS BY ICP4

4.1. ABSTRACT

The herpes simplex virus, type 1 immediate early protein, ICP4, activates the transcription of viral early and late genes and is essential for viral growth. It has been shown to bind DNA and interact with components of the general transcription machinery to activate or repress viral transcription, depending upon promoter context. Since early and late gene promoters have different architectures, and cellular metabolism may be very different at early and late times after infection, the cellular requirements for ICP4-mediated activation of early and late genes may differ. This hypothesis was tested using tk and gC as representative early and late promoters, respectively. Nuclear extracts and phosphocellulose column fractions derived from nuclear extracts were able to reconstitute basal and ICP4-activated transcription of both promoters *in vitro*. When examining the contribution of the general transcription factors on the ability of ICP4 to activate transcription, the fraction containing the general transcription factor TFIIA was not essential for ICP4 activation of the gC promoter, but was required for efficient activation of the tk promoter. The addition of recombinant TFIIA restored the ability of ICP4 to efficiently activate the tk promoter, but had no net effect on activation of the gC promoter. The dispensability of TFIIA for ICP4 activation of the gC promoter required an intact INR element.

In addition, microarray and northern blot analysis indicated that TFIIA abundance may be reduced at late times of infection. This decrease in TFIIA expression during infection and its dispensability for activation of late but not early genes suggests one of the many possible mechanisms for the transition from viral early to late gene expression.

4.2. INTRODUCTION

During the lytic cycle of herpes simplex type 1 (HSV-1) infection, synthesis of viral gene products occurs in three temporally regulated phases, immediate-early (IE or α), early (E or β) and late (L or γ) (92). Each gene contains its own promoter regulatory region and is transcribed by the cellular RNA polymerase II (Pol II) transcriptional machinery (6, 36). Each class of genes, however, differs with respect to its promoter structure, which decreases in complexity from IE to E to L genes (199, 203). These class specific differences in promoter structure may be important in determining the ability to nucleate the assembly of stable preinitiation complexes at various phases of infection, in part mediating kinetic class-specific transcription (200, 216).

Five immediate early genes constitute the first set of genes to be transcribed upon HSV-1 infection. They are maximally expressed approximately 2 to 4 hours post infection (hpi) (92). These genes are expressed without prior viral protein synthesis due to the viral transactivator, VP16 (11, 23). VP16, a viral tegument protein, is released into the cell upon infection, associates with cellular proteins Oct1 and HCF to bind the virus specific TAATGARAT elements found exclusively on immediate-early gene promoters to activate transcription from these promoters (69, 142, 143). In addition to a TATA box and TAATGARAT elements, sites

exist for cellular *cis*-acting factors such as Sp1 and others that contribute to enhanced transcription (72).

Of the immediate early proteins, ICP4 is absolutely required for progression beyond the immediate early phase of gene expression due to its role a transcriptional activator of early and late genes (31, 43, 44, 51, 58, 74, 140, 141). As a transactivator, ICP4 functions to increase the rates of transcription by increasing the rate of transcription complex assembly on promoters (78). ICP4 interacts with components of the basal transcription apparatus to either activate or repress transcription (25, 79-81, 113, 181). Although ICP4 contains a DNA binding region that is essential to activation (47, 63, 137, 151, 175), no specific ICP4 binding sites have been identified on early and late promoters that are responsible for activation (56, 95, 180). Deletion of ICP4 severely impairs expression of early and late genes (44). However, certain mutations in ICP4 that have no effect on early gene transcription do not allow late gene expression, suggesting that ICP4 may act differently on early and late promoters (43, 47).

Efficient transcription of the β or early genes is strictly dependent on the presence of functional α proteins, particularly ICP4 (44, 51, 93, 102, 156, 157). Early promoters differ from IE promoters in that they lack the viral specific TAATGARAT sequences present in IE promoters. However they are similar to IE promoters in that they contain a TATA box and retain upstream cellular activating sequences such as Sp1 and CCAAT boxes, that contribute to activation of these genes (56, 72, 188). Their expression peaks 4 to 6 hpi and is subsequently shut off.

The γ or late genes are the last set of genes that are expressed. They are categorized as either leaky-late (γ1) or strict-late (γ2), depending on their requirement for DNA synthesis for expression (reviewed in 199). The γ1 genes can be suboptimally expressed in the absence of

viral DNA synthesis, whereas the γ2, or true late genes, have a strict requirement for viral DNA synthesis (32, 90-92, 102). True late promoters are clearly distinct from immediate-early and early promoters. These promoters are relatively simple in structure, because while they contain a TATA box, they are devoid of any essential upstream activating sequences (64, 83, 91, 94, 100). Only two known elements downstream from the TATA box are important for late gene expression, the initiator element (INR), which overlaps the transcriptional start site, and the downstream activating sequence (79, 82, 83, 94, 185). ICP4 has been shown to activate transcription from the true late gC promoter maximally when both TATA box and INR are present (79). Mutations in the INR element of this promoter significantly reduce ICP4 activated transcription both *in vitro* and *in vivo* indicating the importance of this element for late gene expression (110).

The basal RNA Pol II transcription machinery is composed of at least seven general transcription factors (GTFs), including TFIIA, -B, -D, -E, -F and -H, and Pol II, which assemble on core promoters in a coordinated manner or, as more recently proposed, as a preassembled holoenzyme complex (213). TFIID is a multiprotein complex containing a TATA binding protein (TBP) and 10 to 14 tightly associated subunits known as TAFs or TBP-associated factors (52, 218). Binding of TFIID to the TATA box, via TBP, is critical for the rate and efficiency of preinitiation complex assembly (18, 94, 214). In reconstituted transcription systems, TBP can function in the absence of TAFs to promote basal levels of transcription from TATA-containing core promoters (19). However, TBP alone is insufficient to support activated transcription by both viral and cellular activators, such as Sp1, VP16, and ICP4. Effective response to these activators requires TAFs in TFIID and many activators have been shown to interact with TFIID through TAFs (21, 73, 77). TAFs also contribute to promoter selectivity by interacting with

other basal transcription factors, other TAFs, and specific DNA sequences, such as the INR element (105, 106). TAF250 is an integral component of the TFIID complex that along with TAF150 and TBP has been shown to interact with the eukaryotic INR element (26, 107, 195). Interestingly, ICP4 was found to interact with TAF250 of TFIID through its C-terminal domain (25).

The GTF, TFIIA, binds TBP and stabilizes TBP binding to the TATA box (18, 35, 97, 115, 123). It is not required for basal transcription when reconstituted with TBP and the remaining GTFs. However, it has been shown that activator-enhanced transcription is not only mediated through the TAFs in TFIID, but is also dependent on TFIIA (40, 111, 122, 147, 189, 211). TFIIA is composed of three subunits (α, β, and γ). The smallest subunit (γ) is required for the ability of TFIIA to stabilize TBP binding to the TATA box and is essential for activated transcription by upstream activators such as Sp1, VP16, NTF-1, and the Epstein-Barr virus activator Zta (40, 118, 148, 211). Mutations in the γ-subunit have been shown to disrupt transcriptional activation by VP16, GAL4-CTF, AP-1 (147). However, *in vitro* transcription studies have shown that TFIIA is dispensable for activation of the HSV gC promoter by ICP4 (79).

The cellular requirements for activation of early promoters by ICP4 have not been studied. Given that HSV infection has a profound effect on cellular gene expression and metabolism, it is possible that the cellular transcription machinery is different at times when early and late genes are transcribed. To this end, we examined features of the general transcription machinery that contribute to ICP4 activation of two structurally distinct promoters, the early (tk) and the true late (gC) promoters, using a reconstituted *in vitro* transcription system. We showed that the general transcription factor TFIIA was required for efficient ICP4-activation

of the early tk promoter, but was not essential for efficient ICP4-activation of the late gC promoter. The dispensability of TFIIA for activation of the gC promoter required a functional INR element. Addition of TFIIA greatly stimulated ICP4's ability to activate transcription of a mutated INR, suggesting that in the absence of an initiator element, activation by ICP4 requires the additional activities of TFIIA to stabilize TFIID binding to the promoter. In addition, we found that TFIIA subunits mRNA abundance was greatly reduced in cells late in infection. These data altogether suggest that at late times of infection the abundance of TFIIA may decline, potentially downregulating ICP4 activation from early promoters. Changes in the abundance of TFIIA would have little effect on the activation of true late promoters by ICP4, since they possess INR elements. This would allow for efficient expression of late genes while turning down expression of early genes.

4.3. MATERIALS AND METHODS

4.3.1. Cells and Viruses

The KOS strain of HSV-1 was used to infect HEL cells for northern blot analysis and Vero cells for ICP4 purification. HeLa cells were used to prepare nuclear extracts and phosphocellulose column fractions.

4.3.2. Preparation of phosphocellulose fractions, human and recombinant TFIIA and ICP4

Phosphocellulose fractions were prepared from 10 L of HeLa nuclear extracts as previously described (25, 50). Approximately 15 ml of nuclear extract at 9.2 mg/ml was loaded over a 15 ml packed column volume of phosphocellulose (P11) resin and eluted as previously described (50). Approximately 96 mgs of total protein eluted, 44% in the A fraction (42 mg in a 20 ml pool), 29% in the B fraction (28 mg in an 8 ml pool), 16% in the C fraction (16 mg in a 6 ml pool), and 11% in the D fraction (10 mg in a 6 ml pool). All fractions were dialyzed against D100 buffer (20 mM HEPES [pH 7.9], 10% glycerol, 100 mM KCl, 2 mM EDTA, 1 mM ditiothreitol, 2 mM phenylmethylsulfonyl fluoride) for use in transcription reactions. Human TFIIA was purified from the first phosphocellulose fraction (the A fraction) by further fractionation on DEAE-Sephacel (25, 160). The 0.5 M KCl eluate (AB fraction) from this column, containing TFIIA, was ammonium sulfate precipitated (0.42 mg/ml), resuspended in buffer D100 and loaded over a Superose 12 gel filtration column equilibrated with D100, as described by Reinberg et al. to purify human TFIIA (160). Recombinant human TFIIA was produced from uncleaved α/β and γ subunits carrying histidine tags from over expressing M15 bacteria harboring pQE-TFIIA$\alpha\beta$ and pQE-TFIIAγ (148). The two polypeptides were independently purified on Ni-NTA agarose columns under denaturing conditions and were renatured separately or in equimolar amounts to produce recombinant TFIIA (rTFIIA) as described elsewhere (111). ICP4 was purified from wild-type (KOS) infected Vero nuclei as previously described (110). Purified rTFIIB used in transcription reactions and purified rTBP used in gel shift analysis were previously described (25).

4.3.3. In vitro transcription and primer extension

The plasmids pLSWT (95), pgCLS1 (79) and pgCLS8 (110) were used as templates for *in vitro* transcription reactions. Reconstituted transcription and primer extension analysis reactions were carried out as previously described (110). Supercoiled DNA templates (100 ng) were incubated with nuclear extract or mixtures of phosphocellulose fractions in the presence or absence of approximately 250 ng ICP4. Phosphocellulose fractions were added back based on their percent contribution to the total protein recovered from the phosphocellulose column to 55 μg per reaction. For each reaction mixture, 11.5 μl of the A fraction, 4.6 μl of the B fraction, 3.4 μl of the C fraction, 3.2 μl of the D fraction and 1 μl of 0.31 mg/ml rTFIIB was used. Where the A fraction was substituted, 3 μl of the AB fraction, 3 μl of Superose 12 purified TFIIA, or the indicated amount of rTFIIA was used.

4.3.4. Electrophoretic mobility shift assay

The EcoRI-BamHI fragment of the plasmid p4 (113) containing a TATA box and an ICP4 binding site was end labeled with ^{32}P using polynucleotide kinase, purified and quantified. DNA binding reaction mixtures (30 μl) contained 12.5 mM HEPES (pH 7.8), 60 mM KCl, 12.5% glycerol, 5 mM MgCl2, 0.5 mg/ml BSA, 20 mM β-mercaptoethanol, 25 μg poly(dG)-poly(dC)/ml and the DNA probe (10^4 cpm) and were incubated with protein samples at 30^{o}C for 30 minutes. The amounts of protein used in the binding reactions were 15 ng TBP, 60 ng of rTFIIAαβ or 15 ng of rTFIIAαβγ. After 30 minutes, 1 μl of anti-TFIIAγ was added where indicated and incubated for an additional 15 min at 30^{o}C. Reactions were resolved on nondenaturing 4% 0.5X Tris-borate-EDTA acrylamide gels and exposed to film.

4.3.5. Northern blot analysis

Poly A+ RNA (2 μg) harvested from uninfected and KOS infected (multiplicity of infection of 10) HEL cells at 4 and 8 hpi was resolved by denaturing formaldehyde-agarose gel electrophoresis, transferred to nitrocellulose membranes, and probed, washed and exposed to film as previously described (53). ^{32}P-labeled probes were generated from pQE-TFIIAαβ and pQE-TFIIAγ (147) by nick translation using [α-^{32}P]dCTP and [α-^{32}P]dGTP.

4.4. RESULTS

4.4.1. Reconstitution of basal and ICP4 activated transcription on early (tk) and late (gC) promoters *in vitro*

It has previously been shown that nuclear extracts prepared from uninfected HeLa cells support basal and ICP4-activated transcription from both an early (tk) and a late (gC) promoter (79). When more purified GTF systems are used instead of nuclear extracts, ICP4 efficiently activates the gC promoter (25, 79). However, ICP4-activated transcription of the tk promoter has not been tested in different GTF systems. To delineate any differences in requirements for cellular transcription factors for the activation of early and late promoters, we first set out to examine the contribution of these factors in a crudely fractionated transcription system. The first step in a commonly utilized strategy for the purification of the GTFs from nuclear extract involves fractionation on phosphocellulose columns (Fig. 9A). Four fractions are generated, A, B, C, and

D, depending on the salt concentration used for elution (0.1, 0.3 M, 0.5 M and 1 M KCl, respectively). One or more of the GTFs can be found in each fraction (TFIIA is found in the A fraction, and TFIID is found in the D fraction). To reconstitute transcription, each fraction was added back as a percentage of the total protein amount recovered (see Materials and Methods). Equivalent amounts of protein were used in reconstitution experiments with both the tk and gC promoters, in the presence and absence of purified ICP4. As observed in Fig. 9B, both basal and ICP4-activated transcription of the tk and gC promoters were efficiently reconstituted using fractions A through D, with the addition of rTFIIB. Apparently, insufficient quantities of TFIIB were recovered in the C fraction to reconstitute transcription.

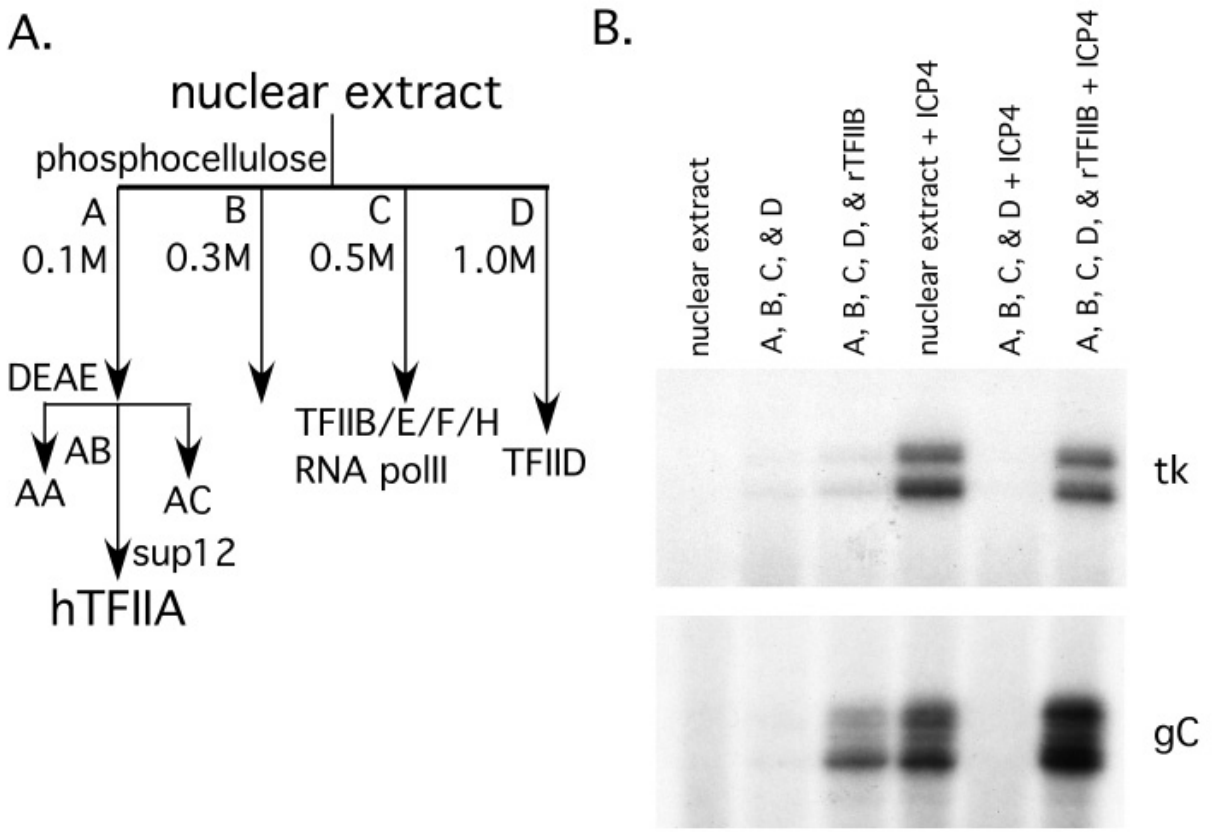


Figure 9. Phosphocellulose column fractions reconstitute basal and ICP4 activated transcription from early (tk) and late (gC) promoters.

A. Scheme for transcription factor fractionation of nuclear extract on phosphocellulose resin column generating four distinct fractions (A, B, C, and D) that contains one or more of the basal transcription factors. The further fractionation of hTFIIA on DEAE and superose 12 columns is also diagrammed. **B.** The ability of phosphocellulose column fractions to reconstitute transcription on representative early and late promoters was assayed for in the presence and absence of purified ICP4. Each fraction was added back based on the present contribution to the total amount of protein recovered from the column to equal 55 ug of protein per each reaction and compared to 60 ug of nuclear extract. Phosphocellulose fractions A through D in the presence of added recombinant TFIIB were able to support basal and ICP4 activated transcription on both tk and gC promoters.

Since each phosphocellulose fraction was enriched for one or more of the basal transcription factors in addition to other factors that play a role in transcription, the contribution of each phosphocellulose fraction to basal and ICP4-activated transcription of both the tk and was promoters was assessed (Fig. 10). Each fraction was individually omitted from the reactions with the tk and gC promoters, and transcription was assessed in the presence and absence of ICP4. The most striking result of this analysis was the differential requirement of the A fraction for ICP4 activation of the tk and gC promoters (Fig. 10). The A fraction was not required for efficient activation of the gC promoter by ICP4. In contrast, activation of the tk promoter by ICP4 in the absence of the A fraction was very inefficient. This suggests that some factor or factors present in the A fraction are crucial for ICP4 activation of the tk promoter, but not for activation of the gC promoter.

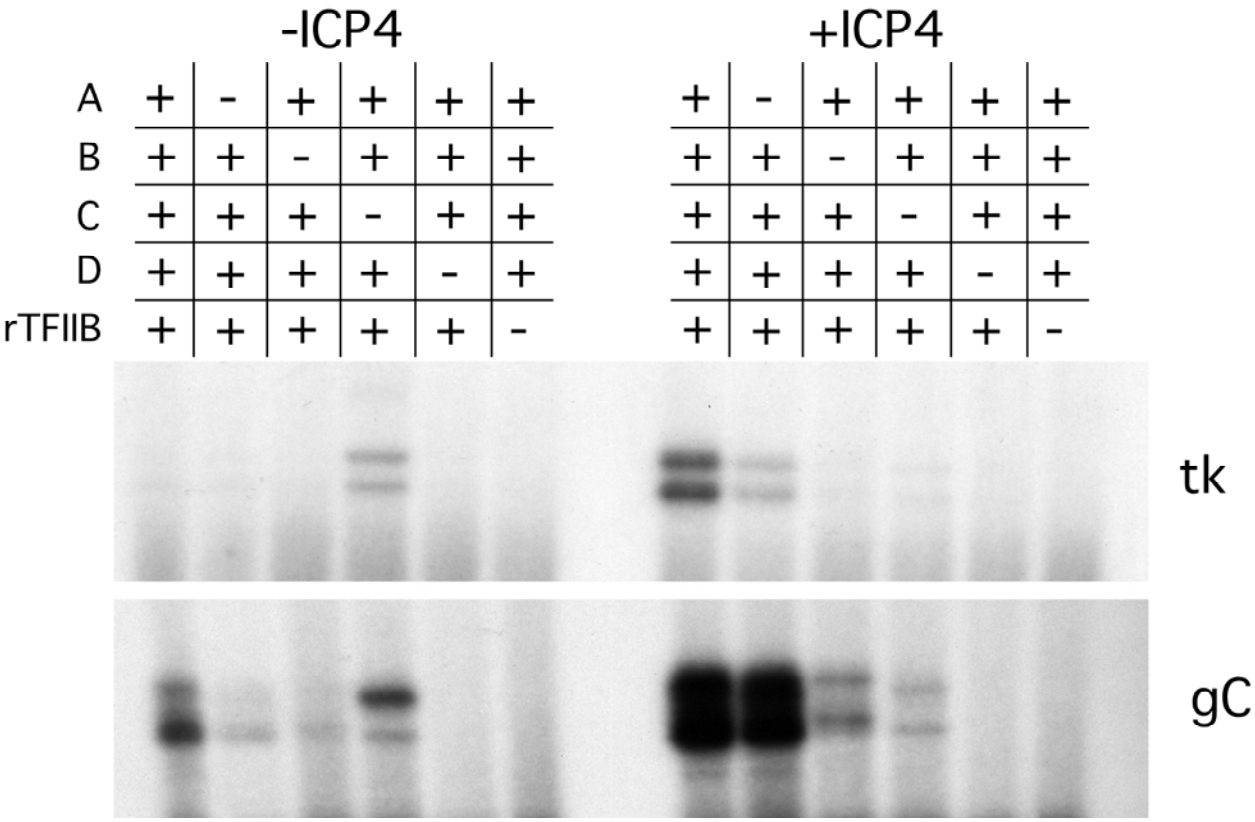


Figure 10. The A fraction is required for efficient ICP4 activation of the tk promoter but is not essential for activation of the gC promoter.

The contribution of each phosphocellulose fraction to basal and ICP4 activated transcription was assayed on both early and late promoters by omitting each fraction from the reaction in the presence and absence of ICP4. Most notable was the differential requirement for the A fraction for ICP4 activation of the early (tk) vs. late (gC) promoter.

4.4.2. Differential requirements for TFIIA in activation of the gC and tk promoters by ICP4

Because the GTF TFIIA is a component of the A fraction, we sought to further fractionate this factor to determine its relevance in the activation of the tk promoter by ICP4. Human TFIIA was further fractionated by chromatography on a DEAE-Sephacel column. The bulk of TFIIA activity eluted in the 0.5 M KCl wash, also known as the AB fraction (Fig 9A). To assess its contribution to ICP4 activation, the AB fraction was substituted for the A fraction in reactions

with both tk and gC promoters (Fig. 11). In the absence of the A fraction, ICP4 efficiently activated the gC promoter, and the addition of AB had little effect on ICP4 activation. In contrast, omission of the A fraction highly diminished the ability of ICP4 to transactivate the tk promoter. Addition of the AB fraction, however, restored the ability of ICP4 to efficiently activate the tk promoter. These data suggests that this fraction, which is known to contain TFIIA, is important for activation of the tk promoter by ICP4 but is dispensable for activation of the gC promoter.

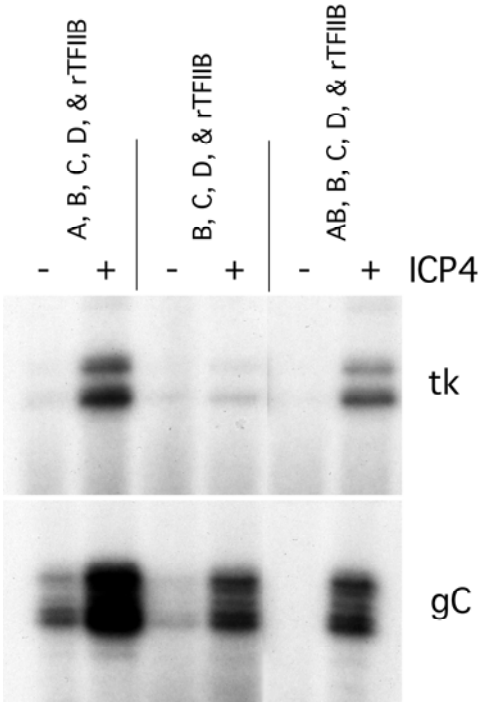


Figure 11. The AB fraction restores ICP4's ability to efficiently activate the early (tk) promoter but has no net effect on activation of the late (gC) promoter.

The phosphocellulose A fraction was further fractionated on DEAE-sepharose (Fig 3A) and the 0.5 M KCl wash (AB fraction) containing the general transcription factor TFIIA, was tested for its ability to substitute for the A fraction in ICP4 activation. The AB fraction was able to efficiently restore ICP4 activation of the tk promoter but had no effect on activation of the gC promoter.

TFIIA was further purified from the AB fraction by gel filtration on Superose 12. Fractions from the Superose 12 column were tested for the ability to compliment ICP4 activation of the early tk promoter (Fig. 12A). Complementing activity was most abundant in fractions 6 and 7, which also corresponded a molecular weight of 82 kDa, in accordance with the previously published molecular mass of the TFIIA complex and its elution profile on Superose 6 (160). The ability of this form of TFIIA to support activation of the tk promoter was compared to that of the AB fraction and found to substitute as well as, or better than the AB fraction in restoring the ability of ICP4 to activate the tk promoter (Fig. 12B). This indicated that TFIIA found in the A fraction was factor responsible for efficient activation of the tk promoter by ICP4.

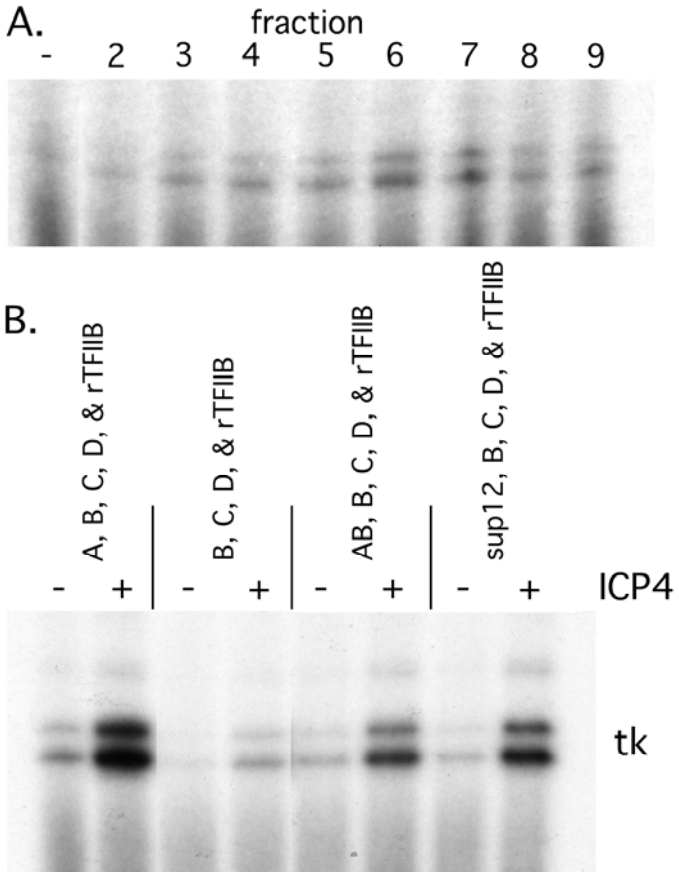


Figure 12. Complementation of ICP4 activation of the tk promoter by superose 12 fractions.

A. Complementing activity was further purified from the AB fraction of DEAE-sepharose by superose 12 gel filtration. Each fraction was assayed for its ability to support ICP4 activation from the tk promoter. The most

abundant complementing activity was found in fractions 6 and 7. This eluted at the size of TFIIA previously reported. **B.** Superose 12 purified TFIIA was substituted for the A or AB fraction and found to efficiently support ICP4 activation of the tk promoter.

To strengthen the interpretation that TFIIA was critical for the activation of tk and to rule out the possibility that some factor co-purifying with TFIIA was contributing to the stimulatory effect on the tk promoter, recombinant TFIIA was produced and used as an alternate source. Human TFIIA is comprised of three subunits, α, β and γ, which have sizes of 35-, 19- and 13-kDa, respectively. The α- and β- subunits of the human form are encoded by one gene (41, 122, 210) while the γ-subunit is encoded on a separate gene (40, 148, 189, 211). rTFIIA was produced from bacteria separately over expressing TFIIAαβ and TFIIAγ, purified under denaturing conditions, and renatured either separately or in a mixture of equal molar amounts. Gel mobility shift assays were conducted on a DNA fragment containing a TATA box to assess the activity of reconstituted rTFIIA (Fig. 13). TBP alone failed to bind the probe, as did either TFIIAαβ or TFIIAαβγ. TBP plus TFIIAαβ also failed to bind the probe, due to the absence of the γ-subunit, which is required for the stabilized binding of TBP to TATA boxes. However, TFIIAαβγ stabilized the binding of TBP to DNA forming the previously observed "DA" complex (40, 148, 189, 211). An antibody specific to the γ-subunit was able to induce a supershift, suggesting the presence of TFIIA in this complex and confirming the successful reconstitution of TFIIA activity.

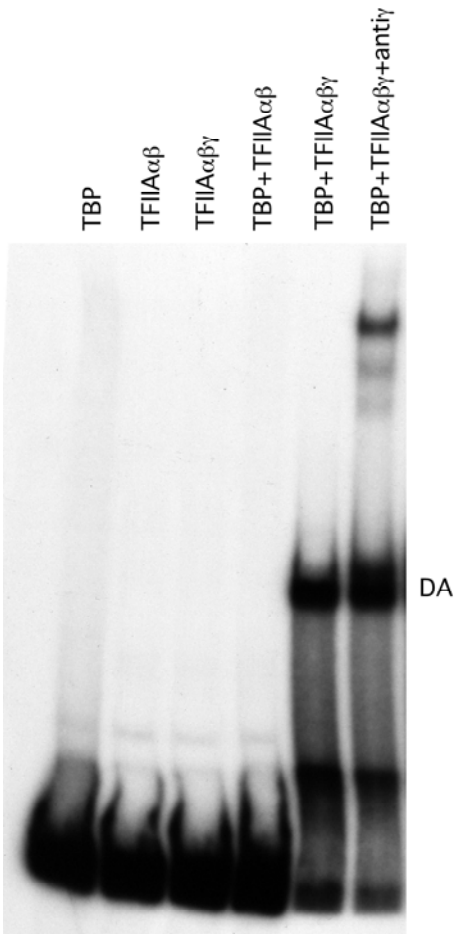


Figure 13. Reconstitution of TFIIA activity using recombinant proteins.

TFIIAαβ and TFIIAγ were expressed in E. coli, purified under denaturing conditions and renatured separately or together. rTFIIA activity was assessed via gel mobility shift analysis with αβ or αβ + γ, and TBP on the TATA motif of the ICP4 promoter. Only TFIIA αβ + γ was able to stabilize binding of TBP to the TATA box and be supershifted by a TFIIA gamma specific antibody suggesting successful reconstitution of TFIIA activity.

Recombinant TFIIA was then compared to the A and Superose 12 fraction for the ability support ICP4-activated transcription of the tk and gC promoters *in vitro* (Fig. 14A). Again, ICP4 efficiently activated the gC promoter independent to a TFIIA-containing preparation (Fig. 14A), and was unable to efficiently activate the tk promoter in the absence of a TFIIA preparation (Fig. 14A). However, rTFIIA, like the A and Superose 12 fractions, restored the ability of ICP4 to efficiently activate the tk promoter. These data support the interpretation that TFIIA is not

essential for activation of the late gC promoter but is required for efficient activation of the early tk promoter.

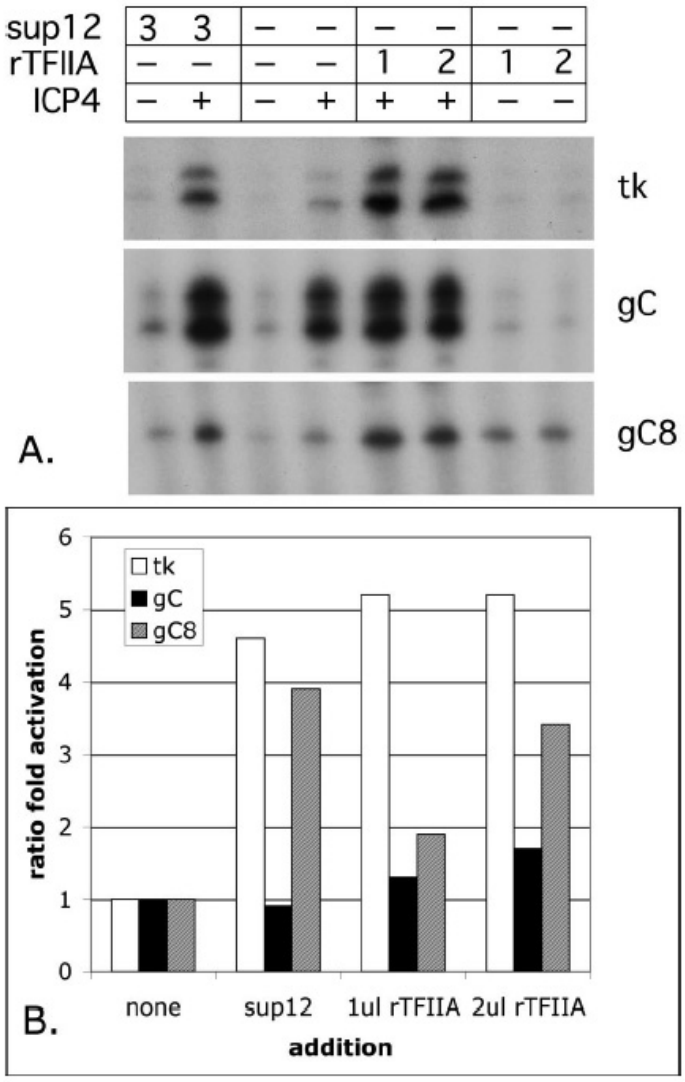


Figure 14. Ability of rTFIIA to enhance ICP4 activation of an early (tk), a late (gC), and an initiator-mutated late (gC8) promoter *in vitro*.

A. The indicated amounts (in microliters) of Superose 12 human TFIIA and rTFIIA were assayed for the ability to support ICP4 activation of the tk, gC, and gC8 promoters *in vitro*. Shown are the autoradiographic images of primer extension products of the *in vitro* transcription reactions. **B.** Quantification of *in vitro* transcription reactions conducted as described in the legend for panel A. Additional *in vitro* transcription reactions were conducted precisely as described for panel A. The dried gel containing the electrophoretically separated primer extension products was analyzed using a Storm 840 PhosphorImager. Under each condition, the fold activation by ICP4 from two determinations was calculated and averaged. Without the addition of a TFIIA-containing fraction, ICP4 activated the tk, gC, and gC8 promoter by five-, nine-, and fourfold, respectively. The values for fold activation under each of the conditions (TFIIA additions) were divided by fold activation for no addition in reactions with the corresponding promoter. Shown are the ratios of fold activation for no addition (none), Superose 12 fraction addition (sup12), and the addition of the indicated amounts of rTFIIA.

True late promoters are relatively simple promoters that do not require upstream activating sequences for efficient expression (199, 203). The efficient activation of the gC promoter by ICP4 requires only an intact TATA box and INR region. The initiator element has shown to be crucial for ICP4's ability to activate gC promoter, since mutations in the initiator reduce its ability to efficiently activate transcription (79, 110). TFIID makes contact with the INR and ICP4 facilitates TFIID binding to promoters (25, 78). TFIIA has previously been shown to be essential for activated transcription in the context of TFIID, yet as our results indicate, TFIIA is not essential for ICP4 to activate the gC promoter. TFIIA has also been shown to stabilize TFIID binding to the TATA box. Therefore, we reasoned that it was possible that in the absence of a functional INR, TFIIA might restore the ability of ICP4 to activate transcription. To this end, we compared the ability of ICP4 to activate the gC promoter to that of a derivate of the gC promoter with a mutated INR element, gC8, in the presence and absence of TFIIA (Fig. 14A). gC8 has three nucleotides mutated in the initiator region and is severely compromised for ICP4 activation (110). In the absence of the A fraction, gC8 was minimally activated by ICP4 and addition of the A fraction somewhat stimulated activation. Substitution of the A fraction with human or recombinant TFIIA significantly stimulated ICP4 activation, suggesting that in the presence of TFIIA the requirement for the INR element for activation by ICP4 is reduced.

A quantitative representation of the effects of TFIIA on ICP4 activation of the tk, gC, and gC8 promoters is given in Fig. 14B. Additional reactions were conducted as described for Fig. 14A. The fold activation under each condition was determined as described in the figure legend. Shown are the ratios for fold activation with the indicated addition relative to that with no addition. Therefore, while gC was strongly activated by ICP4 without TFIIA addition (Fig.

14A), the addition of TFIIA had little effect on the fold activation (Fig. 14B). In contrast, the addition of the Superose 12 TFIIA preparation to reaction mixtures with the tk and gC8 promoters resulted in 4.6- and 3.9- fold-greater activation, respectively, compared to that in reactions without TFIIA addition. Similar results were obtained upon the addition of rTFIIA (Fig. 14B).

4.4.3. Expression of TFIIA during wild-type HSV infection

Because cellular gene expression is significantly changed during the course of lytic infection we were interested in looking at what consequences this had on the expression of components of the basal transcription machinery as a function of time post infection. Microarray analysis conducted on mock-infected cells and cells infected with wild-type virus for 4 and 8 hours revealed a decrease in the abundance of the mRNA for the smallest γ-subunit of TFIIA (data not shown). Northern blot analysis using 2 μg of poly(A)$^+$ RNA from mock- and KOS- infected cells confirmed the decreased expression of this subunit as well as that for the other subunits of TFIIA. By 8 hpi, TFIIA mRNA was undetectable (Fig. 15). These data indicate that TFIIA abundance may decreases as a function of infection.

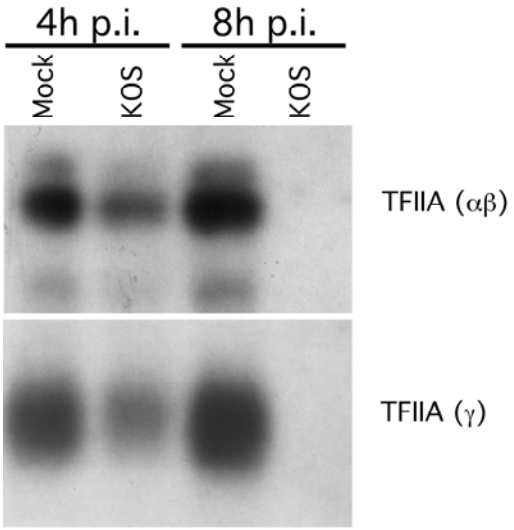


Figure 15. TFIIA mRNa levels as a function of infection.

Poly(A)+ RNA (2 ug) isolated from KOS-infected (multiplicity of infection =10) or mock-infected HEL cells at 4 and 8 hpi was subjected to Northern blot analysis probing for all the TFIIA subunit mRNAs.

4.5. DISCUSSION

Unlike many cellular activators, ICP4 does not have a requirement for specific DNA binding sites for activation and therefore it can activate transcription from a variety of promoters. Looking at two distinct promoters, the tk promoter and the gC promoters, we found different cellular requirements for ICP4-activated transcription. Specifically, the GTF TFIIA was required for efficient ICP4 activation of the tk promoter but was not essential for activation of the late gC promoter. These results indicate that ICP4 has some different requirements for components of the general transcription machinery based on the promoter and possibly on the HSV kinetic class of promoter.

These results suggest that the composition or the assembly of ICP4-activated transcription complexes on the two promoters is different. In the assembly of Pol II transcription

complexes, TFIID is the first factor recruited to the TATA box and is required for subsequent complex assembly (18, 194, 213). By participating in the complex, TFIIA helps stabilize binding of TFIID to the TATA box (35, 97, 115, 123). TFIIA and the TAFs present in TFIID are also required for activated transcription by such activators as VP16, Sp1, CCAAT binding factor and the Epstein-Barr virus transactivator Zta (119, 147, 211). In addition, many of these activators have been shown to interact with TFIIA, TFIID, or both, and these interactions correlate with the ability to stabilize or enhance TFIID-TFIIA-promoter complex assembly and promote transcription (111, 118, 148). In contrast, ICP4 will activate the gC promoter in the absence of TFIIA (25, 79) (Fig. 14B). It has also been shown to interact with TFIID (25), enhance the binding of TFIID to TATA boxes, and promote preinitiation complex (PIC) formation in the absence of TFIIA (78). Therefore, it is likely that ICP4 operationally substitutes for TFIIA in that it independently performs a function normally associated with TFIIA, allowing for efficient activation of the gC promoter.

TFIIA, however, is clear required for efficient activation of the tk promoter. One difference between the tk and gC promoters is the existence of an INR element located at the start site of gC transcription. Core promoters contain a TBP sequence, located 25 to 30 bp upstream of the transcription start site, and some have an initiator element (INR) encompassing the start site. The presence of an INR in addition to a TATA box can serve to enhance the strength of that promoter (55, 120). For promoters that lack a TATA consensus, the initiator element can direct accurate transcription initiation by itself or in conjunction with other promoter elements (125, 177, 178). TAF150, TAF250 and TBP found in TFIID have been shown to stably interact with the INR element and are sufficient for INR activity (26, 195). ICP4 has been shown to bind TFIID through TAF250 and has been shown to stabilize TFIID interaction to the

core promoter (25). The late gC promoter contains both a TATA box and an INR region. Mutations in the initiator element diminish the ability of ICP4 to activate transcription (79, 110). We show here that in the context of an intact INR, ICP4 does not require TFIIA for activated transcription (Fig. 14). This suggests that ICP4 sufficiently stabilizes TFIID through the INR element without the activities of TFIIA. In the presence of a mutated INR, ICP4 alone is no longer able to stabilize TFIID interaction and requires the additional activities of TFIIA. In the presence of TFIIA, ICP4 overcomes the requirement for an intact INR and in conjunction with TFIIA stabilizes TFIID-promoter interactions, thereby promoting activation by ICP4. Although ICP4 interacts with TFIID through TAF250 and TFIIA interacts with TFIID through TBP it is not currently known whether ICP4 directly interacts with TFIIA. It will be interesting to determine whether ICP4 participates in tripartite complexes with TFIIA and TFIID (or TBP) on early and late promoters and to determine the contribution of the INR element on these potential interactions.

4.5.1. Biological significance of the differential requirement for TFIIA in the activation of early and late genes

HSV infection results in dramatic cellular changes, occurring at the levels of mRNA, protein abundance, and post-translational modifications. At late times of infection, the expression of cellular proteins is greatly perturbed suggesting that the composition and/or abundance of components of the Pol II transcriptional machinery may also be significantly altered. Northern blot analysis indicated that the expression of the smallest subunit of TFIIA decreased as a function of infection (Fig. 15). The viral functions that downregulate TFIIA expression are not

currently known. However, this result suggests that TFIIA may not be available for transcription late after infection. This of course would also depend on the stability and activity of the pre-existing three-peptide subunits of TFIIA.

While INR elements have been found on most late promoters, they have not been found or described on early promoters. Therefore, it is possible that a reduction in TFIIA abundance may be one of the mechanisms functioning in the switch from early to late transcription. It is likely that this mechanism is not the only one functioning in this switch. Sp1, which is clearly involved in the transcription of early genes and immediate-early genes, is phosphorylated about the time early genes are shut off. This phosphorylated form of Sp1 is less active in transcription than uninfected cell Sp1 (109). Viral DNA replication is also involved in the switch from early to late gene transcription. In addition, TFIIA is required for activated transcription by VP16, Sp1 and the CCAAT binding protein. Since these activators are involved in immediate-early and early gene transcription, a reduction in TFIIA activity would also reduce the effect of these activators on immediate-early and early transcription.

ICP4 is a large complex molecule with many possible features and functions that have yet to be recognized that allow it to activate transcription from a variety of promoters. In the context of lytic infection, it would follow that if components of the transcription machinery were changing as a function of infection, ICP4 might activate transcription by a variety of mechanisms involving different contacts with different cellular transcription factors. As presented here, TFIIA is required for efficient activation of early promoters but not late promoters. The decreased abundance of TFIIA late in infection may contribute to the switch from early to late transcription.

5. STABILIZED BINDING OF TBP TO THE TATA BOX OF HSV-1 EARLY (tk) AND LATE (gC) PROMOTERS BY TFIIA AND ICP4

5.1. ABSTRACT

Although most activators require all the components of the basal transcription machinery to activate transcription, we have recently shown through an *in vitro* transcription system that ICP4 has a differential requirement for the general transcription factor TFIIA. TFIIA is dispensable for ICP4-activation of a late promoter (gC) but is required for the efficient activation of an early promoter (tk). An intact INR element is required for proficient ICP4-activation of the late promoter in the absence of TFIIA. In the presence of INR-mutated late promoter, ICP4 requires the additional activities of TFIIA to activate transcription suggesting that in the presence of TFIIA, ICP4 can overcome the requirement of an intact INR on late promoters. Because TFIIA is known to stabilize the binding of both TBP or TFIID to the TATA box of core promoters and ICP4 has been shown to interact with TFIID, we tested the ability of ICP4 to stabilize the binding of either TBP or TFIID to the TATA box of representative early, late, and INR-mutated late promoters (tk, gC, and gC8, respectively). Utilizing DNase I footprinting analysis, we found that ICP4 was able to facilitate TFIIA stabilized binding of TBP to the TATA box of the early tk promoter. ICP4 and ICP4-derivatives, n208 but not X25, could stabilize the binding of TBP to both the wild-type and INR-mutated gC promoters. When TFIID was substituted for TBP, ICP4

could stabilize the binding of TFIID to the TATA box of the wild-type gC promoter. ICP4, however, could not effectively stabilize TFIID binding to the TATA box of the INR-mutated late promoter. The additional activities of TFIIA were required to stabilize the binding of TFIID to the INR-mutated late promoter. Collectively, these data suggest that TFIIA may be dispensable for ICP4-activation of the wild-type late promoter because ICP4 can substitute for TFIIA's ability to stabilize the binding of TFIID to the TATA box. In the absence of a functional INR, ICP4 can no longer stabilize TFIID binding to the TATA box of the late promoter and requires the additional activities of TFIIA. The stabilized binding of TFIID by TFIIA may in turn allow ICP4 to more efficiently activate transcription from non-INR containing promoters.

5.2. INTRODUCTION

The expression of Herpes simplex virus type 1 genes relies on the functions of the host cellular transcription machinery (6, 36). Initiation of transcription is a major control point of viral as well as cellular gene expression and requires the proper assembly of the general transcription factors TFIIA, -B, -D, -E, -F, -H and RNA Pol II into preinitiation complexes (PIC) on core promoter elements (reviewed in 124). PIC formation begins with the binding of the general transcription factor TFIID, a multisubunit complex composed of the TATA binding protein (TBP) in association with 10 to 14 TBP-associated factors, known as TAFs (reviewed in 5). The TBP subunit of TFIID recognizes and binds to the TATA box present on core promoters. Although TBP is sufficient for basal transcription initiation, activated transcription requires the TBP-associated factors of TFIID (19). Many cellular as well as viral activators have been shown

to interact with the general transcription machinery through interactions with TAFs (21, 73, 77). TAFs also play an important role in recognition of non-TATA box core promoter elements, such as the INR, that may be present in place of or in addition to a TATA box on core promoters (105, 106). TAF250, TAF150 and TBP of TFIID are sufficient for INR element recognition and function. TFIID binding to the TATA box and/or to the INR is stabilized by TFIIA and TFIIB. Whereas TFIIB is essential for basal transcription directed by either TBP or TFIID, TFIIA is dispensable for TBP-directed basal transcription. Although basal transcription with TBP does not require the general transcription factor TFIIA, TFIIA can stabilize TBP's interaction with the TATA box (18, 35, 97, 115, 123). In the absence of TFIIA, however, activated transcription does not occur suggesting that although it may be dispensable for basal transcription with TBP, TFIIA plays an important role in transcription initiation and activation with TFIID (40, 111, 122, 147, 189, 211). The formation of a TFIID-TFIIA-TFIIB-DNA complex allows the subsequent recruitment of the rest of the general transcription factors, TFIIE, -F, -H, and RNA Pol II, that are recruited individually or as preformed complexes to the core promoter completing PIC formation and thus ready to initiate transcription.

Transcriptional activators physically promote the formation of transcription preinitation complexes by facilitating the recruitment of one or more GTFs to target promoters. Similar to the many transactivators that exert their strength through the cellular transcription machinery, the HSV major transcriptional regulatory protein, ICP4, has been shown to interact with components of the general transcription machinery to either activate or repress transcription (25, 79-81, 113, 181). One of the first gene products produced during infection, the 175 kDa protein ICP4 (153), localizes as a 350 kDa homodimer (135) in discrete foci in the nuclei of infected cells where both viral transcription and replication are thought to originate (60, 61). ICP4 functions as the major

transcriptional activator of early and late genes (45, 58, 72, 74, 142) making it essential for lytic infection (44, 51, 106, 202).

ICP4 as a transcriptional activator contains many conserved regions that include a DNA binding, a nuclear localization, and both N- and C-terminal transactivation regions (47, 151, 175). Although the DNA binding domain is essential to its function as an activator, the operationally defined DNA-binding domain alone is not sufficient for transcriptional induction, as demonstrated by the isolation of ICP4 deletion mutants that bind to ICP4 consensus sites but do not activate transcription. ICP4 does not require any single or collection of ICP4 specific binding sites and no ICP4 sequence specific binding sites responsible for activation have been identified on early and late promoters (29, 54, 56, 180). ICP4 can bind DNA that has a fairly degenerate consensus sequence and has been shown to activate promoters *in vitro* without ICP4 sequence specific DNA binding sites (79).

The N- and C- terminal transactivation domains are essential to ICP4's ability to activate transcription. ICP4 has been shown to interact with the general transcription factors TFIIB through its N-terminal transactivation domain (181) and with TFIID through its C- terminal transactivation domain (25). Like many cellular activators, ICP4 cannot activate transcription when TBP is substituted for TFIID (79). Activation of transcription by ICP4 requires the TAFs present in TFIID. ICP4's interaction with TAF250 of TFIID through its C-terminal transactivation domain serves to increase the rate of transcription preinitiation complex formation on viral early and late promoters (78). ICP4 expressing viruses lacking either the N- or C-terminal transactivation regions express reduced levels of viral early and late genes and are impaired for viral growth in culture (47, 173).

During lytic infection viral gene expression proceeds from the expression of immediate-early to early to late genes (92). The expression of viral genes, which occurs in these three highly regulated phases, are mediated in part by the structural differences within the promoter architectures of each of the three classes of genes (200, 216). Since viral genes are transcribed by the cellular transcription machinery, the one element that most all HSV-1 promoters contain in common is a TATA box. Aside from a TATA box, however, the promoters of each of the three classes of genes are distinct, with a trending decrease in promoter complexity from immediate-early to early to late genes (reviewed in 199, 203). Immediate-early promoters, in addition to containing numerous cellular cis-activating sequences upstream of the TATA box, are the only promoters that contain viral specific activating sequences. Early promoters lack any viral specific sequences but still retain binding sites for cellular specific factors, such as Sp1 and CTF. These cellular activators have been shown to work in concert with ICP4 and the cellular transcription machinery to activate transcription of early genes. Late promoters significantly differ from IE and E promoters in that late promoters lack any influential upstream cis-acting sequences that are binding sites for either cellular or viral specific factors (64, 83, 91, 94, 100). For true late promoters, the sequences downstream from the TATA box are important for late gene regulation (79, 82, 83, 94, 185). The INR element, identified on many late promoters, is one such region downstream from the TATA box. INR elements, which overlap the initiation start site, are common to many cellular core promoters and can promote transcription initiation in the absence of a TATA box or can synergize the effects of a TATA box (reviewed in 177). These elements are specifically recognized by components of the general transcription factor TFIID (105, 106). The INR in addition to a TATA box has been shown to be essential for ICP4 activation of late promoters and, unlike early promoters, ICP4 can activate transcription from

these promoters with a relatively simple set of general transcription factors (79). Mutations in the INR element diminish the ability of ICP4 to activate transcription from these promoters (110).

Because early and late promoters are structurally distinct yet still activated by ICP4, the cellular requirements for the activation of early and late genes by ICP4 may be different. Indeed in an *in vitro* reconstituted transcription system, ICP4 cannot activate transcription of a representative early promoter with a relatively simplified set of general transcription factors. Other cellular factors in addition to ICP4, such as Sp1, are required for activation of early genes. ICP4, on the other hand, can efficiently activate transcription of a representative late promoter with a simple set of general transcription factors dependent on the presence of a functional INR (79). In addition, we have recently reported that there is a differential requirement for the general transcription factor TFIIA for ICP4-activation of early versus late genes *in vitro* (212). TFIIA is required for efficient ICP4-activation of the early tk promoter. Surprisingly however, TFIIA is dispensable for ICP4-activation of the late gC promoter. Dispensability of TFIIA for ICP4 activation of the late promoter requires an intact INR. In the absence of a functional INR, the additional activities of TFIIA allow ICP4 to overcome the necessity for an INR element. In addition TFIIA expression decreases as a function of wild-type infection. Thus TFIIA, which is suggested to be required for all activated transcription, is not essential for ICP4-activation depending on promoter context. Moreover, the decreased expression of TFIIA and its dispensability for late gene activation by ICP4 suggests that functions of ICP4 can substitute for the activities specified by TFIIA in a promoter dependent manner. In an effort to delineate the basis for TFIIA's dispensability for ICP4-activation of late genes, the binding properties of ICP4,

TFIIA, and TBP or TFIID on the tk, gC and INR-mutated gC promoters were analyzed by DNase I footprinting analysis.

5.3. MATERIALS AND METHODS

5.3.1. Proteins

Purified recombinant TFIIA and TBP have been previously described (212). ICP4, n208 and X25 were purified respectively from wild-type (KOS), n208 and X25 producing virus-infected Vero cells as previously described (25). TFIID was immunoaffinity purified from α3 HeLa cells containing a hemagglutinin (HS) epitope-tagged TBP as previously described (25) and analyzed via silver stain analysis and Western blots using antibodies directed against TAF250, TAF150, TAF55, and TBP. The relative amounts of TBP in immunoaffinity-purified TFIID were determined by Western blot analysis using an antibody directed against TBP and comparing the resulting signals to those from known amounts of purified rTBP. For all analyses, 2 ng of rTBP, 12 ng of rTFIIA and 250 pg TBP-equivalent TFIID were used unless otherwise noted.

5.3.2. DNase I footprinting analysis

The SgrA1 – NheI fragments of the pgCLS1 and pgCLS8 plasmids (110) representing the wild-type gC and INR-mutated gC promoters, respectively, and the EcoR1 – Bgl II fragment of the pLSWT plasmid (95) representing the tk promoter, were end-labeled on the coding strand using

polynucleotide kinase and γ^{32} P ATP. The labeled fragment was then purified and quantified. One nanogram of an end-labeled probe (3 X 10^4 to 6 X 10^4 cpm/ng) and the indicated mixture of proteins were incubated for 30 mins at 30°C in a 30 ul reaction buffer consisting of 12.5 mM HEPES (pH 7.8), 60 mM KCl, 12.5% glycerol, 5 mM $MgCl_2$, 0.5 mg/ml of bovine serum albumin, 20 mM B-mercaptoethanol, and 0.006 ug/ul of poly (dG) · poly(gC). Following incubation, 4 ul of 10X DNaseI reaction buffer (50 mM $CaCl_2$, 100 mM $MgCl_2$) was added to each reaction and incubated for an additional 10 min at 30°C. 1 ul of DNase I (0.125 U) was added to each reaction at room temperature for 1 min and stopped by the addition of 40 ul of stop buffer (0.2 M NaCl, 0.02 M EDTA, 1% w/v sodium dodecyl sulfate, 20 mg/ml tRNA, 1 mg/ml proteinase K). Reactions were then incubated at 37°C for 10 mins following phenol and chloroform extraction, and ethanol precipitation. Pellets were resuspended in 95% formamide and run out on denaturing 6% gels for gC promoter analysis or 8% denaturing gels for analysis of the tk promoter. Gels were dried and exposed to Amersham Hyperfilm.

5.4. RESULTS

5.4.1. ICP4, as well as TFIIA, stabilizes the binding of TBP to the TATA box of the wild-type late (gC) and INR-mutated late (gC8) promoters

TFIIA has been shown to stabilize binding of TBP to the TATA box present on cellular promoters (18, 35, 97, 115, 123). To determine whether ICP4 could also stabilize binding of TBP to the TATA box, DNase I footprinting analysis was performed on the viral late gC

promoter. DNase I footprinting analysis is a highly sensitive method utilized to determine the exact binding location of a given protein or group of proteins on a DNA fragment containing sequence specific binding sites. This method has been previously utilized to show TFIIA induced the stabilized binding of TBP to the TATA box (97, 147, 211). The late gC promoter, which is highly activated by ICP4, is a simple promoter that contains only two influential specific DNA binding sites, the TATA box and an INR element (79, 110). The TATA box is recognized by TBP, whereas the INR element is recognized by the TBP-associated factors, TAF250 and TAF150, of TFIID (195). When individual proteins TBP or TFIIA were tested for the ability to bind to the promoter region of the gC promoter, neither TBP nor TFIIA alone bound to the TATA box or any regions surrounding the TATA box (Fig. 16A, lanes 2 and 3). On the other hand, ICP4 alone at concentrations of 10 but not 5 ng, bound to regions surrounding, but not directly at the TATA box as DNase I sensitive sites were present at the TATA box (Fig. 16A, lanes 4 and 5). TFIIA, as previously observed for cellular promoters, stabilized the binding of TBP to the TATA box of the gC promoter, evidenced by DNase I protection of the TATA region (Fig. 16A, lane 6). ICP4 also stabilized the binding of TBP to the TATA box in the absence of TFIIA suggesting that ICP4 alone is sufficient to stabilize TBP binding to the TATA box (Fig. 16A, lane 7 and 8). ICP4, however, had no effect on binding of TFIIA to the gC promoter, as reactions containing TFIIA and ICP4 look similar to ICP4 alone (Fig. 16A, compare lanes 9, 10 with lanes 4, 5). These results suggest that ICP4 can substitute for TFIIA in promoting the stabilized binding of TBP to the TATA box of the gC promoter.

Mutations in the INR significantly reduce the ability of ICP4 to activate transcription (79, 110). The INR-mutated late gC (gC8) promoter is essentially composed of only a TATA box since three nucleotides have been mutated within the INR element (110) rendering this promoter

less responsive to ICP4 activation in the absence of TFIIA (212). When this promoter was substituted for with wild-type gC promoter, TFIIA stabilized the binding of TBP to the TATA box of this promoter (Fig. 16B, lane 18). Similar to that seen for the wild-type promoter, ICP4, like TFIIA, was also able to stabilize binding of TBP to the TATA box (Fig. 16B lane 19 and 20) while having no effect on binding of TFIIA to the promoter (Fig. 16B, lanes 21, 21 compared to lanes 16 and 17). This suggests that ICP4 can substitute for TFIIA in stabilizing the binding of TBP to the TATA box of the late promoter regardless of the presence or absence of a functional INR element.

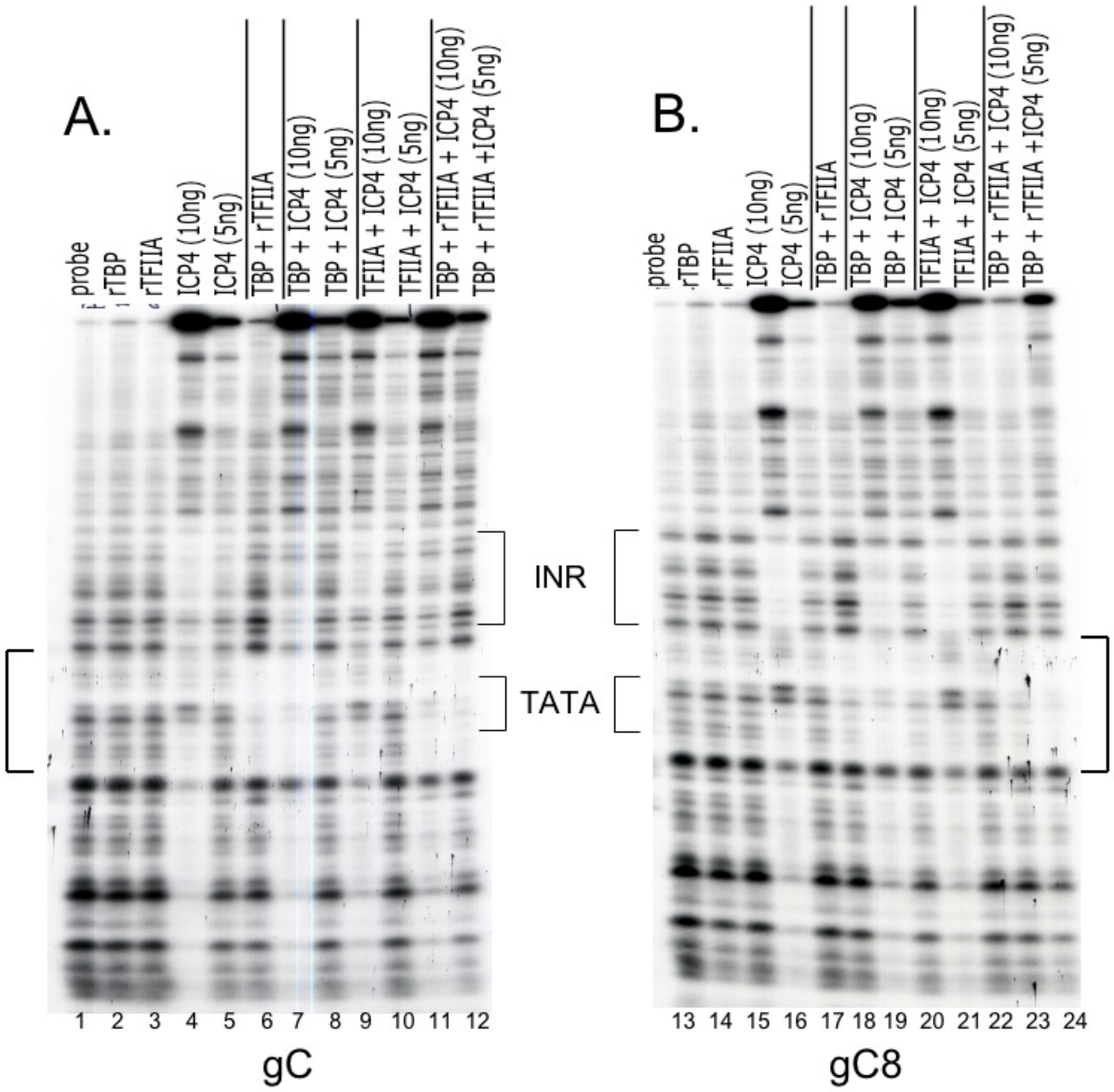


Figure 16. Stabilized TBP-TATA interactions on the wild-type and INR-mutated late gC promoters by either ICP4 or TFIIA.

DNase I footprinting analysis was conducted on (A) the wild-type or (B) the INR-mutated late promoter using TBP, TFIIA and ICP4. Neither TBP nor TFIIA bound to either promoter alone (lanes 2, 3 and lanes 14, 15, respectively). ICP4 alone bound regions outside of the TATA box of both promoters at 10 but not 5 ng (lanes 4, 5 and lanes 16, 17). TFIIA (lanes 6 and 18) as well as ICP4 (lanes 7, 8 and lanes 19, 20) stabilized the binding of TBP to the TATA box of either promoter, while ICP4 had no effect on the binding of TFIIA (lanes 9, 10 and lanes 21, 22). The presence of ICP4 in addition to TFIIA had no net effect on TBP-TATA interactions (lanes 11, 12 and lanes 23, 24).

To determine the lowest concentration of ICP4 able to stabilize the binding of TBP to the TATA box of either the wild-type or the INR-mutated late promoter, without binding to either

promoter itself, varying concentrations of ICP4 were used in the presence or absence of TBP (Fig. 17B). ICP4 at 2.5 ng retained the capacity to efficiently stabilize the binding of TBP to the TATA box of both promoters. The ability of ICP4 at 2.5 ng to stabilize TBP binding to the TATA box was comparable to TFIIA's ability to stabilize the binding of TBP. ICP4 even at 1 ng was able to stabilize the binding of TBP to the TATA box. Thus, ICP4 at concentrations as low as 1 ng could stabilize the binding of TBP to the TATA box without binding to regions surrounding the TATA box of either the wild-type or INR-mutated late promoter. These results also further support that ICP4 does not require the presence of a functional INR to stabilize the binding of TBP to the TATA box of the late promoter.

5.4.2. Regions of ICP4 required to stabilize the binding of TBP to the TATA box of the wild-type and INR-mutated late promoters

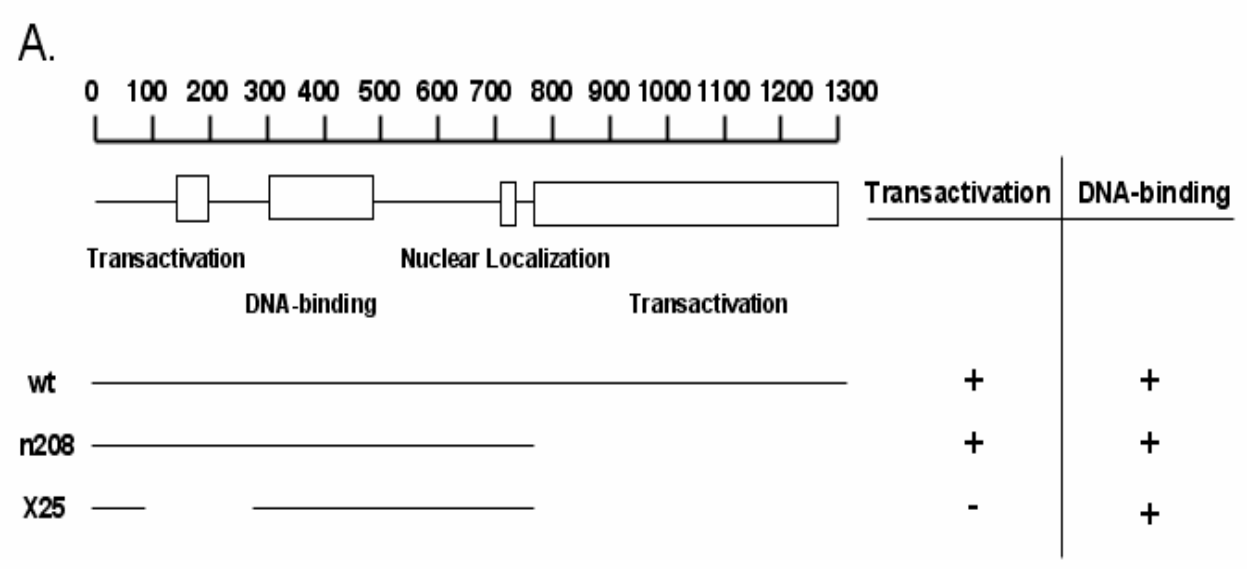
ICP4 is a large structurally complex molecule that contains discrete domains conserved across many ICP4 orthologs. Two ICP4 domains mapped out in Fig. 17A that have been shown to be essential for the ability of ICP4 to activate transcription are the N- and C-terminal transactivation domains (175, 176). n208 is a 774 amino-acid derivative of 1,298 amino-acid ICP4 that lacks the C-terminal transactivation domain (47). This polypeptide still retains a number of physical and functional properties characteristic of the intact molecule such as the N-terminal transactivation domain, the DNA binding and dimerization domain, and the nuclear localization signal (175). Despite the presence of these functional domains, n208 producing viruses are defective for viral growth due to a block at the stage subsequent to viral early gene expression (47). n208 lacks viral DNA synthesis and is impaired for the expression of true late genes,

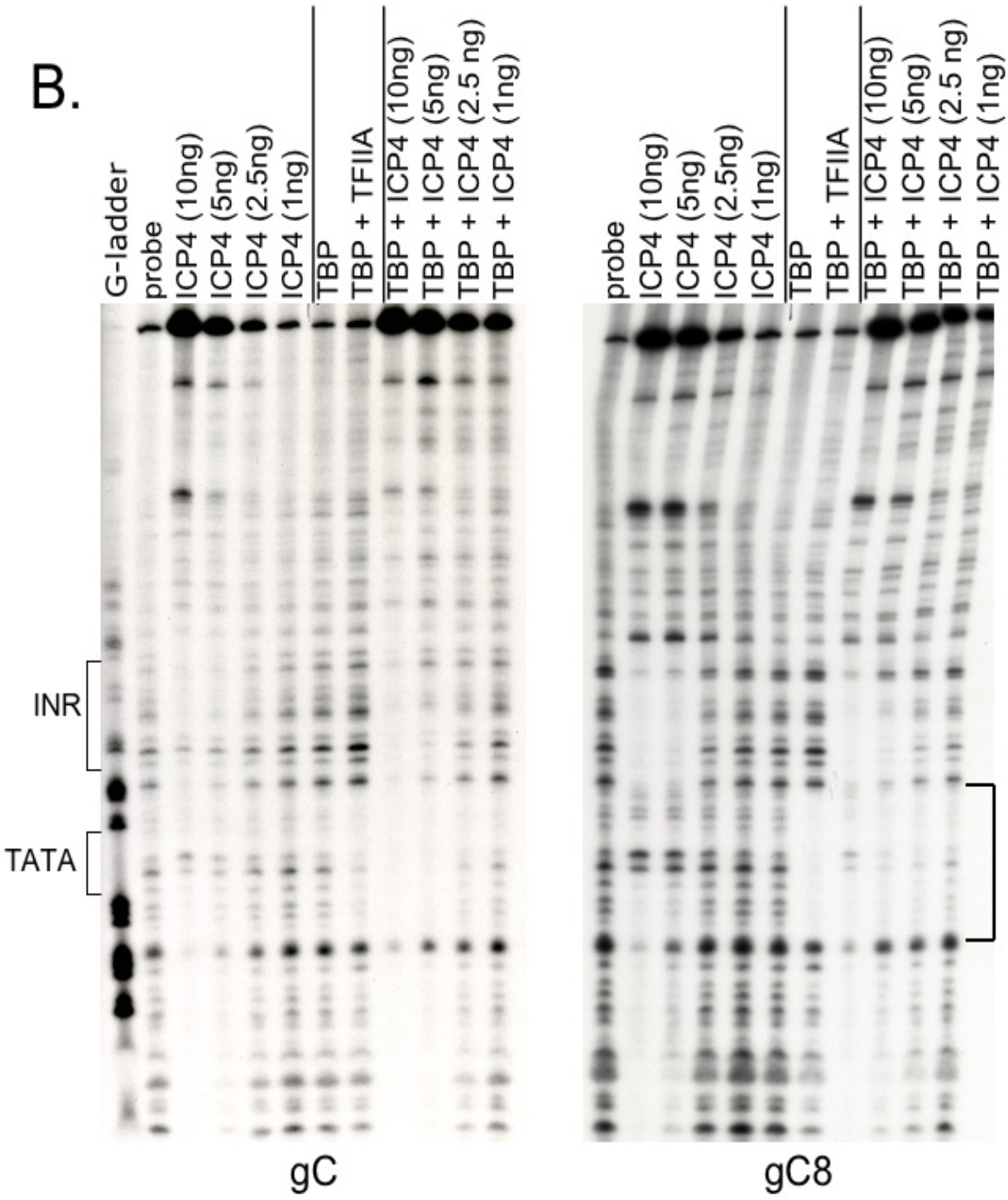
which may be a sole consequence of the deficiency of DNA synthesis. Despite the inability of this virus to replicate, n208 molecules can localize to the nucleus of infected cells, bind ICP4 specific DNA binding sites, and retains the capacity to activate a number of viral early and late genes.

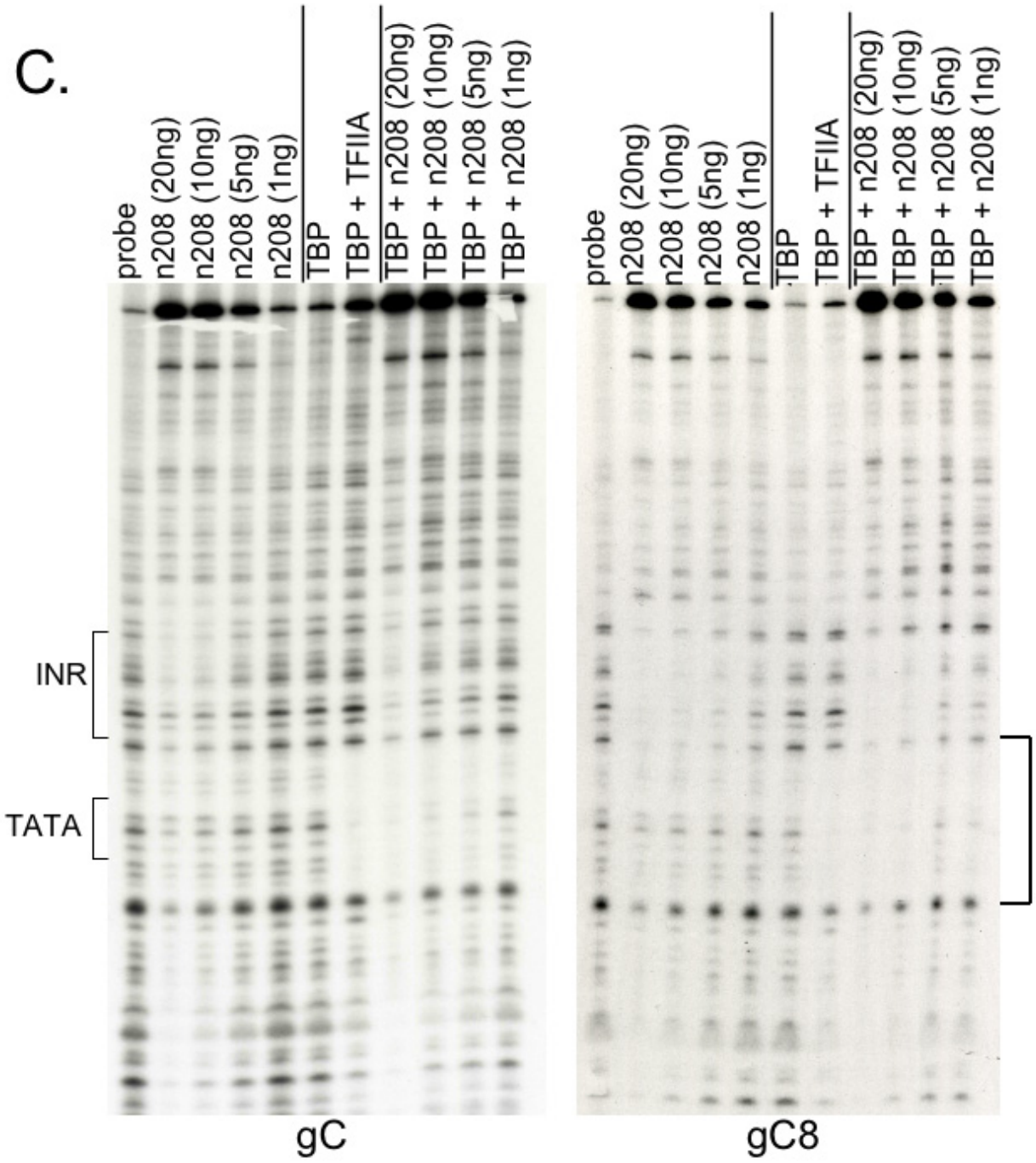
Because n208 retains the ability to bind DNA, autoregulate, and activate a number of viral genes (46, 47), the regions still present in this molecule may contribute to the ability of ICP4 to stabilize binding of TBP to the TATA box. When substituted for full-length ICP4, n208 was able to efficiently stabilize the binding of TBP to the TATA box, at concentrations as low as 1 ng (Fig. 17C). Stabilized binding of TBP to the TATA box by n208 at 1 ng was comparable to TFIIA stabilized binding of TBP. These results suggest that the regions outside of the C-terminal transactivation domain of ICP4 are required to stabilize binding of TBP to the TATA box. In addition no differences were evident in the ability of n208 to stabilize TBP binding to either the wild-type or INR-mutated late promoter, suggesting again that the INR is not required for ICP4 to direct or facilitate binding of TBP to the TATA box.

X25, diagramed in Fig. 17A, is similar to n208 in that it lacks the C-terminal transactivation domain (residues 775 through 1298) but, in addition, also lacks the N-terminal transactivation domain (residues 30 through 274). Removal of both amino acids 30 to 275 and 775 through 1298 results in a mutant protein that still retains sequences sufficient for site-specific DNA binding and multimerization, yet lacks the ability to activate transcription in transient assays (172). To determine if regions specified in the N-terminal transactivation domain were important for stabilized binding of TBP in the background of a C-terminal transactivation domain mutation, X25 was substituted for ICP4. X25 at varying concentrations was unable to stabilize binding of TBP to the TATA box of either the wild-type or INR-mutated

gC promoter (Fig. 17D). This suggests that regions specified within the N-terminal transactivation domain of ICP4 are necessary for both binding to the gC promoter alone and stabilizing the binding of TBP to the TATA box. These data also suggest that the DNA binding activity of ICP4 is not sufficient to facilitate TBP binding.




B.
G-ladder
probe
ICP4 (10ng)
ICP4 (5ng)
ICP4 (2.5ng)
ICP4 (1ng)
TBP
TBP + TFIIA
TBP + ICP4 (10ng)
TBP + ICP4 (5ng)
TBP + ICP4 (2.5 ng)
TBP + ICP4 (1ng)
INR
TATA
gC
probe
ICP4 (10ng)
ICP4 (5ng)
ICP4 (2.5ng)
ICP4 (1ng)
TBP
TBP + TFIIA
TBP + ICP4 (10ng)
TBP + ICP4 (5ng)
TBP + ICP4 (2.5 ng)
TBP + ICP4 (1ng)
gC8


C.
probe
n208 (20ng)
n208 (10ng)
n208 (5ng)
n208 (1ng)
TBP
TBP + TFIIA
TBP + n208 (20ng)
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INR
TATA
gC
gC8

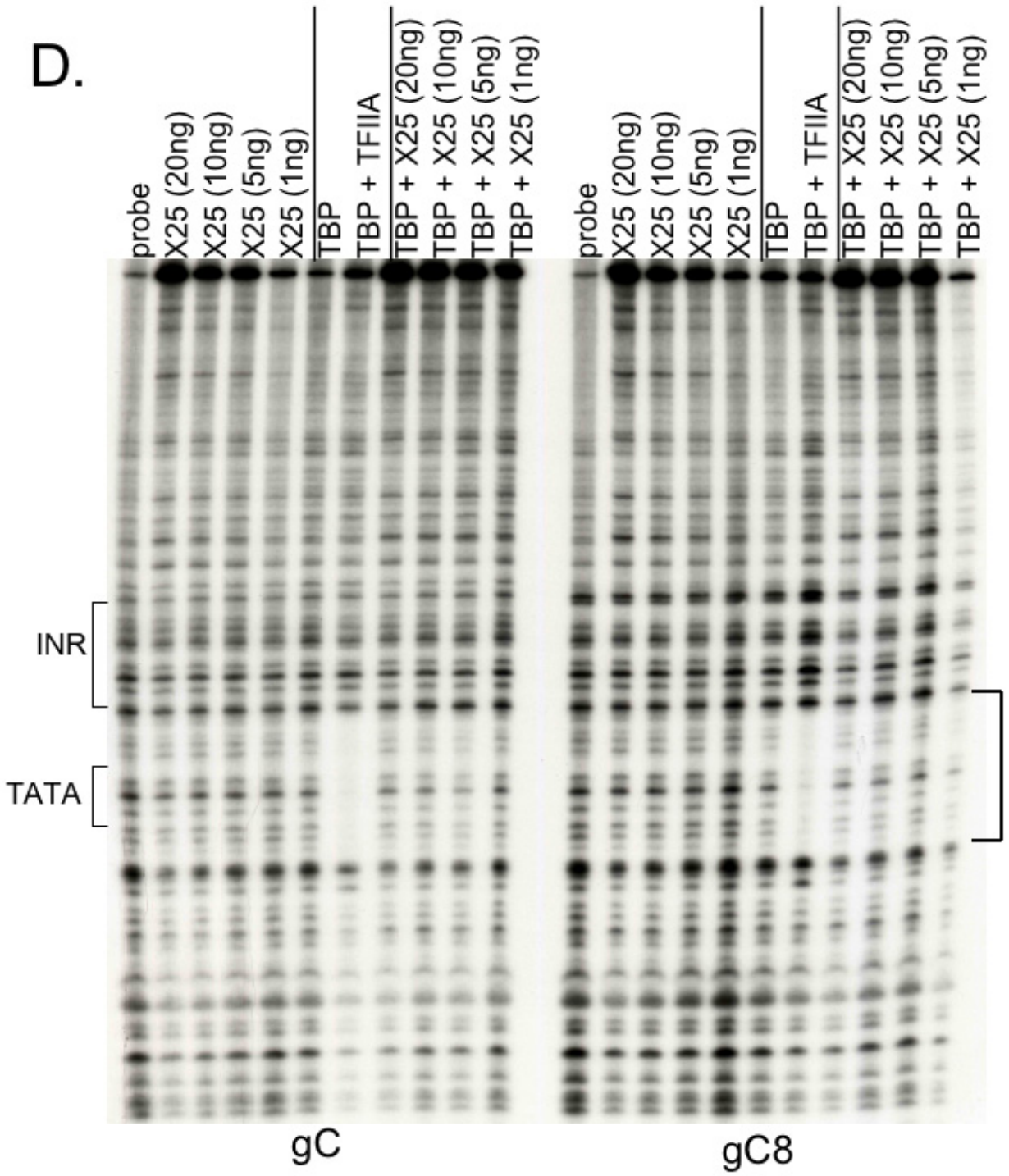


Figure 17. Regions of ICP4 required for stabilized binding of TBP to the TATA box of the wild-type and INR-mutated late gC promoters.

A. Domain map of ICP4 and properties of mutants relative to wild-type ICP4. The important regions of ICP4 are indicated by boxes with their amino acid location indicated on the scale directly above the boxes. The primary structure of each of the mutant molecules relative to the wt protein is indicated. Transactivation *in vivo* was determined by transient transfection assays and/or the transcription rates of selected viral genes in the context of

viral infection. DNA binding was determined by electrophoretic mobility shift assay. This summary was derived from several previous studies. **B.** wild-type ICP4 alone stabilized TBP-TATA interaction on either the wild-type or INR-mutated late promoter. ICP4 alone at 10 but not 2.5 ng bound to regions outside of the TATA box on both the wild-type and INR mutated late promoters. ICP4 at 2.5 and 1 ng, which did not bind either promoter alone, was still able to stabilize TBP-TATA interactions, comparable to TFIIA. **C.** An ICP4 derivate lacking the C-terminal transactivation domain, n208 maintained the ability to bind to regions outside of the TATA box of both promoters. It was also able to stabilize TBP-TATA interactions similar to full-length ICP4 suggesting that the C-terminal transactivtion domain of ICP4 is not required to stabilize binding of TBP to the TATA box. **D.** An ICP4 derivative lacking both the N and C-terminal transactivation domain, X25, could not bind to either promoter alone at varying concentrations nor was it able to stabilize the binding of TBP to the TATA box of either promoter suggesting that the N-terminal transactivation domain of ICP4 is necessary to stabilize TBP-TATA interactions on either the wild-type or INR-mutated late promoter.

5.4.3. ICP4 stabilizes the binding of TFIID to the TATA box of the wild-type but not the INR-mutated late promoter

ICP4 alone is sufficient to stabilize binding of TBP to the TATA box to both the wild-type and INR-mutated late promoter (Fig 16). Because ICP4 and n208, but not X25, have been shown to interact with TBP in solution (25) it is not surprising that both ICP4 and n208, but not X25, are able to stabilize binding of TBP to the TATA box of the wild-type and INR-mutated late promoters. However, in the context of cells, TBP is usually found associated in multi-subunit complexes such as TFIID. To determine if ICP4 was also able to stabilize the binding of TFIID to the TATA box of the wild-type late promoter, TFIID was substituted for TBP. The individual proteins TBP, TFIIA, TFIID, and X25 at 10 ng were unable to bind to the gC promoter alone (Fig. 18, lanes 3 - 6). TFIIA stabilized the binding of either TFIID or TBP to the gC TATA box (Fig. 18, lanes 9 and 10). X25, unable to stabilize the binding of TBP to the TATA box (Fig. 17D), also did not promote TFIID-TATA interactions (Fig. 18, lanes 11 and 14). Full-length ICP4, observed to stabilize the binding of TBP, also stabilized the binding of TFIID to the TATA box of the gC promoter (Fig. 18, lanes 12, 13 and 15, 16, respectively). This occurred regardless of whether ICP4 bound directly to regions on this promoter as ICP4, unbound at 5 ng (Fig. 18,

lane 8), was able to stabilize binding of either TBP or TFIID (Fig. 18, lanes 13 and 16) as effectively as ICP4 bound at 10 ng (Fig. 18, lane 8, 12, and 15). ICP4 stabilized binding of TFIID appeared similar to the ICP4 stabilized binding of TBP (Fig. 18, compare lanes 15 and 16 with lanes 12 and 13) and this was comparable to TFIIA stabilized binding of TFIID and TBP (Fig. 18, compare lanes 12, 13, 15 and 16 with lanes 9 and 10). ICP4, however, had no effect on TFIIA, as TFIIA in the presence of ICP4 looked similar to ICP4 binding alone (Fig. 18, compare lanes 17 – 19 with lanes 6 – 8). These results suggest that ICP4 alone can substitute for TFIIA in the ability to stabilize binding of TFIID to the wild-type gC promoter.

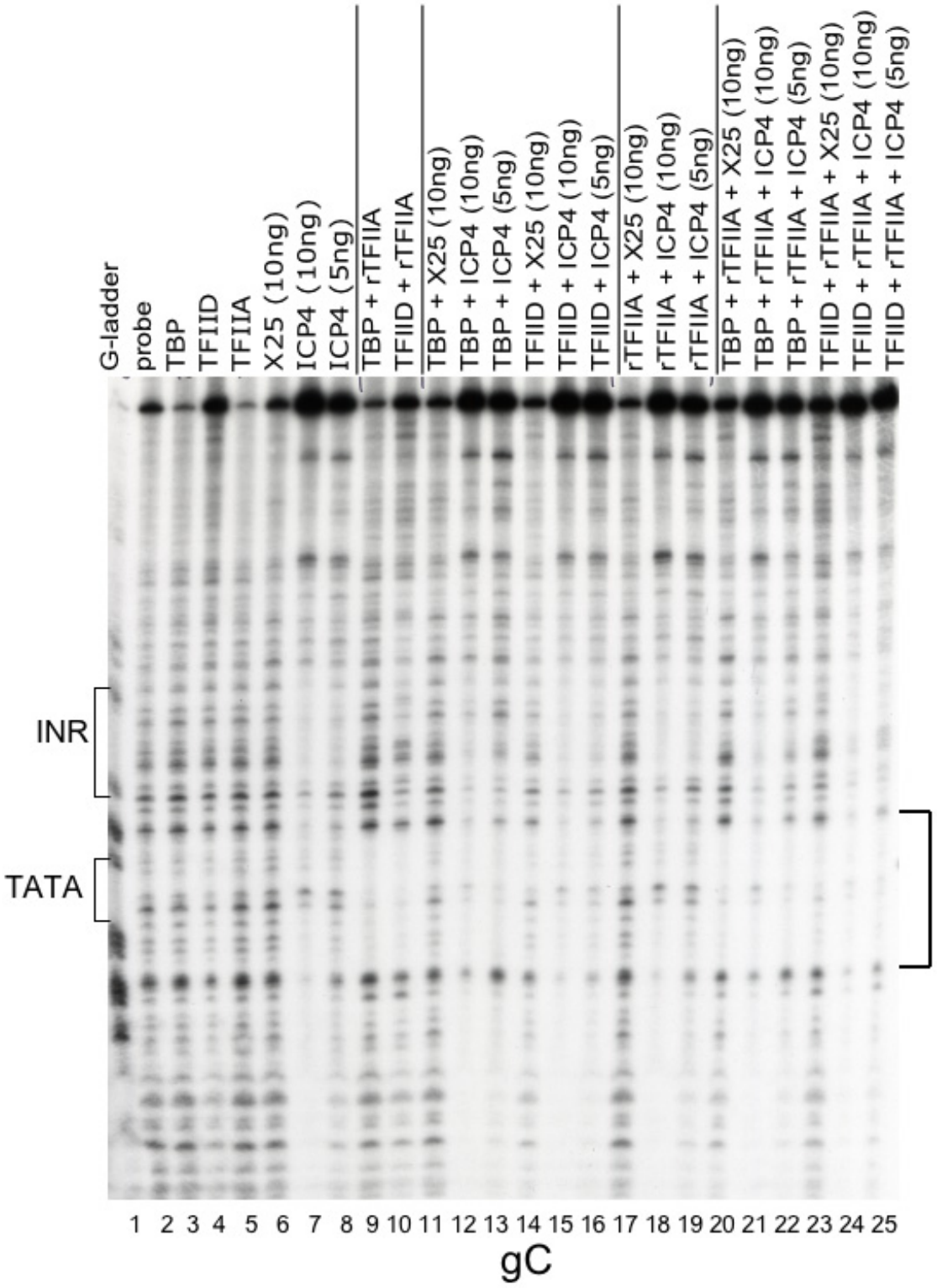


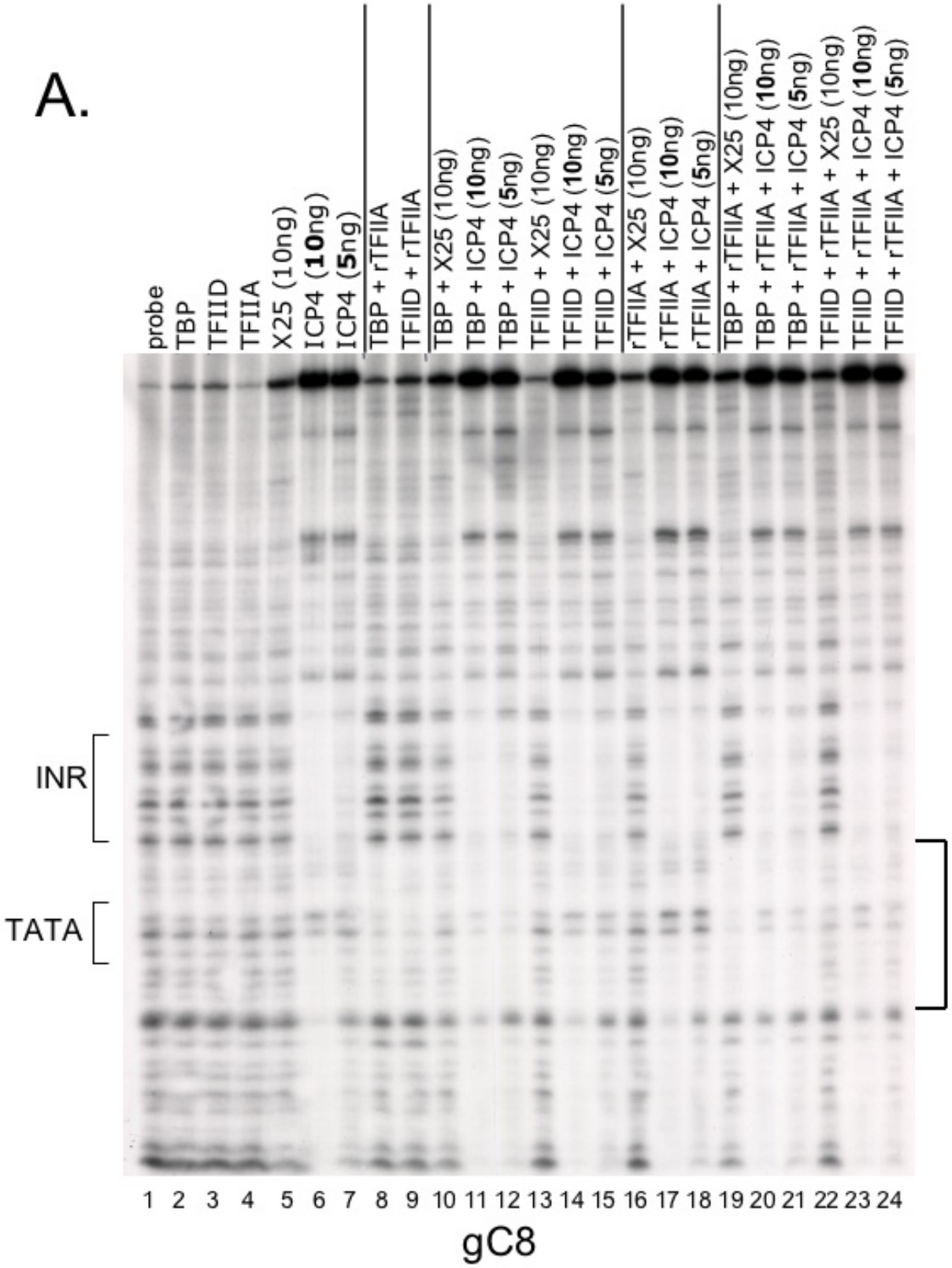
Figure 18. ICP4 alone can stabilize TFIID-TATA interactions on the wild-type INR-containing late promoter.

Affinity purified TFIID was unable to stably bind to the TATA box alone (lane 4). TFIIA was required to stabilize the binding of either TBP or TFIID to the TATA box of the wild-type promoter (lanes 9 and 10). In addition, ICP4

alone, but not X25, could also stabilize the binding of either TBP (lanes 11-13) or TFIID (lanes 14-16) to the promoter at either 10 or 5 ng. ICP4 had no effect on binding of TFIIA (lanes 17-20), as binding appeared similar to ICP4 alone (lanes 6-8). TFIIA in addition to ICP4 had no net effect on either TBP-TATA (lanes 22 and 23) or TFIID-TATA (lanes 25 and 26) interactions. However, TFIIA did promote stabilized binding of either TBP (lane 21) or TFIID (lane 24) in the presence of the ICP4 derivative, X25.

Figure 16B and 17B suggest that ICP4 stabilizes the binding of TBP to the TATA box in the absence of a functional INR. However, ICP4's ability to stabilize the binding of TFIID to the TATA box may differ and may require the presence of a functional INR element. To determine whether an intact INR element was required for the ability of ICP4 to stabilize the binding of TFIID to the TATA, the INR-mutated late gC8 promoter was analyzed (Fig. 19). Similar to the wild-type promoter, neither TBP, TFIIA, affinity-purified TFIID, or the ICP4-derivate X25, alone were able to bind to the INR-mutated late promoter (Fig. 19A, lane 5; B, lane 6). ICP4 alone at both concentrations of 10 and 5 ng bound to the gC8 promoter (Fig. 19A, lanes 6 and 7). Due to this, a lower concentration of ICP4 at 2.5 ng that did not bind the gC8 promoter was also analyzed (Fig. 19B, lane 8). As observed with the wild-type late promoter, TFIIA stabilized binding of either TBP or TFIID to the TATA box of the INR-mutated late promoter (Fig. 19A, lanes 8 and 9, 7B, lanes 9 and 10). X25 again had no effect on the ability of either TBP or TFIID to bind the TATA box (Fig. 19A, lanes 10 and 13; 7B, lanes 11 and 14). Although ICP4 could stabilize TBP binding to the TATA box of this INR-mutated promoter at concentrations ranging from 10 to 2.5 ng (Fig. 19A, lanes 11 and 12; B. lanes 12 and 13), ICP4 at both high and low concentrations, was unable to efficiently stabilize the binding of TFIID to the TATA box of this late promoter (Fig. 19A, lanes 14 and 15; B. lanes 15 and 16). TFIID in the presence of ICP4 appeared similar to ICP4 alone indicating that ICP4 could not efficiently stabilize the binding of TFIID to the TATA box of an INR deficient late promoter (Fig. 19B, compare lanes 15 and 16 with lanes 7 and 8). The additional activities of TFIIA were required to stabilize TFIID-TATA

interactions (Fig. 19B, lanes 24 and 25). These results suggest that although ICP4 can stabilize the binding of TBP to a promoter consisting of only a TATA box, ICP4 alone is not sufficient to stabilize the binding of TFIID to a promoter containing only a TATA box. Stabilized binding of TFIID to a promoter containing only a TATA box required the activities of TFIIA. Thus in the absence of a functional INR element on a late promoter, ICP4 cannot substitute for TFIIA to stabilize TFIID binding to the TATA box.


A.
probe
TBP
TFIID
TFIIA
X25 (10ng)
ICP4 (10ng)
ICP4 (5ng)
TBP + rTFIIA
TFIID + rTFIIA
TBP + X25 (10ng)
TBP + ICP4 (10ng)
TBP + ICP4 (5ng)
TFIID + X25 (10ng)
TFIID + ICP4 (10ng)
TFIID + ICP4 (5ng)
rTFIIA + X25 (10ng)
rTFIIA + ICP4 (10ng)
rTFIIA + ICP4 (5ng)
TBP + rTFIIA + X25 (10ng)
TBP + rTFIIA + ICP4 (10ng)
TBP + rTFIIA + ICP4 (5ng)
TFIID + rTFIIA + X25 (10ng)
TFIID + rTFIIA + ICP4 (10ng)
TFIID + rTFIIA + ICP4 (5ng)
INR
TATA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
gC8

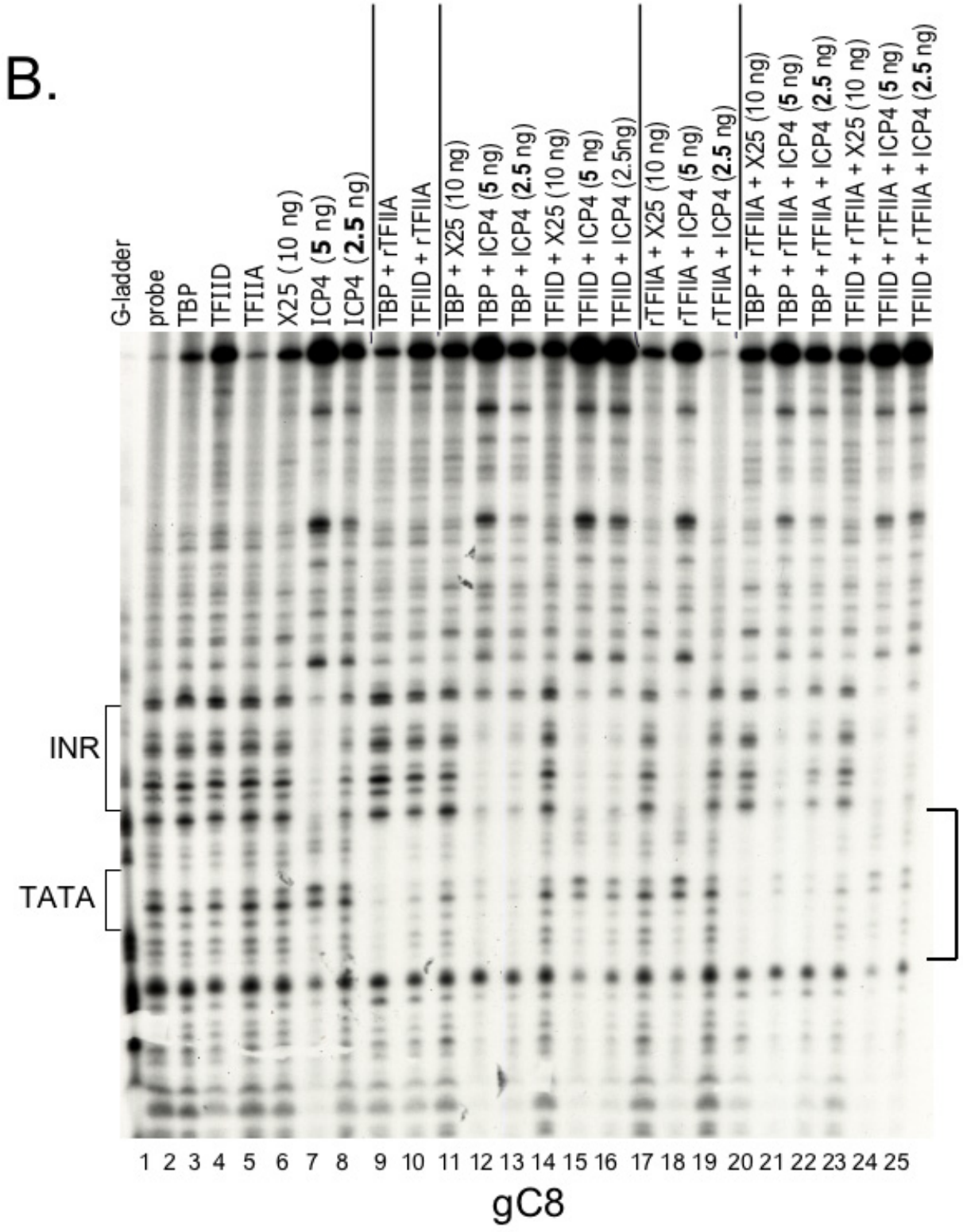


Figure 19. ICP4 does not efficiently stabilize binding of TFIID to the TATA box of the INR-mutated late promoter in the absence of TFIIA.

Footprinting analysis of the INR-mutated late promoter was conducted with 10 and 5 ng of ICP4 (**A**.) and with 5 and 2.5 ng of ICP4 (**B**.). Due to the ability of ICP4 to bind to INR-mutated late promoter regions alone at both 10 and 5 ng (A, lanes 6 and 7) a lower concentration of ICP4 at 2.5 ng that did not bind alone (B, lane 8) was also analyzed. Individual proteins, rTBP, rTFIIA or affinity purified TFIID were unable to bind to the INR-mutated late promoter alone (A, lanes 2 – 4 or B, lanes 3 – 5). TFIIA stabilized the binding of either TBP or TFIID to the TATA box (A, lanes 8 and 9 and B, lanes 9 and 10). Although, ICP4 alone stabilized the binding of TBP to the TATA box (A, lanes 11 and 12, B, lanes 12 and 13), ICP4 at concentrations of 10, 5 and 2.5 ng did not efficiently stabilize TFIID-TATA box interactions on the INR-mutated late promoter (A, lanes 14 and 15 and B, lanes 15 and 16). TFIIA was required to effectively stabilize the binding of TFIID to the TATA box of this promoter in the presence of ICP4 (lanes 24 and 25). In addition the ICP4 mutant, X25, had no effect on binding of either TBP or TFIID (A. lanes 11 and 14).

5.4.4. ICP4 facilitates TFIIA in stabilizing the binding of TBP to the early promoter TATA box

Unlike the gC promoter which contains a fairly strong TATA box with a sequence TATTAA, the tk promoter has a lower affinity TATA consensus sequence (34). Whereas 3 ng of TBP alone could bind the gC TATA box (data not shown), the same concentration of TBP alone could not bind to the TATA box of the tk promoter. Increasing concentrations of TBP showed that 40 ng of TBP alone was required to bind to the tk TATA box (Fig. 20). TFIIA reduced the required concentration to 20 ng. Addition of ICP4 to TFIIA further reduced the amount of TBP required to bind the tk promoter to 10 ng. Thus higher concentrations of TBP are required to bind to the TATA box of the tk promoter and TFIIA significantly reduced the amount required. ICP4 further reduced the amount of TBP required, probably by facilitating the ability of TFIIA to stabilize the binding of TBP to the TATA box.

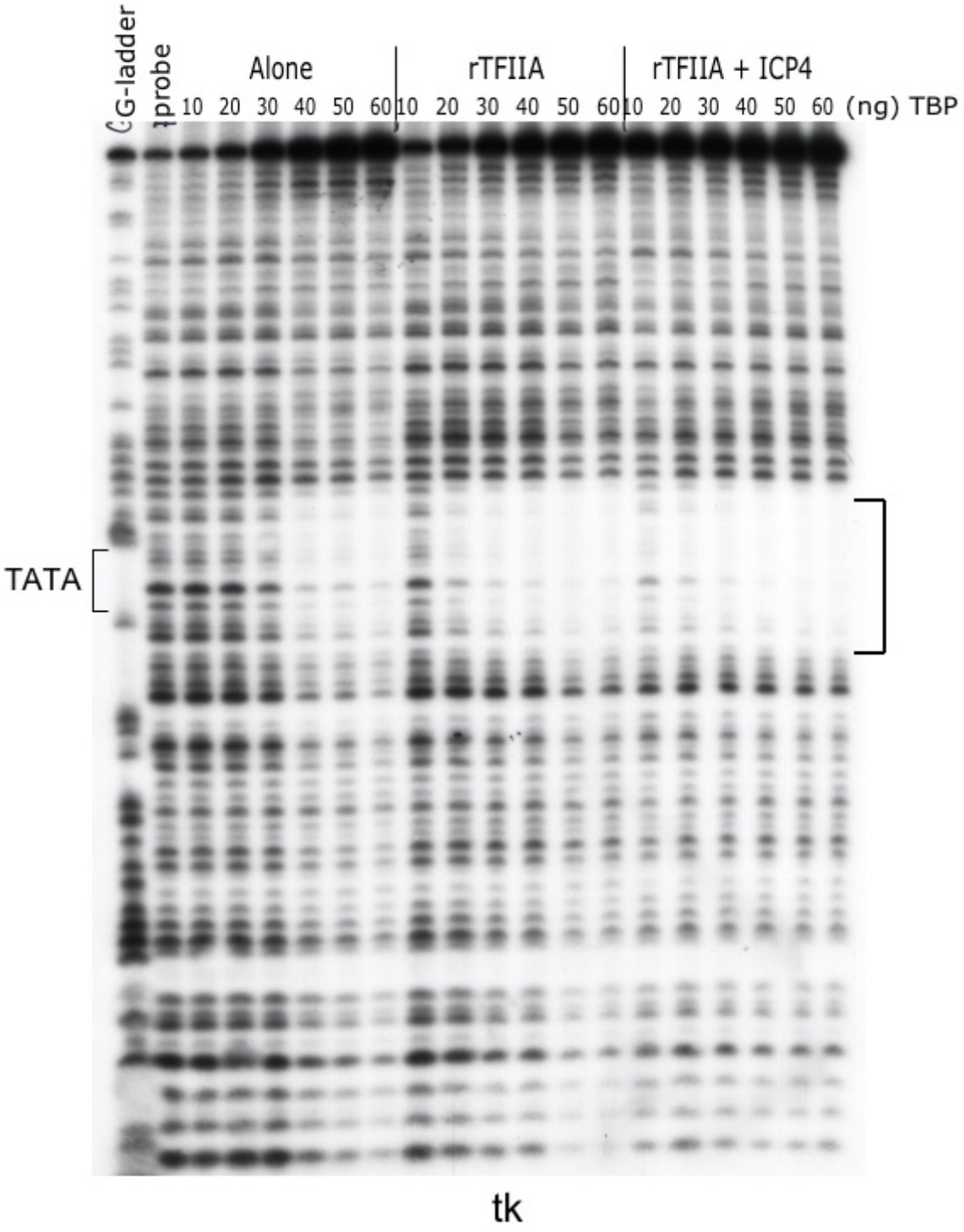


Figure 20. ICP4 facilitates the ability of TFIIA to stabilize the binding of TBP to the TATA box of the tk promoter.

Whereas 3 ng of TBP alone bound to the gC promoter (data not shown), 40 ng of TBP alone was required to bind to the TATA box of the early tk promoter. TFIIA reduced the amount of TBP required for binding to the TATA box to 20 ng and addition of ICP4 further reduced the amount of TBP to 10 ng.

5.5. DISCUSSION

In this study the binding properties of TFIIA, ICP4 and TBP/TFIID were examined on representative early, late, and INR-mutated late promoters. Our results indicate that ICP4 could substitute for TFIIA in stabilizing the binding of TBP to both the wild-type and INR-mutated late promoters. For the early promoter, ICP4 facilitated TFIIA's ability to stabilize the binding of TBP to the TATA box. When the TBP-containing complex TFIID was substituted for TBP, ICP4 was only able to stabilize TFIID binding to the TATA box of the wild-type late promoter, not to the INR-mutated late promoter. Stabilized binding of TFIID to this promoter required TFIIA. These results suggest that the efficient ICP4-activation of the wild-type gC promoter in the absence of TFIIA may be explained by the ability of ICP4 to substitute for TFIIA in stabilizing the binding of TFIID to the TATA box in the presence of a functional INR element. In the absence of a functional INR, ICP4's ability to activate transcription is severely compromised due to the inability of ICP4 to efficiently stabilize TFIID binding. TFIIA is required to stabilize TFIID-TATA box interactions on this INR-mutated promoter alleviating ICP4's requirement for an intact INR, allowing ICP4 to more effectively activate transcription from this promoter. Thus, ICP4 can substitute for TFIIA in stabilizing the binding of TFIID to a late promoter containing a functional INR element but cannot substitute for TFIIA in stabilizing the binding of TFIID in the absence of a functional INR element.

5.5.1. The regions of ICP4 required for the stabilized binding of TBP to the TATA box

The observation that ICP4 and n208 directly stabilize the binding of TBP to the TATA box of the gC promoter is not unexpected since both forms of ICP4 have been shown to interact with

TBP in solution (25). The ability of n208, which lacks amino acids beyond 778, to both interact with TBP in solution and to stabilize binding of TBP to the TATA box of the late promoter (Fig. 17C), suggests that the C-terminal transactivation domain is dispensable for TBP interaction and TATA box stabilization. The inability of X25, which lacks amino acids 30 – 274 in addition to amino acids beyond 778, to stabilize TBP-TATA or TFIID-TATA interaction strongly suggests that regions specified within amino acids 30 – 274, which include the N-terminal transactivation domain, are necessary for ICP4's ability to stabilize TBP-TATA interactions and may be necessary for direct interaction with TBP. Moreover, this suggest that the DNA binding domain alone, which is still intact in X25, is not sufficient to stabilize TBP binding to the TATA box.

Although both ICP4 and n208 are able to interact with TBP in solution (25) and can efficiently stabilize binding of TBP to the TATA box (Fig. 17B and C), neither one of these molecules are able to activate transcription with TBP (79). Activated transcription by ICP4 requires the TBP-associated factors of TFIID. ICP4's C-terminal domain has been shown to interact with TAF250 of TFIID to promote transcription preinitiation complex formation (25). Mutants that lack the C-terminal transactivation domain, such as n208 and X25, do not interact with TFIID in solution (25). The fact that n208 interacts with TBP in solution but not with TFIID implies that TBP is inaccessible in the context of TFIID in solution. Interestingly however, n208, but not X25, is still able to recruit and stabilize the binding of TFIID to an immobilized gC promoter template suggesting that in the presence of a TATA box, n208 can still stabilize TFIID binding (78). Results presented here support the idea that the N-terminal transactivation domain of ICP4 is required for this effect. n208 bound to promoter DNA, may encourage a rate limiting conformational change during weak TFIID-TATA interactions,

exposing TBP and allowing for interactions with the N-terminal domain of n208 which in turn may serve to stabilize TFIID binding to the TATA box.

Activated transcription not only requires the TAFs of TFIID, it also requires the general transcription factor TFIIA (40, 111, 122, 147, 189). Although TFIIA has a weak stimulatory effect on basal transcription lacking exogenous activators and is dispensable for TBP-directed basal transcription in purified systems *in vitro*, TFIIA is essential for activator-mediated transcription. In the absence of TFIIA, many activators lose the ability to activate transcription (40, 118, 148, 211). Activators interact directly with TFIIA to mediate stabilized interaction with TBP and the preinitiation complex. TFIIA, as well as TAFs, directly interact with and stabilize the binding of TBP within TFIID to the TATA box of core promoters (18, 35, 97, 123). Dynamic interplay exists between TFIIA and TAFs that regulate the binding of TFIID to promoter DNA. TFIIA mediates an activator-induced conformational change in the transcription factor TFIID that significantly alters the interaction of TAFs with promoter DNA (55, 147). The amino-terminal domain of TAF250 contains a negative regulatory element that inhibits TBP-TATA complex formation, demonstrating that at least one TAF can preclude the interaction of TBP with the TATA box. TFIIA, as a positive acting general factor, has been shown to competitively derepresses the inhibitory effect of TAF250 by destabilizing the interactions between TAF250 and TBP (55, 147). Thus it is of interest that ICP4 interacts with TAF250 of TFIID and that it can substitute for TFIIA in stabilizing the binding of TFIID to the TATA box of an INR containing promoter. ICP4 may function in a manner analogous to TFIIA in that it may alleviate the inhibitory effects of TAF250. The regions of TAF250 that interact with ICP4 have yet to be determined. However, it is plausible that ICP4, through its C-terminal transactivation domain, can competitively interact with TAF250's N-terminal negative

regulatory domain to destabilize its interaction with TBP. This may in turn allow the N-terminal region of ICP4 to interact with TBP to stabilize its interaction with the TATA box in the context of TFIID.

5.5.2. Late promoter requirements for TFIID stabilized binding to the TATA box by ICP4

Previous analysis of the promoter requirements for ICP4-activation of viral late genes has determined that the INR element, present on most true late promoters, is the only other element required in addition to the TATA box for efficient ICP4-activation, suggesting that late promoters are quite simple and unusual in nature since these are the only two promoter elements required for activation. Mutations within the INR element of a representative late promoter severely compromise the ability of ICP4 to activate transcription from this gene both *in vitro* and *in vivo* (79, 110). Like cellular promoters, immediate-early and early promoters, in addition to core promoter elements, contain numerous cis-acting sequences for transactivators that work synergistically to recruit and stabilize the transcription machinery through interactions with different components of the general transcription factors. However, ICP4 alone can activate transcription with a relatively simple set of general transcription factors without the requirement of other cellular or viral specific activators in the presence of only a TATA box and a functional INR element (79). In addition, the general transcription factor TFIIA is not required for ICP4 activation of a late promoter containing an INR and a TATA box (212). This is quite intriguing since TFIIA has previously been shown to play an unusually important role in INR-mediated transcription (87, 126, 189). TFIIA has been shown to selectively enhance TFIID transcription

from a promoter that contains both a TATA box and an INR (55). TFIID alone has been shown to have similar affinities for TATA and TATA-INR promoters. TFIIA strongly enhances TFIID binding to a TATA-INR promoter while having little effect on binding to a TATA box only promoter. This suggests that TFIIA-induced conformational changes are essential for the sequence-specific TFIID interaction with the INR. ICP4 may be functioning in a similar manner to TFIIA. Through protein-protein interactions and potential conformational changes that occur within both ICP4 and TFIID, ICP4 may relieve the inhibitory effect of TAF250 and induce interactions of TAFs with the INR in a manner similar to TFIIA.

ICP4's interaction with TFIID has been shown to be critical for ICP4 activation late in infection. The ability of ICP4 to interact with TFIIA has not been tested. Due to the declined expression of all three subunits of TFIIA during infection and its dispensability for late gene activation, it is likely that if ICP4 and TFIIA do interact, interactions at late times of gene expression are not essential. Since the promoter architecture for early and late genes differs, the interaction of ICP4 with TFIIA may be promoter dependent. ICP4 may interact with TFIIA early during infection, on early gene promoters that require TFIIA for efficient expression. Late in infection when the expression of TFIIA is declining, ICP4 may perform some of the functions intrinsic to TFIIA and TFIIA is not required for efficient late gene expression.

In the context of HSV infection, TFIIA is present early during infection and is required for activation of early genes. ICP4 in cooperation other cellular activators that require TFIIA, work together to recruit and promote stabilized binding of TFIID to the TATA box of early promoters. As infection proceeds, TFIIA expression decreases, coincident with its dispensability for INR-containing late gene expression as ICP4 can substitute for TFIIA in stabilizing the binding of TFIID to the TATA box, as well as induce interactions of TAFs with the INR. This

study clarifies one of the many viral mechanisms that exist for the attenuation of early gene expression while allowing succession of late gene expression.

6. SUMMARY OF THESIS

Although HSV genes, like cellular genes, rely on the functions of the host cellular transcription machinery for expression (6, 36), we hypothesized that infection also impacted the expression of components that make up the cellular transcription machinery. cDNA microarray chips containing all the components of the general transcription factors were constructed in-house and used to examine changes in the expression patterns of these components at early and late times of HSV infection. While moderate changes in the mRNA abundance of some of the components of the general transcription factors were observed at early times post wild-type infection (4 hpi) these changes were more profound at late times of infection (8 hpi). Only a small number of these components were observed to be upregulated during infection, such as the largest subunit of RNA Pol II, while a larger number of these factors were observed to be downregulated during infection, such as the smaller subunits of RNA pol II, TFIIA and TAF55. The HSV mechanisms existing early during infection that contribute to the shutoff of cellular genes are also likely to contribute to the disrupted expression of components of the cellular transcription machinery. Such mechanisms include early translation disruption by polyribosome dispersal and mRNA destabilization induced by the viral specific factor *vhs* (reviewed in 179), inhibition of host cellular splicing by the IE protein ICP27 (reviewed in 170), and viral induced nuclear reorganizations by such viral factors as the IE protein ICP0 (reviewed in 57, 84). Because the normal composition of these factors is essential to the transcription of cellular genes, changes

observed in the expression of these factors would certainly contribute to the downregulation of cellular gene expression.

Although alterations in the normal composition of the general transcription machinery during infection may disrupt cellular gene expression, disruption of the transcription machinery would also have a large impact on viral gene expression. Immediate-early (IE) and early (E) promoters contain binding sites for cellular activators that work in concert with viral activators, such as VP16 and ICP4, that promote expression of these genes (reviewed in 199, 203). Many of these viral and cellular activators interact with and recruit components of the basal transcription machinery to IE and E promoters making these interactions essential to activated transcription. Because these genes rely on cellular transactivators that require the normal composition of the cellular transcription machinery for expression, upsetting the normal balance of the cellular transcription machinery early during infection would not be highly beneficial to their expression. Most of the observed changes in the expression of general transcription machinery occur later in infection (beyond 4 hpi) when early and late genes are being expressed, suggesting that if any components of the general transcription machinery were altered during infection it would largely impact E and L gene expression. Coincidently, beyond 4 hpi early gene expression is attenuated signifying that changes in the general transcription machinery may contribute to the declined expression of early genes. However, viral late genes are efficiently expressed late in infection, suggesting that viral transcriptional regulatory mechanisms must exist that cooperate with these changes to allow for the continued high-level expression of late genes. Rather than containing cis-acting sequences that are the binding site for cellular activator, late promoters have sequences, such as the INR and downstream activating sequences (reviewed in 199, 203), that are recognized by components of the general transcription factors (26, 82, 106, 107, 154).

Recruitment of the GTFs and the activation of late genes is primarily accomplished through the viral transactivator ICP4. Because late genes do not require cellular activators that rely on the normal composition of the general transcription machinery, late genes may still be efficiently activated by ICP4 with an altered set of transcription factors. Unlike most transcription factors, ICP4 does not require DNA specific binding sites on early and late promoters to activate transcription. Given that ICP4 is a fairly large protein that can potentially interact with the general transcription machinery in different ways, ICP4 may have differential requirements for the general transcription machinery depending on promoter context. Thus although many of the components of the general transcription machinery are differentially expressed late in infection trending toward declining expression, ICP4 may substitute, or may not require some of the functions of the general transcription factors that are altered or are no longer available at the time late genes are expressed.

Through microarray and Northern blot analysis, the expression of the largest subunit of the 12-subunit RNA Pol II enzyme was observed to increase while the smaller subunits were observed to decrease as a function of wild-type infection. Additionally, the largest subunit, which contains the carboxy-terminal domain essential to transcription initiation and elongation, is aberrantly phosphorylated during infection (164). A combination of these changes must have some effect on cellular and/or viral gene expression. It would be of interest to compare the protein ratios of the smallest subunits of RNA Pol II as well as the largest subunit of RNA Pol II and its phosphorylation pattern, from wild-type infected and uninfected cells at various times of infection to determine any differences in the protein composition of Pol II during infection. Purifying and comparing the activities of infected and uninfected Pol II in reconstituted transcription on cellular and viral early and late promoters in the presence and absence of ICP4

to determine any differences in ICP4-mediated activation would also be a future worthwhile endeavor. Perhaps infected aberrantly phosphorylated Pol II works with ICP4 to initiate transcription from selective promoters in part mediating class-specific differences in promoter activation.

Another factor to further examine is TAF55, a component of the general transcription factor TFIID. Both microarray and northern blot analysis suggested that the expression of TAF55 was decreased during infection. With the newly defined function of TAF55 to interact with and inhibit TAF250-dependent transcription (70), it would be of interest to determine if the decreased expression of TAF55 during infection affects TAF250-dependent viral promoter activity. Given that late but not early promotes contain an INR element that relies on TAF250 for function, the presence of TAF55 at early times of infection could be hypothesized to block TAF250-dependent late gene expression early during infection while having no effect on TAF250-independent early gene expression. Late in infection, the decreased abundance of TAF55 may lift the block in TAF250 activity resulting in an increase in the expression TAF250 dependent late promoters. This may be a mechanism that prevents the expression of late genes during early times of infection.

One observed microarray change that we have correlated with *in vitro* biochemical analysis was the decreased expression of the general transcription factor TFIIA. All three subunits of the TFIIA complex were observed to be downregulated at late times of wild-type infection through both microarray and northern blot analysis. Reconstituted transcription analysis on representative early and late promoters showed that while TFIIA was essential for efficient ICP4-activation of the early tk promoter, it was not required for ICP4-activation of the late gC promoter. Dispensability of TFIIA for ICP4-activation of the late promoter required an

intact INR. From previous studies we showed that mutations within the INR of the gC promoter severely compromised the ability of ICP4 to activate transcription from this promoter (110). However, here we show that the additionally activities of TFIIA allow ICP4 to overcome the requirement for a functional INR to efficiently activate transcription from an INR-mutated late gC promoter.

Early promoters, which contain upstream activating sequences that are binding sites for cellular activators, require TFIIA in addition to ICP4 to activate transcription. Late in infection, however, TFIIA expression is decreased. This may compromise early gene expression because, cellular activators in conjunction with ICP4, can no longer function in the absence of TFIIA to activate early genes. Thus the decreased availability of TFIIA late in infection may contribute to early gene attenuation. Late genes that contain an intact INR do not require TFIIA for ICP4-activation and therefore the decreased expression of TFIIA would not impact late gene expression. This suggests a mechanism for the switch from early to late gene expression.

TFIIA is known to stabilize the binding of both TBP and TFIID to the TATA box on core promoters (35, 97, 111, 115, 160, 210). TFIIA has been termed a co-activator of transcription because although it stabilizes the binding of TBP to the TATA box, TFIIA is not required for TBP-mediated transcription. The TBP-associated factors of the general transcription factor TFIID are also not required for basal transcription and have also been termed co-activators of transcription. Yet in the context of eukaryotic cells with the exception of yeast, TBP is rarely found in free form. TBP is usually associated in multi-subunit complexes, such as general transcription factor TFIID (reviewed in 77). For TFIID-mediated basal and activated transcription, TFIIA is required (35, 40, 66, 111, 118, 119, 122, 189, 211). TFIIA is thought to counteract the repressive effects of TAF250 of TFIID. TAF250 inhibits the binding activities of

TBP through interactions with the DNA binding surface of TBP. TFIIA interacts with repressive domain of TAF250 relieving its interaction with TBP, allowing TBP to bind to the TATA box of DNA (147).

For activated transcription, both TFIIA and the TAFs of TFIID are required (reviewed in 124, 145). Although these factors seem to be dispensable for basal transcription, they play very important roles in activated transcription since most cellular as well as viral activators interact with TFIIA and TAFs. ICP4 requires the TAFs of TFIID to activate transcription (79). However, ICP4 seems to have a differential requirement for TFIIA depending on the promoter context. This is not completely unusual giving that ICP4 can activate transcription of randomly different genes. Unlike other activators that bind to promoter specific sequences to activate transcription, ICP4 does not require binding to a DNA-specific sequence to activate transcription. This is beneficial to viral gene expression, because this allows ICP4 to activate transcription of vastly different viral promoters. Perhaps differential interactions of ICP4 with the cellular transcription machinery and/or alteration of this machinery may also allow for regulation of ICP4-activated transcription of different classes of genes at different points during infection.

What causes TFIIA to be dispensable for ICP4 activation in the presence of an INR element in addition to a TATA box? When we examined the binding properties of ICP4, TFIIA and TBP/TFIID on the tk, gC and INR-mutated gC promoter, footprinting analysis showed that ICP4 like TFIIA stabilized the binding of TBP on the TATA box of the gC and INR-mutated gC promoters. An ICP4-derivative n208, which lacked the C-terminal activation domain, was also able to stabilize TBP to the TATA box. However, another ICP4-derivativeX25, which lacked both the N- and C-terminal transactivation domain, was not able to stabilize the binding of TBP

to the TATA box. The fact that n208, lacking the C-terminal domain, but not X25, lacking both the C- and N-terminal domains, was able to stabilize binding of TBP to the TATA box suggested that the N-terminal activation domain played an important role in this event. Although ICP4's ability to stabilize TBP was noteworthy, as mentioned above, ICP4 requires the activities of the TAFs of TFIID to activate transcription. When TFIID was substituted for TBP, ICP4 could only efficiently stabilize TFIID binding to the wild-type gC promoter but not the INR-mutated gC promoter. TFIIA was required to stabilize binding of TFIID to the INR-mutated gC promoter. These results suggest that ICP4 could substitute for TFIIA in stabilizing the binding of TBP or TFIID to the TATA box of the wild-type INR containing gC promoter. However, ICP4 was unable to effectively stabilize the binding of TFIID to the TATA box in the absence of a functional INR and required the additional activities of TFIIA.

In piecing together the data that TFIIA is required for ICP4-mediated activation of early genes, but late in infection TFIIA expression is downregulated and is dispensable for ICP4-mediated activation of INR-containing but not INR-mutated late genes, with the result that ICP4 can substitute for TFIIA in stabilizing the binding TFIID to the wild-type but not INR-mutated late promoter a picture emerges of the possible mechanism by which ICP4 might activate transcription of INR-containing late genes, later in infection without the requirement for TFIIA. ICP4 may function in a manner similar to TFIIA only on INR-containing promoters. TFIIA normally relieves TAF250's inhibitory effect on TBP by displacing TAF250's repressive domain from the DNA-binding surface of TBP (147). TFIIA also directly interacts with TBP within TFIID to stabilize its binding to the TATA box. Additionally, TFIIA strongly enhances TFIID binding to a TATA-INR promoter (55) and a TAF250-TAF150-TBP complex is known to be sufficient for INR activity (105, 107, 195). TFIIA can bind to and alter the organization of TAFs

in the TFIID complex and this reorganization, particularly through TAF250 interaction can enhance the ability of TFIID to interact with the INR. ICP4 interacts with TAF250 through its C-terminal activation domain (25). It is possible that the C-terminal domain of ICP4 also interacts with the repressive domain of TAF250 to alleviate its inhibitory effect on TBP. The exposure of TBP may then allow the N-terminal domain of ICP4 to interact with TBP and stabilize its binding to the TATA box. ICP4 induced conformational changes of TAF250 may in turn enhance and stabilize TFIID's interaction with the INR on late promoters leading to efficient activation of late genes in the absence of TFIIA. ICP4 induced conformational changes of TAF250 may not be sufficient enough to stabilize TFIID binding in the absence of a functional INR shifting the equilibrium of TAF250 back toward its TBP-inhibitory state. Future experiments should be aimed at determine which regions of TAF250 and TBP interact with ICP4. Regardless, this results in the inability of ICP4 to efficiently activate transcription of an INR-mutated late promoter. The addition activities of TFIIA are required to secure TFIID binding to the TATA box of the INR-mutated late promoter. This suggests why TFIIA allows ICP4 to overcome the requirement of a functional-INR on late promoters.

In summary we have shown that the expression of many components of the general transcription machinery are dysregulated during infection. One of the observed changes that correlated with transcription analysis was the decreased expression of all three subunits of the TFIIA complex. Although the decreased expression of TFIIA during infection most likely contributes to the suppression of cellular genes, TFIIA also impacts the expression of viral genes. We showed that TFIIA was required for the efficient ICP4-mediated activation of a representative early gene but that it was dispensable for the activation of a representative INR-containing late gene. Early promoters do not contain INR elements. These promoters do

however contain binding sites for cellular activators that work with ICP4 to activate transcription. Cellular activators require TFIIA for function. In addition, ICP4 in the absence of an INR also requires the TFIIA for function because as our data suggests, ICP4 cannot effectively stabilize the binding of TFIID to a TATA box alone. This suggests that ICP4 activates transcription of early genes in a distinctly different manner from the way it activates INR-containing late genes. We further showed that ICP4 did not require TFIIA for the efficient activation of an INR-containing late gene because ICP4 could substitute for TFIIA in stabilizing the binding of TFIID to this promoter. In contrast, ICP4 required the additional activities of TFIIA to activate transcription from an INR-mutated late promoter because it could not effectively substitute for TFIIA in stabilizing TFIID binding to a TATA box in the absence of an INR. This correlates with previous observations that ICP4 activates early and late genes by different mechanisms. Putting all of this together in the context of cells, at early times of infection TFIIA is available and is required for the expression of early genes. Because these promoters contain cis-acting sequences but do not contain any INR elements, ICP4 as well as cellular activators function through TFIIA to stabilize TFIID binding to the TATA box to activate transcription. Late in infection the TFIIA expression is decreased and may no longer be available for ICP4 and other cellular activators to activate transcription of early genes, leading to early gene attenuation. The decreased expression of TFIIA does not effect late gene expression because for late promoters that contain an INR, TFIIA is no longer required for ICP4-activation. ICP4 can efficiently stabilize TFIID binding to the TATA box in the presence of an INR, which serves to activate transcription of these genes. Collectively all this data provide evidence that the decreased expression of TFIIA during infection is one mechanism that contributes to the shutoff of early gene expression allowing the shift to late gene expression.

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