INTERACTIONS BETWEEN ICP4 AND THE CELLULAR TRANSCRIPTION MACHINERY THAT MEDIATE HSV-1 GENE EXPRESSION

by

Jonathan Trevor Lester

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SCHOOL OF MEDICINE

This dissertation was presented

by

Jonathan Trevor Lester

It was defended on

February 26th, 2009

and approved by

Fred Homa, Associate Professor,
Department of Microbiology and Molecular Genetics

Paul R. Kinchington, Associate Professor,
Departments of Ophthalmology and Microbiology and Molecular Genetics

Martin Schmidt, Associate Professor,
Department of Microbiology and Molecular Genetics

Tom Smithgall, Professor and Chair,
Department of Microbiology and Molecular Genetics

Dissertation Advisor: Neal DeLuca, Professor,
Department of Microbiology and Molecular Genetics

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Jonathan Trevor Lester, PhD

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Herpes simplex virus type 1 (HSV-1) infected cell polypeptide 4 (ICP4) is a critical regulator of viral gene expression that is required for productive infection. ICP4 has been shown to act, depending on the promoter structure, as both an activator or repressor of viral genes. ICP4 has two broad transcriptional regulatory domains, the N-terminal domain, which exhibits both repression and transactivation, and the C-terminal domain, which is involved exclusively in transactivation. ICP4 regulates transcription through interactions between ICP4's transcriptional regulatory domains and cellular general transcription factors including components of TFIID. Although it has been shown that a region in the amino-terminus of ICP4 corresponding to aa 30-210 is necessary for appropriate transactivation and repression of viral genes, specific domains responsible for these activities remain uncharacterized. Using deletion mutants spanning this region, we show that the entire region is necessary for ICP4 function, but that loss of aa 30-142 has a greater detrimental impact on the ability of ICP4 to transactivate E and L genes. Neither deletion had a significant impact on ICP4 repression, however.

The protein interactions made by ICP4 *in vivo* during infection were also studied. We show ICP4 forms complexes with TFIID, thus verifying previous *in vitro* data. Novel interactions between TFII-I and components of the Mediator complex were also identified. In addition, Mediator was found to colocalize with ICP4 starting at early and continuing into late times of infection. Mediator was also recruited to viral promoters in an ICP4-dependent manner,

showing a direct role for Mediator in ICP4-mediated transcription. Together, the data show that ICP4 regulates transcription through interactions between its complex regulatory domains and a diverse set of cellular protein complexes, including components of the basal transcription machinery and coactivator complexes.

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1.0 INTRODUCTION

1.1 HERPESVIRIDAE

The order *Herpesvirales* comprises a diverse group of DNA viruses that are highly disseminated throughout our environment with hosts in nearly all vertebrate species. Traditionally, these viruses have been identified based on morphology. A herpesvirion consists of a core with a linear, double-stranded genome ranging from 124-230 kilobases. Surrounding the core is an icosahedral capsid that is approximately 125 nm in width and comprised of 162 capsomeres. Surrounding the capsid is an amorphous, proteinacious tegument layer. Finally, surrounding the tegument is a lipid bilayer envelope with glycoprotein spikes (210). Further subdivision of the order defines three families: *Alloherpesvirdae* which infect fish and amphibians, *Malacoherpesvirdae* which infect bivalves, and *Herpesviridae* which infect mammals, birds, and reptiles (175). Over 200 members of *Herpesviridae* have been identified with most animal species susceptible to infection by one or more members. In humans, there are currently eight known herpesviruses: herpes simplex virus 1 and 2 (HSV-1, -2) also known as human herpesvirus 1 and 2 (HHV-1 and HHV-1), Varicella zoster virus (VZV, HHV-3), Epstein-Barr virus (EBV, HHV-4), cytomegalovirus (CMV, HHV-5), HHV-6, HHV-7, and Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) (210).

Herpesviridae contains three sub-families (alpha, beta and gamma). These were historically based on the host-range and tissue specificity, the duration of the replicative cycle, and the cell-type that harbors latent infection (235) but are now based on genomic organization and gene sequence. Hallmarks of the Alpha-herpesviruses are a relatively broad host-range, short replicative cycle, and latency in sensory neurons. Members that infect humans consist of HSV-1 and -2 as well as VZV. The Beta-herpesviruses have a longer replication cycle and a restricted host-range compared to the alpha-herpesviruses. This sub-family establishes latency in secretory glands, lymphoreticular cells, and the kidneys. Members of the Beta-herpesviruses that infect humans include CMV, HHV-6, and HHV-7. The third sub-amily are the Gamma-herpesviruses. Members have a host-range restricted to the family or order to which the natural host belongs, have a variable replication rate, and are also lymphotrophic. Gamma-herpesviruses include EBV and HHV-8 (210).

The human herpesviruses are significant pathogens with many members being endemic to the human population. The clinical manifestations of infection are diverse. HSV-1 and -2 primarily cause orolabial and genital epithelial lesions and can rarely lead to encephalitis. VZV is the causative agent of chickenpox and zoster, which can lead to a painful and potentially debilitating neuralgia. Recurrent reactivations from latency of HSV leading to infections of the eye can also cause herpes keratitis, the leading cause of infectious blindness in the developed world. CMV causes a wide-range of diseases predominately in immunocompromised individuals. Manifestations are most severe in congenital infections potentially leading to mental retardation or vision and hearing loss. Pathology of CMV infection also includes CMV mononucleosis, CMV retinitis, and GI disease. HHV-6 and -7 cause roseola infantumin in infants and can lead to more significant complications in immunosuppressed individuals. EBV is

the causative agent of mononucleosis and B cell lymphomas while KSHV is associated with Kaposi's sarcoma (236).

1.2 HSV PATHOGENESIS

HSV enters the host via infection of skin and mucosa epithelial cells primarily in the orolabial or genital region and is spread by close person-person contact. The virus replicates its DNA in the nucleus of infected cells, producing progeny virions that spread to neighboring cells. Egress results in the death of the infected cell. This presents as lesions or blisters at the site of infection (236). During the course of infection, the virus enters the sensory neurons that inervate the site of infection. The virus infects the neurons and the capsid travels via retrograde transport to the cell bodies of the sensory ganglia. Given the areas typically involved with infection, the corresponding sensory ganglia include the trigeminal or sacral ganglia. In these ganglia, the genome establishes an asymptomatic latent infection in the nuclei (40, 274).

During latency, the viral genome persists in a quiescent state with little gene expression other than the abundant expression of the latency-associated transcripts (LATs) (218, 244, 288). The genome associates with histones in a repressive nucleosomal structure and endures in an episomal, circular configuration (53, 181). Dynamic interactions between HSV, the neuron, and the host immune system contribute to the maintenance of latency (44). CD8+ T-cells associate with latently infected neurons and may inhibit viral reactivation through the release of IFN-γ and lytic granules that cleave viral proteins important in viral lytic replication (137, 141). Environmental and physiological stresses, including UV-light, fever, malnutrition, fatigue, or anxiety lead to a de-repression of the viral genome, and also immunosuppression and loss of the

CD8+ T-cell functions involved in surveillance of the infected neuron, which results in reactivation (79, 288). Once reactivated, the virus enters the lytic replication cycle, producing progeny virions that travel down the axon via anterograde transport. This reactivation is often asymptomatic but can lead to recurrence of clinical manifestations at the site of the primary infection and allows for the dissemination of the virus to new hosts (40).

1.3 HSV GENOME CONFIGURATION

The HSV genome consists of a large (~152 kb), linear double-stranded DNA molecule with a G + C content of approximately 68% (138). Within the virion, the DNA molecule contains nicks and gaps, which may serve to activate host DNA repair processes upon entering the nucleus (80, 234). The genome is divided into two covalently linked subunits, the L and S regions (figure 1) that contain unique sequences (U_L and U_S) of about 108 and 13 kb, respectively. These regions are flanked by inverted repeats (R_L and R_S) (reviewed in (173)). The terminal repeats are in a direct orientation with the internal repeats inverted; thus, R_L is described by ab and b'a' while the R_S is a'c' and ca (162). The structure of the a sequence is highly conserved. However, it contains a variable number of repeats depending on the HSV strain, and so it ranges in length between 250-500 bp (234). There is a constant single copy of the a sequence at the S terminus but variation in the number of repeats abounds at the L-S junction and the L terminus (162). Different isolates of HSV can therefore have genomes that range in size up to 10 kb (287).

The presence of the repeat elements allows for recombination between the terminal direct repeats and the internal inverted repeats. This causes the inversion of the unique and repeat sequences relative to one another and leads to four distinct isomers of the genome in equimolar

proportions: no inversion, U_L inversion, U_S inversion, and both U_L -U_S inversion (45, 111). All four isomers can arise from infection with a single isomer. Deletion of the internal repeat sequences, which prevents production of the various isomers, has no discernable effect on viral replication in tissue culture but is important for viral pathogenesis (234). As opposed to the expendable internal a sequences, the terminal a sequences are important for both concatemerization and packaging of the genome, and thus are required for replication (3, 117). The genome also includes three origins of replication with one in the U_L region (ori_L) and one repeated copy in the R_S (ori_S) (236).

Figure 1: Schematic representation of the structure of the HSV genome.
The HSV genome is divided into two unique regions, Unique long (U_L) and Unique short (U_S). These regions are bracketed by repeats R_L and R_S indicated by arrows. The R_L repeat is composed of elements: the a (red) and b (blue) sequences while the R_S is composed of a and c (green) sequences. The terminal direct repeats (ab and ca) are inverted in the junction (b'a' and a'c'). A single copy of the a sequence is found at the U_S terminus, while varying copy numbers are found at the the U_L terminus and junction region.

The HSV genome encodes approximately 80 open reading frames utilizing both DNA strands (173). The U_L region contains at least 56 genes associated with viral DNA replication, virion structure, and ICP27. The U_S region encodes 12 glycoproteins, ICP22, and ICP47 among others. The R_S contains the ORF for ICP4 while R_L contains the ICP0 ORF. Thus there are two copies of both the ICP4 and ICP0 genes per genome (236). HSV genes are similar in structure to cellular genes with a promoter, an ORF, 3' UTR and a polyadenlyation signal (10). However, only 5 viral genes contain introns which are abundant in cellular genes (213, 236). Exploiting this difference is one way the virus promotes viral over cellular gene expression.

1.4 HSV PRODUCTIVE LIFE CYCLE

The HSV productive cycle begins with entry of the virus into the cell via binding of the virion to the cell surface through specific receptor interactions. This binding leads to fusion of the envelope with the cell membrane (reviewed in (270)). The de-enveloped virion is then transported to the nuclear pore complex (NPC) where the viral DNA is injected into the nucleus (267). The viral gene expression program then begins in the nucleus and proceeds in a tightly regulated cascade consisting of three broad stages, the Immediate Early (IE, or α), Early (E or β), and Late (L or γ) (120, 121). The IE genes primarily coordinate the viral gene expression program; E genes are involved in DNA replication; and L genes encode mostly structural proteins. Once sufficient E proteins are present, viral DNA replication commences. Following DNA replication, L genes are expressed, allowing for capsid assembly, encapsidation, and egress.

1.4.1 Viral entry

Viral entry proceeds through two distinct pathways. The first pathway involves fusion of the viral envelope with the plasma membrane whereas the second involves endocytosis of the enveloped virion and subsequent fusion of the envelope in endocytic vesicles (reviewed in (236)). Although the second pathway is well characterized in certain tissue culture models, its importance during clinical infection is unclear. The first step of entry is the attachment of the virion to the cell surface through interactions between gC and gB in the viral envelope and cell surface glycosoaminoglycans (GAGs) (113, 259, 272, 301). gB is essential for viral replication whereas gC is nonessential in tissue culture although the deletion of gC results in reduced viral

entry (113). Attachment of the virus to the cell surface triggers the interaction of gD with one of its receptors and, in conjunction with gB, gH, and gL, fusion of the envelope with the cellular membrane (reviewed in (271)). Receptors for gD include intercellular adhesion molecules called nectins, herpes virus entry mediator (HVEM) -a member of the tumor necrosis factor receptor family- and 3-*O*-sulfated heparan sulfate (34, 85, 187, 260). Once the capsid is released into the cytoplasm, it is transported via the microtuble network to the NPC through interactions with the cellular motor protein dynein (57, 267). Once at the NPC, the capsid binds to the pore and injects the viral DNA into the nucleus in a poorly understood mechanism that is dependent on both energy and importin β (56). Viral gene expression commences shortly after the genome gains access to the nucleoplasm.

1.4.2 Viral DNA replication

There are seven HSV genes required for viral DNA replication (19). These include the DNA polymerase holoenzyme heterodimer comprised of U_L30 and U_L42, the origin-binding protein U_L9, single-stranded DNA binding protein ICP8, and the DNA helicase-primase complex comprised of U_L5, U_L8, and U_L52 (reviewed in (236)). DNA replication can begin from any of the three origins of replication while none of them are specifically required (125, 215). Once the viral gene expression program proceeds adequately to accumulate sufficient levels of E proteins, viral DNA replication commences.

Traditionally, the viral genome was thought to circularize shortly after entering the nucleus and serve as the template for DNA replication while in the circular form (214). Based on this circular model, viral replication was thought to proceed first through theta replication followed by a switch to rolling circle replication resulting in long end-to-end concatemers (19).

More recently, it has been shown that ICP0 expression inhibits the circularization and so the linear molecule persists to serve as the DNA replication template (130). Recombination-dependent replication has been suggested as a possible alternative mechanism for viral DNA replication that would proceed from a linear template (196, 297). The structure of the replication template and the mode of replication, however, remain controversial.

1.4.3 Viral encapsidation and egress

Late genes are expressed to maximal levels after the onset of DNA replication. These genes encode the structural proteins required for the encapsidation, envelopment, and ultimate egress of the mature virion. The viral capsid is composed of four proteins, the major capsid protein VP5, VP23, VP19C, and VP26 (reviewed in(117)). Capsids are assembled around a scaffold. A viral protease removes the scaffold and concurrently the viral DNA enters the capsid. DNA is added in unit length genomes through cleavage of concatemers by the terminase complex made up of U_L15, U_L28, and U_L33 (3, 14). Cleavage and removal of the scaffold and addition of the DNA triggers maturation into C-capsids, which are then tegumented, enveloped, and released from the cell.

Viral egress from the cell is a complicated, multi-step process that is not fully understood. First, mature C-capsids exit the nucleus through an envelopment-deenvelopment process at the nuclear membrane (261). The capsid is enveloped at the inner nuclear membrane, passes through the perinuclear space and subsequently fuses with the outer nuclear membrane (183). The deenveloped capsid is released and transported to the golgi where it is reenveloped. The golgi vesicle containing the enveloped capsid then fuses with the outer membrane, releasing the mature virion from the cell. How and when the capsid acquires the tegument is not well

understood but appears to be a dynamic process during egress and may play a role in coordinating the egress of the mature virion (51, 52, 182, 183).

1.5 GENERAL OVERVIEW OF CELLULAR TRANSCRIPTION

Understanding cellular transcription is essential for determining how HSV utilizes the machinery for its own transcription. The successful regulation of gene expression is necessary for all forms of life. The human genome is estimated to have between 20,000 - 25,000 protein-encoding genes, making the regulatory system overseeing their proper expression necessarily very complex (1). There are several regulatory stages, including but certainly not limited to controlling access to the DNA template itself, recruitment of RNA polymerase to the gene of interest, initiation of transcription, the elongation of the transcript, splicing, polyadenylation, export of the mRNA from the nucleus, mRNA stability, and translation. HSV encodes proteins that target each stage to enhance its own gene expression at the expense of the host cell. A major site of regulation both for the cell and HSV is the recruitment and stabilization of RNA pol II to promoters. This proceeds through the formation of a preinitation complex (PIC) on the core promoter.

The PIC consists of the cellular general transcription factors (GTFs) TFIID, TFIIA, TFIIB, TFIIF, TFIIE, and TFIIH and RNA polymerase II (pol II) (18, 134, 139). TFIID, a large complex consisting of TBP and its associated factors (TAFs), is the first GTF to bind to the promoter through interactions with the TATA box and other core promoter elements. Following TFIID binding, the remaining GTFs and pol II are recruited (21, 193). Upstream activators act through general cofactors to both recruit and stabilize TFIID binding to the promoter as well as

recruit and stabilize the other GTFs and pol II to TFIID to form the final PIC (278). General cofactors, such as TAFs and the Mediator complex, act as intermediaries bridging the upstream activators with the GTFs to stabilize PIC formation (reviewed in (278)). Thus, activated transcription is a result of complex interactions between upstream activators, general cofactors, and GTFs to form stable PICs at promoters.

1.5.1 Core promoter elements

The promoter can be divided into two different regions, the core promoter region and the regulatory elements (reviewed in (278)). The core promoter generally consists of those regulatory elements that influence transcription within 40 or 50 base-pairs (bp) both up- and downstream of the transcription start site. Regulatory elements are those cis-acting elements that bind repressors and enhancers of transcription and fall outside of the core promoter region. The core promoter functions primarily to interpret the regulatory input from distal enhancers and repressors as well as orient the transcription machinery for proper transcription initiation through binding of the general transcription machinery. Viral IE and E promoters include both core promoter regions and regulatory elements whereas L promoters consist of just a core promoter. Several elements and the proteins that recognize them have been defined, however only those elements relevant to HSV infection will be discussed further.

The first core promoter element characterized, and one shared by all HSV promoters, is the TATA box. The TATA box has a consensus sequence of TATA(A/T)A(A/T)(A/G) and is localized -31 to -24 bp relative to the start site. The TATA box binding protein (TBP) binds to the TATA box and is one of the first proteins recruited to the promoter (reviewed in (264)). Although the TATA box was initially thought to be a feature of all promoters and the key way

they were recognized by the general transcription machinery, recent genome-wide analyses show that as little as 10% of human promoters include this element (10, 54). Interestingly, promoters that contain the TATA box element tend to have a strictly defined transcription start site within a few base-pairs and are tightly regulated (21, 173).

The other major promoter element found on HSV promoters is the Inr element. It has a consensus sequence of YYA+1N(T/A) YY (where Y is pyrimidine and N is any nucleotide) and also serves as a binding site for TAF1/TAF2 components of TFIID (23, 181). It can direct transcription either alone or in conjunction with other core elements such as the TATA box (63, 161, 262). In addition to components of TFIID, the cellular factor, TFII-I, has also been shown to recognize and bind the Inr element and play a role in Inr-driven transcription (168, 238). While not found on all HSV promoters, the Inr sequence is a common element of L promoters and is required for their proper expression (93, 140).

1.5.2 General transcription factors

Transcription initiation proceeds through an ordered recruitment of several different protein factors, termed the general transcription factors (GTFs) (21, 190, 202). Initially defined as those crude fractions separated by column chromatography that are required to reconstitute site-specific transcription *in vitro* using purified RNA pol II, these six factors were termed TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH based on their chromatographical characteristics (reviewed in (278)). RNA pol II by itself is unable to recognize promoters and initiate transcription. The GTFs, through interactions with core promoter elements described above and other upstream activators, organize on the promoter to initiate transcription. These factors are recruited in a stepwise fashion, beginning with TFIID binding first, followed by TFIIA, TFIIB,

TFIIF, which recruits RNA pol II, TFIIE, and TFIIH.

TFIID is a large, multi-protein complex consisting of TBP and several TBP-associated factors (TAFs), which is highly conserved from yeast to humans (7). TFIID recognizes the promoter through interactions with core promoter elements. TBP binds to the TATA box in the minor groove of the DNA, significantly bending the DNA upon binding (194). In addition, TAF1/TAF2 recognize the Inr sequence (28), TAF6/TAF9 recognize the downstream promoter element (DPE) (253), and TAF1 binds the down stream core element (DCE) (221). These additional interactions with the core promoter allow TFIID to recognize both TATA-less and TATA-containing promoters.

In addition to promoter recognition, TAFs are important for activated transcription. Purified TBP alone is able to reconstitute basal transcription *in vitro* but is unable to support activated transcription (22). For activated transcription, the adddtion of TAFs in TFIID is required. TAF1 exhibits several enzymatic activites, including histone acetyltransferase, kinase, and ubiquitin activating/conjugating activity, directed at both histones and other members of the cellular transcription machinery (reviewed in (292)). In addition, several upstream activators have been shown to interact with various different TAFs to mediate their function.

Binding of TFIID is stabilized by TFIIA and TFIIB (reviewed in (278)). The C-terminus of TFIIB interacts with both TBP and the TFIIB-recognition element (BRE) on the core promoter. These interactions stabilize the TBP:TFIIB:DNA ternary complex either in the presence or absence of TFIIA (195, 281). The N-terminus of TFIIB facilitates the entrance of the TFIIF/RNA pol II complex via interactions with the RPB1 and RPB2 subunits of RNA pol II (23, 29, 100). TFIIB also plays a role in positioning RNA pol II for proper transcription initiation. TFIIA stabilizes binding of TFIID through interactions with TBP and DNA upstream

of the TATA box (63, 203). TFIIA also inhibits TAF1-mediated repression of TBP binding to the TATA box (203). This is borne out by the requirement for TFIIA for basal transcription driven by TFIID in reconstituted transcription systems. Interestingly, TFIIA has been shown to be required for ICP4-mediated HSV E gene expression but dispensable for L gene expression in an Inr-dependent manner (312).

The TFIID-TFIIA-TFIIB complex then recruits TFIIF/RNA pol II to the promoter via interactions between TFIIB and both TFIIF and RNA pol II (23, 29, 100). TFIIF has multiple functions in the PIC, including stabilizing RNA pol II binding to the TFIID-TFIIB promoter complex, repressing spurious initiation through inhibition of RNA pol II binding to non-promoter regions, and recruitment of TFIIE and TFIIH (reviewed in (278)). TFIIF also facilitates in subsequent steps of transcription, including promoter clearance and elongation.

RNA pol II is a large protein complex made up of 12 subunits, RPB1 to RPB12, that are highly conserved from yeast to humans with some sharing similarity with bacterial RNA polymerases. X-ray crystallography has elucidated the structure of the RNA pol II enzyme, showing in part that the complex forms a clamp-like structure that closes on the DNA during transcription (43, 299). A major site of RNA pol II regulation is the C-terminal domain (CTD) found in the largest subunit, RPB1 (reviewed in (59, 278)). The CTD consists of a heptapeptide tandem repeat of tyrosine-serine-proline-threonine-serine-proline-serine (YSPTSPS) with 52 repeats in humans, 42 times in *Drosophila*, and 26-29 times in yeast. This region is heavily post-translationally modified via phosphorylation at serines 2 and 5 and glycosylation of the serine or threonine residues. The phosphorylation of serines 2 and 5 act as a marker indicating the stage of transcription. As such, there are two general forms, RNA pol II_O, RNA pol II_A. The II_A form represents the hypo- or unphosphorylated CTD and is generally involved in PIC formation and

initiation. Serine 5 phosphorylation occurs at the promoter during the inititation and clearance phases. Serine 2 is then phosphorylated during the elongation phase, thus constituting the hyperphosphorylated form II_O where both serine 5 and serine 2 is phosphorylated. Several cellular kinases that target the CTD have been identified, including the TFIIH-associated CDK7, mediator-associated CDK8, and CDK9, which is associated with elongation factors. Interestingly, HSV infection induces an abberant form of phosphorylated RNA pol II, II_i, where only serine 5 is phosphorylated (228).

The final two GTFs, TFIIE and TFIIH, are important for promoter melting and initiation of transcription (116). Experimental systems involving pre-melted templates do not require their presence. TFIIE aids in the recruitment and regulation of TFIIH. TFIIE interacts with RNA pol II, TFIIB, and promoter DNA to facilitate binding (74, 75, 171, 201, 293, 310). It also recruits TFIIH and augments its ATPase, DNA helicase, and CTD kinase activities (153, 199, 200, 251). Through these functions, TFIIH aids in initation of transcription through promoter melting and conversion of RNA pol II from its PIC form to a stable elongating form.

1.5.3 Mediator

Another large complex involved in nearly all cellular transcription is the Mediator complex. It was initially described as a cofactor required for the activities of various activators, including thyroid receptor (TRAP complex), vitamin D receptor-interacting protein complex (DRIP), activator recruited complex (ARC) and Sp1 (CRSP) (reviewed in (278)). In humans, two general forms have been characterized based on chromatographic properties: the large complex called Mediator P.5 and the small complex, Mediator P.85. The larger complex corresponds to TRAP/SMCC and ARC/DRIP and contains a dissociable kinase module generally linked with

repression. The smaller complex P.85, such as CRSP, lack this module. Structural studies reveal a common organization of the different Mediator complexes that consist of a head, middle, and tail module. The head module primarily interacts with RNA pol II and undergoes a dramatic conformational shift upon association with pol II. The middle and tail modules also make some contact with RNA pol II but mostly are involved in interactions with various cellular activators (18, 27, 38).

The primary function of the Mediator complex is to serve as a bridge between activators and the GTFs and RNA pol II (129, 154, 276, 279). Mediator alone can enhance both basal and activated transcription in *in vitro* reconstituted systems. It does this mostly through interactions with RNA pol II and upstream activators. Mediator can also enhance TBP binding to the TATA box and stabilize the promoter-bound scaffold complex, thus enhancing reinitiation of transcription from the same promoter.

1.5.4 TAF-containing complexes

In addition to TFIID, there are other large, TAF-containing complexes that are important for transcription. These TAF-complexes, including TBP-free TAF-containing complex (TFTC), the PCAF/GCN5 complexes, the yeast Spt-Ada-Gcn5 acetlytransferase (SAGA) and its human homolog (STAGA), all lack TBP and can drive transcription from both $TATA^{+}$ and $TATA^{-}$ promoters (reviewed in (192)). Despite being made up of different subunits, these complexes do share a subset of TAFs including TAF6, -9, -10, and -12. Similar to TFIID, these complexes all have HAT activity, and TFTC and TFIID form a similar clamp-like structure with varying number of lobes surrounding a solvent-accessible grove for DNA-binding. These complexes can also recognize some core promoter elements through shared TAF subunits, and SAGA has been

shown to interact with and recruit TBP to some $TATA^+$ promoters (291). The similarities shared between TFIID and these TAF-containing complexes along with the observation that loss of TFIID-specific TAF1 function only results in a partial loss of global transcription suggest these complexes may play a similar role in promoter recognition and transcription as TFIID.

1.6 CASCADE OF VIRAL GENE EXPRESSION

The viral gene expression program proceeds as a tightly regulated cascade consisting of three broad stages called kinetic clases: the Immediate Early (IE, or α), Early (E or β), and Late (L or γ)(figure 2)(112, 120, 121, 170, 211). The stages are defined both by their temporal expression and their expression in the presence of various inhibitors. The IE genes are the first class to be expressed, can be detected as early as 30 minutes post-infection, reach peak expression levels between 2 and 4 hours post-infection, and do not require prior viral protein synthesis for transcription (120, 121). There are six IE proteins, including ICP0, ICP4, ICP22, ICP27, ICP47, and $U_S1.5$, whose orf is contained entirely within the ICP22 locus (33, 120, 211). Five of the six IE proteins, ICP0, 4, 22, 27, and $U_S1.5$ coordinate the expression of the remaining classes of expression while ICP47 inhibits antigen presentation to CD8 T cells (47, 55, 172, 241). Accumulation of sufficient IE protein leads to the expression of E genes and the subsequent reduction in IE expression.

The second class of genes, the E genes, reaches peak expression between four and six hours post-infection (120, 121). These genes require *de novo* viral protein synthesis but reach maximal expression in the absence of viral DNA replication. This class consists primarily of DNA replication machinery and modulators of nucleotide metabolism, including the viral DNA

polymerase (U_L30), origin binding protein (U_L9), ICP8, and thymidine kinase (tk) (236). Expression of E genes leads to the onset of DNA replication at around four hours post-infection and continuing as late as 15 hours post-infection (120, 121).

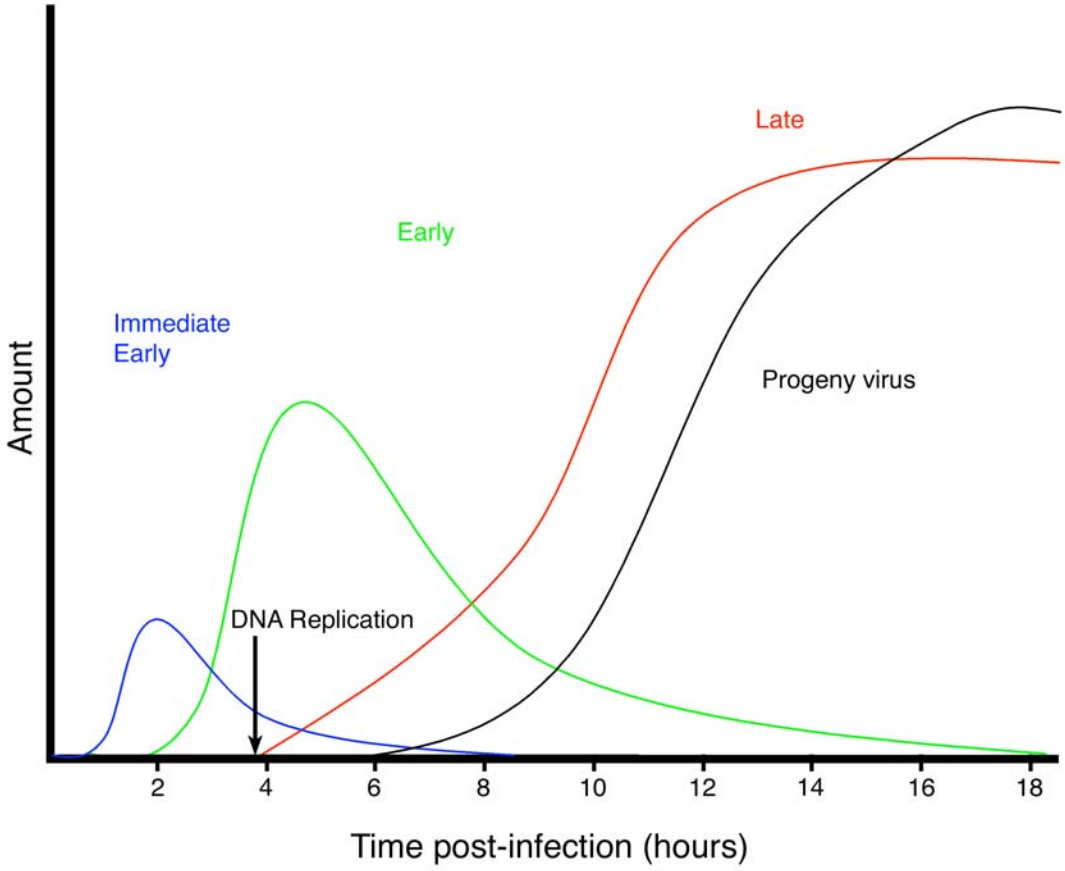

Figure 2: The temporal cascade of viral gene expression.
Viral transcription can be divided into three temporal phases, Immediate early (blue), Early (green), and Late (red). The immediate early genes reach peak expression between 2 and 4 hours post-infection (hpi) and are involved in coordinating the expression of the remaining classes of genes. The E genes are maximally expressed between 4 and 6 hpi and encode the viral DNA replication machinery. After sufficient E gene expression, viral DNA replication commences. The Late genes require DNA replication for their expression and encode the structural components of the virus.

The final class of genes, the L genes, is not expressed to maximal levels until after DNA replication begins (86, 124, 289, 295). They can be subdivided into two groups, leaky late or γ_1, which are expressed in low levels prior to DNA replication but require it for maximal expression (313), and true late or γ_2, which are not expressed until after DNA replication commences. This

class encodes proteins required for the assembly and egress of progeny virions as well as many of the tegument proteins. Robust expression of late genes leads to production of detectable progeny virions by eight hours post-infection. This progeny production increases exponentially resulting in the eventual death of the host cell.

Regulation of the viral gene expression cascade continues to require additional investigation; however, it is clear that the orderly expression of gene classes is mediated through the dynamic interaction between viral and cellular factors at both the transcriptional and translational level. Accumulation of mRNA determines the pattern of expression and this accumulation is chiefly regulated at transcription initiation (289, 290). Thus the initiation of transcription serves as a pivotal site of regulation. Each viral ORF is driven by its own promoter containing a TATA box that is recognized by the cellular transcription machinery (8, 41). As such, viral promoters have many similarities with their cellular counterparts including other cellular promoter elements and binding sites for cellular transcription factors. Each kinetic class has its own general promoter structure, which allows the viral and cellular transcription factors to either positively or negatively regulate each class (figure 3)(35, 119, 178, 273).

The virus also alters the cellular environment to divert resources from expression of cellular to viral genes and to aid in the switch between different viral classes. Entering with the infecting virion as part of the tegument, vhs is an endonuclease that indiscriminately degrades both cellular and viral mRNA species (147-149). This serves to inhibit production of cellular proteins while viral mRNA translation continues due to their rapid and robust transcription. The decreased RNA stability also allows for a tighter transition between viral temporal classes and underscores the role transcription rates play in determining gene expression.

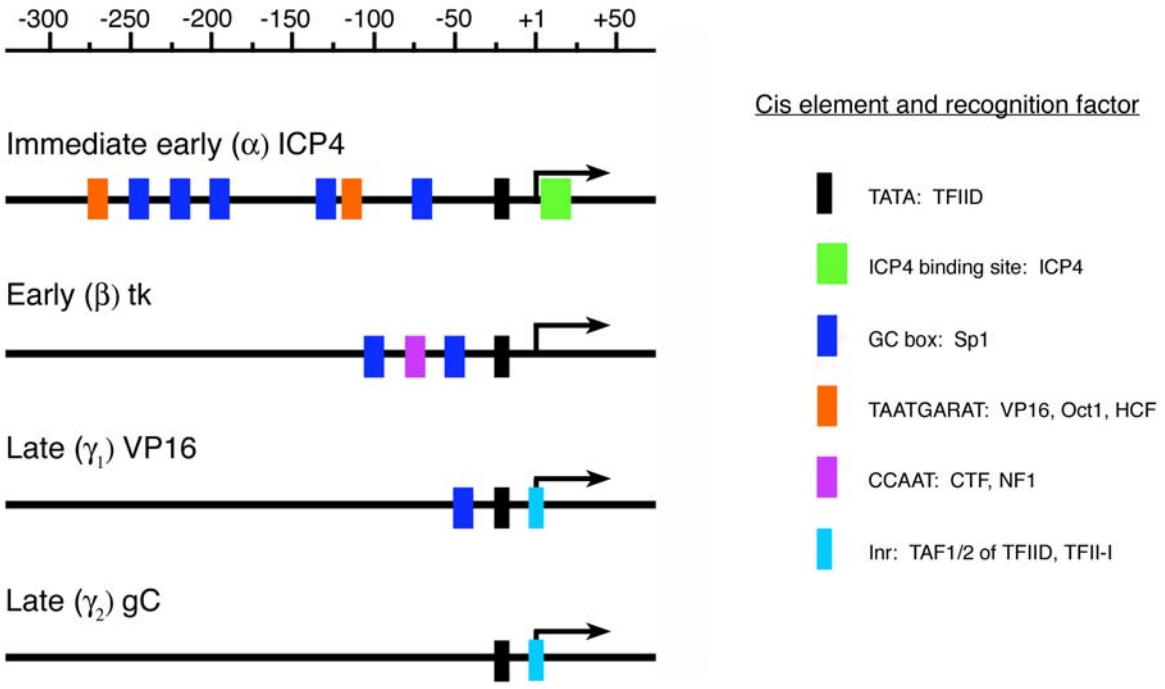

Figure 3: Schematic representation of representative Immediate Early, Early, and Late gene promoters. The cis-acting elements and their spatial arrangement are shown for the ICP4, tk, VP16, and gC promoters. Common to all HSV promoters is the TATA box, which serves as a major binding site for the cellular transcription machinery. The number and arrangement of the cis-elements varies from promoter to promoter, wit h an overall trend of decreasing complexity from IE to L promoters.

In addition to RNA stability, the virus also impedes RNA-splicing through the action of the viral IE protein ICP27 (105, 106). This serves to repress the expression of intron-containing cellular genes while allowing expression of the generally intron-less viral genes to proceed. Indeed, there are only four lytically expressed viral genes that contain introns, and three of them, ICP0, ICP22, and ICP47 are IE genes that would be transcribed and spliced prior to ICP27's inhibition (reviewed in (236)). The virus also alters the transcription environment by altering histone modifications (96), phosphorylation of transcription factors (139) and RNA polymerase II (164), and the abundance and localization of the transcription machinery (223, 312), all in the pursuit of diverting cellular resources to maximize viral transcription and replication. The

regulation of the different classes as well as the contribution of specific viral proteins to the regulation will be discussed in detail in the following sections.

1.6.1 Regulation of Immediate Early genes

The IE genes are expressed within the first hour of infection making them the first class to be expressed (122, 123). The promoter structure of the IE genes are the most complex of the three classes with binding sites for many cellular and viral factors (figure 3). IE genes are expressed prior to viral protein synthesis and are dependent on cellular factors for efficient transcription. A hallmark of the IE genes is the TAATGARAAT promoter element found upstream of the TATA box and is responsive to a complex consisting of viral VP16 and the cellular HCF and Oct-1 (13, 166, 167). The tegument protein VP16 enters the cell with the infecting virion and associates with HCF as it traffics into the nucleus (150, 151). The VP16-HCF complex then interacts with Oct-1 at TAATGARAAT motifs to stimulate transcription (142, 144, 177). VP16 contains a potent acidic transcription activation domain that interacts with several cellular factors to stimulate transcription (91, 243). These stimulating factors include members of the general transcription factors (87, 160, 275, 304), chromatin remodeling complexes (114), and the Mediator complex (306). In addition to the TAATGARAAT elements, IE promoters contain binding sites for other cellular transcription factors. The GC box and CCAAT motif act as binding sites for Sp1 and CTF or NF1, respectively, that act in concert with VP16 to activate transcription (132).

The expression of IE genes peaks approximately two to three hours post-infection then tapers over time. One of the contributing factors in this decrease is the IE protein ICP4. ICP4 inhibits activation through the cooperative formation of a stable, tripartite complex (TPC) with

TBP and TFIIB on promoters containing the high-affinity ICP4 binding site (145, 266). The only IE promoter that contains a properly positioned ICP4 binding site is ICP4, itself. It is unclear to what extent ICP4 participates in the repression of the other IE genes. However, deletion of ICP4 results in their overexpression. This may due to a loss of a repressive function played by ICP4 or the loss of E gene expression. ICP27's interference in RNA splicing may also play a role in shutting down IE gene expression, albeit at a post-transcription initiation step. As mentioned above, three IE genes contain introns and thus require the cellular splicing machinery for their expression.

1.6.2 Regulation of Early genes

Efficient expression of the E genes requires the IE proteins, especially ICP4. E promoters retain many of the same binding sites for cellular factors as the IE promoters but lack the TAATGARAAT binding site thus delaying their activation. E promoters do contain a TATA box and several upstream binding sites for Sp1 and other cellular transcription factors (132). These binding sites are not as numerous as on the IE promoters but still play an important role in E gene activation. Sp1 is a common transcription factor that interacts with the general transcription factors, specifically TAF10 of TFIID (115, 277), and Mediator (239) to activate transcription. ICP4 recruits general transcription factors to E promoters through non-specific DNA binding (26, 92). ICP4 binding is required at E promoters for efficient recruitment of both TBP and pol II (247).

E gene expression peaks between four and six hours post-infection and declines as robust DNA replication commences. The mechanism of E gene transcriptional downregulation is not fully understood, but evidence suggests viral DNA replication and viral-induced changes in the

transcription machinery is involved. Sp1 is phosphorylated at later times of infection, and this phosphorylation temporally corresponds to reduced E gene expression (139). The phosphorylated form of Sp1 harvested from infected cells retains efficient DNA-binding ability but exhibits reduced transactivation of the tk E promoter *in vitro* (139). Viral DNA replication also links to repression of E genes, mainly through the observation that inhibitors to viral DNA replication lead to an over-expression of E genes (156). These same inhibitors also partially block Sp1 phosphorylation suggesting a correlation between these observations (139). HSV infection also induces a reduction in the general transcription factor (GTF) TFIIA at late times of infection (312). Interestingly, TFIIA is required for efficient activation of E but not L genes, suggesting its loss may play a critical role in the switch from E to L expression.

1.6.3 Regulation of Late genes

The late genes are the last class to be expressed. As mentioned previously, late genes require viral replication for maximal expression and can be divided into two groups based on the degree of dependence. Low levels of leaky late genes can be detected prior to DNA replication while true late genes are entirely dependent on replication for expression (39, 120, 121, 313). The foundation for the difference between the 2 subgroups is unclear. Inherent differences in late gene promoter strength, progressive dilution of inhibitors by continued DNA synthesis, or post-replication changes in the genome could play a role. The repressive mechanism appears to act at least in part at a post-preinitiation complex formation step, as recent evidence has shown the ICP4-dependent recruitment of TBP and RNA pol II to the gC promoter in the absence of DNA replication (247). Therefore the locus of regulation of late gene transcription seems not to be

exclusively related to the binding of factors to the promoter, but also to later steps in transcription.

The L promoters are the simplest of the three classes in that they contain very few if any upstream cis-acting sequences (figure 3) (118, 119). Indeed, true late promoters usually have none while leaky late may have one or two binding sites for cellular activators (290, 295). These sites most likely explain their low levels of expression prior to DNA synthesis. Instead of upstream sites, L promoters are dependent on cis-acting sites downstream of the TATA box and the non-specific DNA-binding of ICP4 for efficient expression. These sites include the down stream activating sequence (DAS) and the Initiator element (Inr) (97, 98, 273, 298). The DAS element is essential for the proper expression of some late genes (U_L38) but not others (gC) (93, 140).

The Inr is a common cellular promoter element that is recognized by various cellular factors, including TAF1/2 of TFIID and TFII-I (28, 238, 263). Efficient activation of gC requires an intact Inr element. As Inr is a known binding site for TAF1/2, and TAF1/2 are specific to the TFIID complex, the recruitment of TFIID to late promoters is critical for expression (93, 140). Deletion of the Inr results in loss of gC expression by decreased TFIID binding and a corresponding decrease in the recruitment of TBP and RNA pol II to the promoter (247). Inr deletion also results in a decrease in the amount of ICP4 bound to the promoter, suggesting ICP4 and TFIID binding may be cooperative, thus facilitating ICP4's non-specific binding to DNA (247). The presence of the Inr also alleviates the requirement of TFIIA for efficient transactivation. This may be part of the mechanism that underlies the switch from E to L gene expression, as the amount of TFIIA is reduced at L times of infection.

1.7 IMMEDIATE EARLY VIRAL PROTEINS INVOLVED IN TRANSCRIPTION

HSV encodes several proteins that are involved in transcription. Two proteins, VP16 and *vhs*, are important in initiating the viral transcription program and were discussed earlier. As mentioned above, the IE proteins' primary role is to facilitate viral transcription. Four of five IE genes are involved at either transcription or post-transcription stages of gene expression. The expression of these proteins serves to enhance viral replication by activating viral gene expression and inhibiting host processes.

1.7.1 ICP0

Despite playing a key role in several processes, ICP0 is considered non-essential in tissue culture with mutants being impaired for growth, especially at low MOIs (242). ICP0 is a 110 kDa phosphoprotein that contains a RING finger domain with E3 ubiquitin ligase activity that is essential for ICP0 function (65, 66, 211, 212). ICP0 has been shown to be a critical determinant of viral latency, playing key roles in both lytic replication and establishment of and reactivation from latency (24, 32, 107, 244).

ICP0 is considered a promiscuous transactivator due to its ability to activate expression in transient assays from a broad range of promoters with no sequence specificity (67, 84, 197). ICP0 facilitates lytic replication and the expression of all three classes of genes through increased transcription rates (25, 133, 246). In transient expression assays, ICP0 and ICP4 synergistically increase expression from responsive promoters to greater levels than either achieve individually (67, 197). They have also been shown to interact, *in vitro* (309). ICP0 targets additional cellular processes to promote viral replication. Early in infection, ICP0 co-

localizes with and disrupts cellular ND-10 bodies (68, 169). These structures contain several different cellular proteins that are involved in a variety of cellular functions. ICP0 also inhibits the cellular DNA-damage response and the antiviral interferon response (60, 155, 206).

1.7.2 ICP22

ICP22 is also dispensable for viral replication in a cell-type specific manner. It is a 68 kDa phosphoprotein that facilitates expression of a subset of late genes (249). ICP22, in conjunction with U_L13 protein kinase, induces the stabilization of cdc2 and the degradation of cyclins A and B (4). Free cdc2 then interacts with U_L42 to recruit topoisomerase IIa to newly synthesized viral DNA to aid in resolution of tangled concatemeric DNA and aid in L gene expression (5, 6). ICP22 and U_L13 also phosphorylate the RNA pol II CTD to form the aberrantly phosphorylated form of RNA pol II_i found during HSV infection (164). The effect of this phosphorylation is unclear but is thought to aid in diverting transcription from cellular to viral genes (228, 229).

1.7.3 ICP27

ICP27 is multifunctional 63 kDA protein that is required for viral replication. ICP27 co-localizes with ICP4 in viral replication compartments where it alters the cellular RNA splicing machinery, aids in the export of viral mRNA, and facilitates the function of both ICP0 and ICP4 (180, 226, 245). ICP27 is required for the efficient expression of a subset of E genes, specifically those involved in DNA replication, making it indirectly necessary for DNA replication (226, 241, 284). It is also necessary for efficient expression of at least some L genes, both due to its role in promoting DNA replication and through direct activation of L gene

expression (131, 226, 227). As mentioned earlier, ICP27 inhibits RNA splicing to the benefit of the mostly intronless viral genes (20, 106). It also aids in the export from the nucleus of unspliced RNA (106, 248). In addition, ICP27 effects the elongation, termination, and polyadenylation site selection during transcription through its interaction with the CTD of RNA pol II (103, 176, 179). In cellular transcription, the processes of elongation, splicing, and polyadenlyation are linked both temporally and spatially through various protein interactions. Similar to cellular transcription where proteins involved in a specific stage of transcription regulate the recruitment and function of those proteins involved in other stages, ICP27 modulates the function and phosphoryation pattern of ICP4, and ICP4 and ICP27 have been shown to interact *in vitro* (204, 226, 245).

1.7.4 ICP4

ICP27 functions primarily in the post-transcription initiation phase while ICP4 activates transcription at the preinitation stage. ICP4 is the major regulator of viral transcription and is required for productive infection (47, 55, 217, 294). It acts both as a transcriptional activator and repressor, activating E and L genes while repressing its own transcription (48, 67, 84, 86, 197, 198, 224). ICP4's regulatory function is primarily through interactions with the cellular general transcription machinery at the promoter. HSV-1 expressing nonfunctional mutants of ICP4 exhibits an increased expression of IE genes while failing to express E or L, thus ICP4 is critical for the switch from IE to E and subsequently L gene expression (47, 55, 217).

1.7.4.1 ICP4 localization

ICP4 can first be detected less than one hour post-infection in small, discreet foci lining the nuclear rim (42, 211). These foci localize adjacent to ICP0 and ND-10 structures and contain one or more viral genomes (70). As infection proceeds, a portion of these foci develop into replication compartments acting as sites of both viral transcription and DNA replication (269). As such, ICP4 co-localizes in these compartments with both viral and cellular factors involved in these processes, including TBP, RNA pol II, ICP27, ICP22, ICP8, and the viral DNA polymerase (69, 165, 223, 283). At later times in infection, a proportion of ICP4 is found in the cytoplasm in an ICP27 dependent manner (314). The majority of ICP4 staining, however, remains in the nucleus in replication compartments. The function of the ICP4 found in the cytoplasm is unclear although, ICP4 is packaged in the viral tegument also in an ICP27 dependent manner (250, 308).

1.7.4.2 ICP4 structure

ICP4 is a large, structurally complex molecule that exists in cells as an elongated, 350 kDa homodimeric phosphoprotein (42, 184, 211, 257). The IE3 locus, which encodes ICP4, is located in the U_S terminal repeats making two copies per genome (188). The protein is 1298 amino acids with a corresponding molecular weight of 175 kDa (42). Due primarily to its heavily phosphorylated state, ICP4 presents as three different species on SDS-PAGE gels and as many as seven on 2-D gels (2, 73, 211, 296, 302). In addition to phosphorylation, ICP4 exhibits several other post-translational modifications, including adenylation, guanlyation, and ADP-ribsoylation (16, 17). To date, however, the only modification that has been shown to effect ICP4 function is phosphorylation (12, 186, 205). Gel filtration analysis has shown the ICP4 molecule adopts an elongated structure with a Stokes radius of approximately 90 Å and an axial

ratio of 20 to 1, possibly allowing it to exert influence over long distances (184, 257).

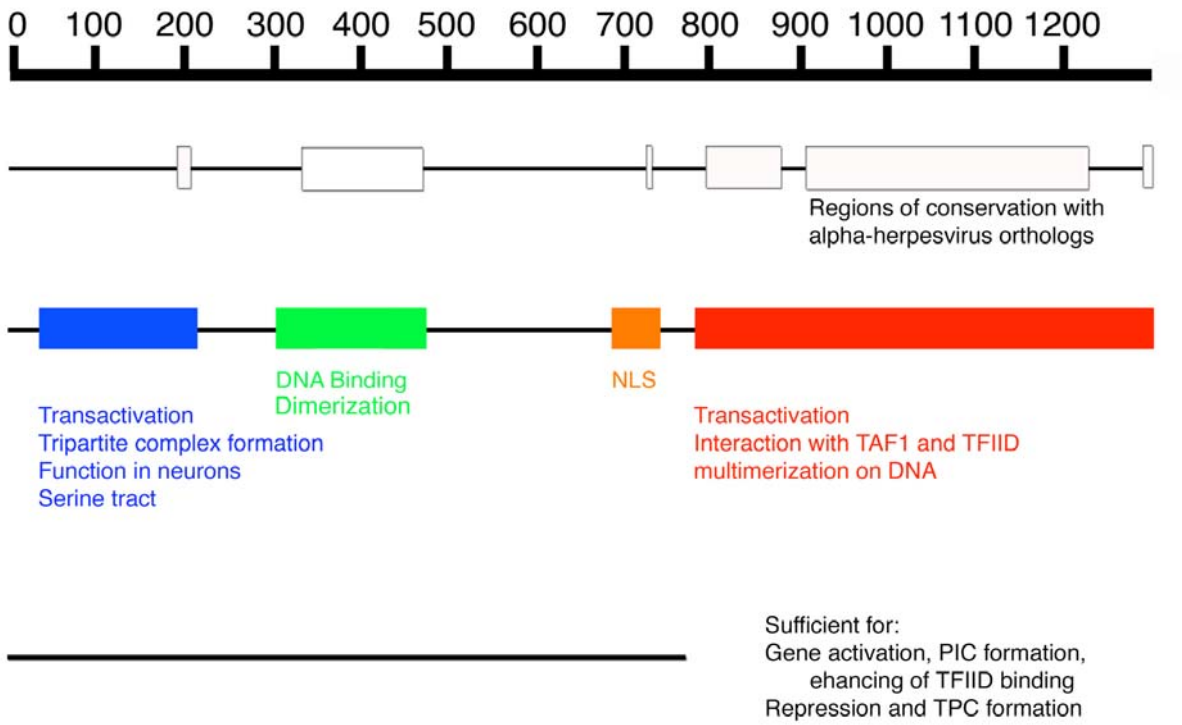

Figure 4: Functional domains of ICP4.
The 1298 amino acid primary sequence of ICP4 is shown. Below the primary sequence, white boxes indicate regions of similarity between ICP4 and its VZV homolog IE62. Colored boxes and text describe the various domains of ICP4 and their function.

ICP4 has been divided into several discreet regions based on various functional properties (figure 4). These functional domains correlate strongly with regions of significant amino acid similarity between ICP4 and its orthologs from other alpha-herpesvirus family members including pseudorabies virue IE180, VZV IE62, and the equine herpesvirus immediate-early protein (30, 174). They include two transcriptional regulatory regions localized in the N- and C-terminal regions separated by a dimerization domain, nuclear localization sequence (NLS), and a DNA-binding domain that contains a helix-turn-helix motif (49, 50, 81, 207, 208, 256). The ability of ICP4 to bind DNA, dimerize, and localize to the nucleus all contribute to the functions attributed to its transcriptional regulatory domains.

1.7.4.3 The N-terminal regulatory domain of ICP4.

The transcriptional regulatory domain found in the N-terminus of ICP4 spans amino acids 30-210 and can perform many of the regulatory functions of ICP4. A mutant termed n208 that is truncated at amino acid 774 and lacks the C-terminal regulatory domain retains the ability to repress its own transcription and transactivate a subset of E genes (50). It does not, however, efficiently replicate its DNA, most likely due to its partial deficiency in E gene expression, or express L genes. Deletions of both the transcriptional regulatory domains result in non-functional mutants that are trans-dominant (257). The N-terminal region has been shown to be important for ICP4's formation of a tripartite complex with TBP and TFIIB at strong ICP4 binding sites (145, 266). TPC has been shown to be involved in ICP4-mediated repression (94, 145). In addition, this region is important for ICP4-mediated stabilization of TFIID on some viral promoters (92).

1.7.4.4 Serine tract

The polyserine tract found in the amino terminus of ICP4 is a conserved stretch of 24 residues, 13 of which are serines. It can be found in all of the neurotropic alpha-herpesviruses including HSV-1 and -2, VZV, pseudorabies virus, Marek's disease virus, equine herpesvirus 1 and bovine herpesvirus 1 (30, 174). It is heavily phosphorylated, mediates phosphorylation of other regions of ICP4, and plays a unique role in viral growth in neurons.

The serine tract also has homology to a small cellular protein, P15, which supports VP16 activation during infection (12). The serine tract has consensus sites for phosphorylation by the cellular kinases PKA, PKC, and casein kinase II (302, 303). It is phosphorylated in vitro by PKA and PKC (302, 303). Interestingly, deletion of the serine tract results in loss of phosphoryation not only of those residues that are deleted but also residues in other areas of

ICP4. This suggests phosphorylation of the serine tract leads to successive phosphorylation of other regions of ICP4 (302, 303). Interestingly, deletion of the serine tract has only a small impact on viral growth in tissue culture and in the corneas of mice, but these viruses show significant impairment in growth in neurons. This virus lacking the serine-tract immediately enters into a latent state in mouse trigeminal ganglia and reactivates much less efficiently (12). In neurons, lytic infection with this mutant virus is halted just prior to viral DNA synthesis with low levels of expression of IE genes and some E genes, and very low expression of some late genes (12). This may be due to the reduced phosphorylation of the ICP4 mutant, considering the wild-type virus exhibits similar growth deficiencies in PC12 cells that lack PKA. This parallel indicates a potential role for PKA in viral replication through the phosphorylation of the serine-tract of ICP4 (303). Changes in the post-translational modifications may represent the difference in activity of this obviously vital region of ICP4.

1.7.4.5 DNA binding

ICP4 is both a sequence-specific and non-specific DNA binding protein, both abilities localizing to a region between amino acids 263 to 487 (207, 256). This region is highly conserved between ICP4 and its VZV homolog IE62 and includes a predicted helix-turn-helix motif commonly found in DNA-binding proteins. DNA-binding is required for both ICP4-mediated transactivation and repression although the requirement for binding is different. Repression requires specific ICP4 binding sites at or near the start site of transcription whereas no specific binding site has been characterized for transactivation (94, 95, 145, 207, 208, 232, 256). ICP4 binds to the degenerate sequence RTCGTCNNYNYSG, where R is purine, Y is pyrimidine, S is C or G, and N is any base (72, 78). For proper repression of a promoter, the ICP4 binding site must be present both in the correct orientation and in close proximity to the transcription

initiation site (145, 231, 232). While there are ICP4 sites of varying strengths scattered throughout the genome, only a few promoters, LAT, ORF P, ORF O, and ICP4, have properly positioned sequences for efficient repression (94, 145, 185, 232).

The role DNA-binding plays in ICP4-mediated transactivation is less clear. Although no binding sites have been associated with transactivation, ICP4 mutants unable to bind DNA are also nearly universally unable to activate transcription (35, 61, 64, 102, 265). In addition, recruitment of TBP and RNA pol II to viral promoters requires ICP4 to also be present, showing the importance of DNA-binding (247). Mutants have been developed, however, that lack efficient DNA-binding but retain some level of transactivation efficiency (128, 209, 254). Interpretation of these results is complicated by ICP4's sequence and non-sequence specific DNA-binding. These mutants were unable to bind to the ICP4 consensus sequence but may retain the ability to non-specifically bind DNA with low affinity. The C-terminus of ICP4 also mediates ICP4 multimerization on DNA and the cooperative recruitment of ICP4 and TFIID to promoters containing the Inr element, both of which may function to increase its overall affinity for DNA (146, 247). This cooperative recruitment may serve to increase ICP4's affinity for DNA, enriching non-specific ICP4 binding to promoter regions.

1.7.4.6 Nuclear localization

ICP4 localizes primarily to the nucleus during infection. This ability comes from the ICP4 nuclear localization sequence (NLS) between aa 723 and 732 (50, 207). This NLS has similarities to the SV40 virus large T-antigen protein, and thus may operate in a similar fashion. The large T-antigen NLS is recognized by the importin 58/97 complex, which targets proteins to the nuclear pore complex (88, 89, 134). At late times of infection, a fraction of the total cell ICP4 localizes to the cytoplasm. The mechanism for ICP4's retention in the cytoplasm is

unclear. The VZV homolog IE62 displays a similar though more dramatic localization pattern with a higher proportion localizing to the cytoplasm in late times of infection. Interestingly, IE62's localization is regulated by a phosphorylation event near its NLS by the viral kinase Orf66 (62).

1.7.4.7 The C-terminal domain

The C-terminus of ICP4 contains the largest region of homology with ICP4 orthologs. Despite the extensive similarity, truncation mutants that lack the last 500 amino acids retain all the functions associated with ICP4, including DNA-binding and transcription repression and activation (50). The truncation mutants are defective for growth, however, due to deficient DNA replication and L gene expression. In regards to L gene expression, the C-terminus has been shown to interact with TAF1 of TFIID, an interaction important for efficient activation of the Inr-containing L genes (26). This interaction, and possibly the multimerization of ICP4 on DNA also attributed to the C-terminus, is important for the recruitment of not only TFIID but also ICP4 to L, but not E, promoters (247). Interestingly, some C-terminus mutants show temperature sensitive loss of ICP4 function. This suggests the c-terminus plays a role in global ICP4 protein folding. In addition, some temperature sensitive mutants retain DNA-binding and transactivation but lack the repression function generally attributed to the N-terminus. This could also be due to defects in protein folding however, the retention of DNA-binding and transactivation suggests otherwise. The C-terminus is clearly required for efficient viral replication and may mediate intra-molecular interactions to mediate ICP4's full function (37, 46, 55, 209, 219).

1.7.4.8 ICP4-mediated transactivation and repression

ICP4 is the major activator of early and late genes during HSV-1 lytic infection. It transactivates expression at the promoter by both recruiting the cellular transcription machinery to the promoter and stabilizing their binding (26, 92). Although ICP4 activates both E and L genes, it does so through different mechanisms. Mutational analysis of ICP4 has shown that both its N- and C-terminal regulatory domains are important in transactivation; although each plays a different role (12, 26, 92). Expression of E genes requires primarily the N-terminal domain while efficient expression of L genes requires the C-terminus.

Transactivation functions via the GTF TFIID (26, 92). TFIID, itself, is a multi-protein complex containing the TATA-binding protein (TBP) and several additional subunits known as TBP associated factors (TAFs) (7, 101). This complex serves as the first GTF recruited to the promoter, and facilitates the formation of the pre-initation complex (202). Studies of TFIID and ICP4 interactions show that the C-terminus of ICP4 specifically interacts with the TAF1 subunit and facilitates TFIID binding to the TATA box (26, 92). TAF1 has been shown to be important in facilitating TFIID interaction with Inr elements (286). Although ICP4 can efficiently activate transcription with only a TATA box, *in vitro* transcription reactions using the late gene gC promoter show that the presence of an Inr sequence in the promoter greatly enhances transcription (93). The known interactions of ICP4 and the TFIID complex coupled with the observation that the TATA box and Inr sequence are the only cis-acting elements required for efficient activation of gC *in vitro*, underscore ICP4's role as the major transcriptional activator of late genes (93, 140).

As in transactivation, interactions with both DNA and the transcriptional machinery are required for efficient repression (145). A consensus ICP4 binding site located proximally to the

start site of transcription is necessary for efficient repression (145). This presents a distinct difference between ICP4 repression and transactivation given that transactivation appears to require no specific ICP4 DNA binding site. *In vitro* transcription assays show that ICP4 binding to its consensus site specifically represses activated transcription to basal levels. Because basal transcription is achieved, ICP4 does not simply exclude the binding of GTFs, indicating possible protein interactions may also be involved in transcriptional repression (94, 95). Gel electromobility shift assays using the ICP4 promoter show that ICP4 forms a tripartite complex (TPC) with TFIIB and TBP. The presence of all three proteins greatly increases the affinity of ICP4 and TBP for DNA (266). Additionally, ICP4's ability to form this TPC correlates with its ability to repress activated expression from its own promoter (145, 266). Thus, the process of ICP4-mediated repression requires DNA binding to consensus ICP4-binding sequences and interactions with TFIIB and TBP.

The ability to form TPCs and repress transcription has been localized to the N-terminal domain. A truncation mutant lacking the entire carboxy-terminus, n208, still exhibits efficient binding to and repression of transcription from the ICP4 promoter (49, 256). Interestingly, smaller, temperature sensitive mutants in the c-terminus lose the ability to repress transcription but retain both the ability to bind ICP4 consensus sites and activate transcription from E promoters (37). This implies there may be some cooperativity between the N- and C-terminal regulatory domains.

2.0 RATIONALE

HSV gene expression proceeds in a highly regulated cascade that is mediated through interactions between viral and cellular factors. ICP4 is required for the proper expression of all three classes of viral genes, making it the major transcriptional regulator of HSV transcription (47, 55, 217, 294). ICP4's role in both E and L gene expression and the significant difference between E and L promoter structures suggest ICP4 must be able to activate transcription in diverse environments. In addition, HSV has been shown to alter the makeup of the cellular transcription machinery during the course of infection (77, 139, 229, 312). Thus, regulation of global transcription in an infected cell and the subsequent cascade of viral gene expression are orchestrated by the complex interaction between a virally induced, evolving cellular transcription milieu, specifically influenced by ICP4 and other viral proteins.

ICP4's large size and complex structure may facilitate its role at diverse promoters and in changing environments, providing large surfaces for interactions with cellular and viral proteins to orchestrate the gene expression cascade. There are several regions of ICP4 that are conserved amongst other alpha-herpesvirus (30, 174). These regions correspond to functional domains of ICP4 that are necessary for its activity, including the NLS and DNA-binding domains. Of the two broad transcriptional regulatory domains, the C-terminus is the much more highly conserved. This conservation exists despite the fact that the N-terminal domain exhibits both transactivation and repression while the C-terminal domain is involved exclusively in

transactivation. Interestingly, small temperature sensitive mutations in the C-terminus can abrogate ICP4 function, including those functions attributed to the N-terminus (37, 46, 55, 209, 219). This suggests that the C-terminus plays a role in the function of the N-terminus or on global ICP4 folding. The fact that these mutants retain the ability to bind DNA suggests it is the former.

The sole area of partial homology within the N-terminal domain with IE62 is a conserved serine tract. Mutants lacking this region exhibit reduced viral replication in tissue culture and specifically show impaired growth in neurons (12). Deletion mutants that lack the serine tract as well as additional sequences do show reduced transactivation in transient transfection assays using a CAT reporter construct driven by the tk promoter (256). Additional studies fusing ICP4 sequences to the GAL4 DNA-binding domain, thus separating the ICP4 transactivation and DNA-binding, showed that sequences between aa 97-109 may be important for activation (305). Taken together, the transactivation domains of ICP4 appear to not be simple linear stretches of primary sequence but more likely a complex tertiary structure that forms surfaces for interactions with the transcription machinery.

ICP4 interacts with the general transcription machinery to recruit and stabilize the PIC at the promoter. This function is through contacts with TFIID (26, 92). It also forms TPC with TBP and TFIIB on the DNA to exert a suppressive effect on transcription (145, 266). In addition to the cellular transcription machinery, ICP4 has been shown to interact *in vitro* with ICP0 and ICP27 as well as with the cellular L22 in a yeast two-hybrid screen (158, 204, 309).

As discussed above, ICP4 is a large protein with complicated structural domains. It functions to activate a diverse set of promoters in a changing cellular environment. The evolution from IE to E to L gene expression has been extensively studied and is partly due to changing

availability of cellular factors. The changing cellular mileu likely leads to changing interactions of ICP4 and GTF through the course of IE to E to L gene expression. The complexity of the ICP4 functional domains and the extensive role ICP4 plays in the regulation of the cascade of gene expression leads us to hypothesize that ICP4's transcriptional regulatory domains form a complex tertiary structure that acts as a platform to mediate multiple protein-protein contacts with a diverse set of cellular protein complexes. This would allow ICP4 to activate transcription from a diverse set of promoters while employing different cellular protein complexes.

The goals of this study were to

1) **Characterize the N-terminal transcriptional regulatory domains** that are necessary for ICP4 function and

2) **Identify the proteins that interact with ICP4 during infection**.

To characterize the functional domains, mutant viruses that express ICP4 deletion mutants with loss of the N-terminal transcription domain were made. The d3-8 virus expresses an ICP4 molecule that lacks aa 30-142, which include the 90-110 region mentioned above, and the d8-10 mutant lacks aa 143-210. In addition, the regions in the C-terminus of ICP4 have been shown to be important for functions attributed to the N-terminal regulatory domain. With this in mind, the deletion mutants above were made in two forms. The d3-8 and d8-10 which express the deletions in the context of full length ICP4 and nd3-8 and nd8-10 which contain a stop codon at aa 774. These deletion-truncation mutants lack not only the specified deletion but also the C-terminus of ICP4. This allows for the examination of the separate and combined contributions of the N- and C-terminal transactivation domains of ICP4. These comparisons are

accomplished through metabolic protein labeling to assess protein expression and northern blot analysis to investigate accumulation of specific viral mRNA transcripts representative of the three classes of gene expression. Viral growth curves assessed the effect these mutations have on viral growth while DNA-binding studies ensure any effect on regulation is due directly to the regulatoy domains and not to changes in DNA-binding affinity. These viruses express the mutant ICP4 in the context of infection and allow for the study of ICP4 function in its native context.

Tandem affinity purification (TAP) was used to investigate further the protein interactions made by ICP4 during infection. TAP is a method of isolating protein complexes from the cell and has been used successfully to characterize several large protein complexes. Again, it is important to study ICP4 function in the context of infection, thus a virus that expresses an N-terminally TAP-tagged ICP4 will be developed. Mass Spectrometry (MS) and western blot analysis will be used to characterize ICP4-containing protein complexes isolated by TAP. Immunofluorescence will be used to verify colocalization between ICP4 and potential binding partners *in vivo.* Finally, because ICP4 functions at promoters to recruit and stabilize PICs, chromatin immunoprecipitation (ChIP) studies were performed to investigate whether potential interactors are specifically recruited to viral promoters, *in vivo.*

In addition to gaining a better understanding of the molecular biology of a ubiquitous human pathogen, this study will also help to understand the basic process of cellular transcription. HSV provides a unique model system to study how different promoter elements respond to a changing cellular transcription mileu. Studying the role ICP4 plays in activating or repressing the different viral promoters in the context of changing GTFs provides an

understanding of how the activity of cellular transcription factors can be modulated based on cellular conditions.

3.0 ROLE OF THE AMINO-TERMINAL SEQUENCES OF HSV-1 ICP4 IN TRANSCRIPTIONAL ACTIVATION AND REPRESSION

3.1 ABSTRACT

Herpes simplex virus type 1 (HSV-1) infected cell polypeptide 4 (ICP4) is a critical regulator of viral gene expression that is required for productive infection. Depending on the promoter structure, ICP4 has been shown to act as both an activator and repressor of viral genes through interactions with both DNA and cellular general transcription factors including TATA-binding protein (TBP), TFIIB, and TFIID. Although it has been shown that a region in the amino-terminus of ICP4 corresponding to aa 30-210 is necessary for appropriate transactivation and repression of viral genes, specific domains responsible for these activities remain uncharacterized. We hypothesize that the transactivation and repression activities attributed to the amino-terminus of ICP4 are a result of or are regulated by discreet functional domains found in the amino-terminus. In this study, we use a combination of deletion and truncation mutants of HSV-1 ICP4 to isolate the transactivation and repression functional domains of the N-terminus. Using growth curve analysis, ^{35}S-methionine-labeled protein SDS-PAGE, and northern blot analysis we show that while deletion of either aa 30-142 or 142-210 reduces ICP4 function, loss of aa 30-142 is more detrimental. We also show the reduced transactivation efficiency of these mutants is not due to a corresponding reduction in DNA-binding. Furthermore, the

transactivation deficient mutant still retains the ability to form tripartite complexes with TBP and TFIIB on DNA, a function necessary for efficient repression as shown by electromobility shift assays using purified protein. Our results suggest that the transactivation and repression functions of ICP4 are indeed mediated by separate mechanisms and that ICP4's transcriptional regulatory function may not be a discrete linear array of amino acids, or a domain, as with many other transcription factors.

3.2 INTRODUCTION

The immediate early protein of HSV-1, the infected cell polypeptide 4 (ICP4), is the major regulator of viral transcription and is required for productive infection (47, 55, 217, 294). It acts both as a transcriptional activator and repressor, activating E and L genes while repressing its own transcription (48, 67, 84, 86, 197, 198, 224). HSV-1 expressing nonfunctional mutants of ICP4 exhibit an increased expression of IE genes while failing to express E or L, thus ICP4 is critical for the switch from IE to E and subsequently L gene expression (47, 55, 217).

ICP4 is a large, structurally complex molecule that exists in cells as an elongated, 350 kDa homodimeric phosphoprotein (42, 184, 211, 257). ICP4 binds DNA both non-specifically and specifically to the consensus sequence ATCGTCNNNNYCGRC where R = purine, Y = pyrimidine, and N = any base (72, 78). DNA-binding is required for ICP4-mediated transactivation although specific ICP4-binding sites have not been demonstrated (35, 61, 64, 102, 265). Binding to strong sites at or near the start site of transcription associated with repression (94, 95, 145, 207, 208, 232, 256). The significance of this disparity is unclear.

ICP4 regulates transcription through interactions with DNA and a variety of the cellular general transcription factors (GTFs). All HSV-1 genes are controlled by their own promoter and transcribed by the cellular RNA polymerase II (RNA pol II) transcription machinery (8, 41, 290). Regulation of viral gene expression is achieved through cellular and/or viral cis-acting elements found in the promoter structures of each class of genes as well as viral and cellular trans-acting proteins (64, 83, 120, 139, 290). The complexity of the promoter structures and their dependence on trans-acting cellular factors for activation decreases as infection proceeds with a corresponding increase in dependence on viral factors (290).

ICP4 has been shown to stabilize the formation of preinitiation complexes (PICs) on viral promoters *in vitro* via enhancement of TFIID binding to the TATA box and to directly interact *in vitro* with both TBP and TAF1 of the TFIID complex (26, 92). In addition, ICP4 cooperatively forms a stable, tripartite complex (TPC) *in vitro* with TBP and TFIIB on promoters containing the ICP4 binding site (145, 266). The formation of the TPC localizes to regions of ICP4 that are also required for efficient repression of activated transcription suggesting the TPC has a suppressive effect on promoters (94, 145). ICP4 interaction with TBP and TFIIB specifically inhibits activated transcription at a step prior to elongation while allowing basal transcription to proceed implying it does not simply preclude binding of the PIC to the promoter (95). This suggests ICP4 interferes with the action of activators through formation of TPCs to negatively regulate transcription from these promoters. Thus, ICP4 mediates both positive and negative transcriptional regulation through complex interactions with DNA and several members of the cellular transcriptional machinery.

In order to better determine how ICP4 regulates transcription, several studies have been performed to try to isolate and characterize the specific functional domains of ICP4. Several

functional domains have been described including two transcriptional regulatory regions localized in the N- and C-terminal regions separated by a dimerization domain, nuclear localization sequence, and a DNA-binding domain that contains a helix-turn-helix motif (49, 50, 81, 207, 208, 256). These functional domains correlate strongly with regions of significant amino acid similarity between ICP4 and its orthologs from other alpha-herpesvirus family members (174). Interestingly, truncation mutants that lack the C-terminal transactivation domain retain autoregulation as well as transactivation of viral genes, albeit to a reduced level (26, 49, 50). These mutants are lacking, however, efficient L gene expression and thus show significant growth deficiency (50).

The broad transcriptional regulatory domain found between aa 30-210 of the N-terminus of ICP4 exhibits both transactivation and autoregulation function. In order to more fully characterize these activities, viruses that express deletion and truncation mutants of the ICP4 N-terminal transcriptional regulatory domain were developed. This study examines the contributions of the N-terminal regulatory domain in the presence and absence of the C-terminal region of ICP4. It utilizes viruses that express mutant ICP4 in the context of infection and more directly studies gene expression via *in vivo* and *in vitro* methods at both the protein and mRNA levels. In addition, the DNA-binding and tripartite complex formation of the mutant ICP4 molecules were also studied.

3.3 MATERIALS AND METHODS

3.3.1 Cells and viruses

Vero and E5 cell lines were maintained by standard procedures. E5 cells were derived from vero cells and express complementing levels of ICP4 (47, 49). The HSV-1 wildtype strain, KOS, was propagated in vero cells while the ICP4 mutant viruses n12, n208 (50), nd3-8, d3-8, nd8-10, and d8-10 (50, 256, 266) were propagated on E5 cells. Viral growth curves were performed on vero cells for all viruses. 5 x 10^5 cells were infected on ice with KOS or mutant HSV-1 at an MOI of 5. Virus was allowed to adsorb for 1 hour on ice, then cells were washed twice with cold Tris-buffered saline (TBS) containing 0.25 mM Tricine, 137 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, and 0.68 mM $CaCl_2$, pH 7.35. The infection was allowed to proceed at 37^{o}C for 2, 4, 8, 12, 24, or 36 hours as indicated. Cells were then scraped into the media, freeze-thawed twice, and sonicated. Virus was titered on E5 cells via plaque assay.

3.3.2 ^{35}S-methionine peptide labeling

Confluent monolayers of 5 x 10^5 Vero cells were infected in the presence or absence of 400 ug/ml phosphonoacetic acid (PAA; Lancaster Synthesis, Inc) with either KOS or mutant HSV-1 at an MOI of 10. The infection was allowed to proceed in DMEM media either with or without 400 ug/ml PAA for 6 or 12 hours at 37^{o}C. After 5 or 11 hours, the cells were washed once with warm TBS, then incubated with 1 ml warm TBS containing 50 uCi ^{35}S-methionine for 1 hour at 37^{o}C. Cells were then washed with cold TBS containing 0.1 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and scraped into 0.2 ml 2x Laemmlli Sample buffer (BioRad).

Samples were boiled for five minutes, and the proteins were separated via SDS-PAGE. The gel was dried and exposed to Hyperfilm (Amersham) to visualize the radiolabeled proteins.

3.3.3 Southern blot analysis

2 x 10^5 E5 cells were infected with virus and allowed to grow at 37^{o}C until extensive CPE was observed. Cells were then freeze-thawed twice and then centrifuged at 12,500 rpm for 15 min. Pellets were washed once in cold TBS and repelleted as above. Pellets were resuspended in TE containing 0.6% SDS and 200 ug proteinase K (Roche) and incubated overnight at 37^{o}C. DNA was extracted twice in a phenol:chlorofom:isoamyl alcohol (25:24:1) mixture, followed by 1 volume chloroform:isoamyl alcohol (24:1). DNA was then precipitated in ethanol, pelleted, and washed with 70% ethanol. Pellets were dried and resuspended in TE. Samples were then double digested with *Bam*H1 and *Pst*I overnight at 37^{o}C, loaded onto a 1.2% TBE-agarose gel, and separated overnight at 70 volts. The samples were analyzed via Southern blot as described previously (244). The radiolabeled probe was made with the Nick Translation system (Invitrogen) following manufacturer's protocol using 0.5 ug of the template pKBY containing the *Bam*H1-Y fragment that contains part of the HSV-1 ICP4 gene and 5 μl each of [α-^{32}P]dCTP and [α-^{32}P]dGTP. The unincorporated nucleotides were removed using Centricep columns (Princeton Separations) as per the manufacturers protocol. The membrane was then exposed to Hyperfilm.

3.3.4 Northern blot analysis

$5x10^6$ vero cells were infected with wildtype or mutant HSV-1 at MOI of 10 in the presence or absence of 400 μg/ml PAA. 6 or 12 hours post-infection, total RNA was harvested using Ultraspec RNA Isolation System (Biotecx Laboratories) following manufacturer's protocol. RNA was quantified using Ribogreen RNA Quantitation (Invitrogen) per manufacturers specifications and a Storm 840 (Molecular Dynamics) using the Scanner Control version 4.1 and ImageQuant version 1.2 software (Molecular Dynamics). 20 ug of each RNA sample was loaded onto a 1.3% formaldehyde-agarose gel and separated at 50 volts overnight and assayed via Northern blot as described previously (126). The radiolabeled probe was made with the Nick Translation system (Invitrogen) following manufacturer's protocol using 0.5 ug template pKBY BamH1-Y fragment (ICP4) (245), pw3ΔHS8 (ICP0) (49), pLSWT (tk) (127), and pSXgC (gC) (140) and 5 ul each of [α-^{32}P]dCTP and [α-^{32}P]dGTP. The unincorporated nucleotides were removed using Centricep columns (Princeton Separations) as per the manufacturers protocol. The membrane was then exposed to Hyperfilm.

3.3.5 DNA-binding studies

The ability of the ICP4 mutants to bind DNA was assayed via electromobility shift assay using whole cell protein extracts. 1 x 10^6 vero cells were infected with the indicated virus at an MOI of 10. Six hours post-infection, the cells were washed twice with cold TBS containing 0.1 mM TLCK, then scraped into 1 ml TBS with 0.1 mM TLCK and centrifuged at 12,500 rpm for 1 minute. The cell pellet was resuspended in 50 μl lysis buffer containing 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 2% NP-40, and 0.1 mM TLCK and incubated on ice for 30 minutes. The

lysate was clarified via centrifugation at 12,500 rpm for 15 minutes. The supernatant was then used in electromobility shift assays as described previously (128). The probe used was a γ^{32}P-ATP end-labeled *Eco*RI-*Bam*HI fragment from the P4 plasmid. This fragment spans sequences from -108 to +27 of the ICP4 promoter relative to the transcription initiation site and includes the TATA box and the ICP4 binding site (145).

The ability of the ICP4 to form tripartite complexes with TBP and TFIIB was assayed via electromoblity shift assays with purified ICP4, TBP, and TFIIB. Wildtype, n208, nd3-8, and d3-8 ICP4 was purified from infected vero cells as described previously (128). Recombinant TBP and TFIIB was purified as previously described (99, 135, 266). 15 ng ICP4, 60 ng TBP, and 500 ng TFIIB were incubated with the labeled probe detailed above, as described previously (266). The binding reactions were then resolved in a native, 4% polyacrylamide gel at 200 V for 2 hours. The gel was dried and exposed to Hyperfilm.

3.3.6 Western blot analysis

5 x 10^5 vero cells were infected with the indicated virus at an MOI of 10. The infection was allowed to proceed at 37^{o}C for 6 hours. The cells were washed in cold TBS containing 0.1 mM TLCK and then scraped into 0.2 ml 2x Laemmlli Sample buffer. Samples were boiled for five minutes, separated via SDS-PAGE and analyzed via Western blot as previously described (255). The primary antibody used was a 1:5000 dilution of N15, a rabbit polyclonal specific to the N-terminus of ICP4 as previously characterized (255) in 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween-20 (TBS-T) and 1% milk. The secondary antibody used was a 1:5000 dilution of an anti-rabbit polyclonal IgG-HRP conjugate (Promega) in TBS-T. Bound antibody was

visualized using the ECL Plus Western Blotting Detection System (Amersham) per manufacturer's protocol.

3.4 RESULTS

ICP4 is a critical regulator of HSV-1 gene expression, acting to both repress and activate viral genes. Despite expansive study, the mechanism of this regulation and the functional domains responsible are not fully understood. Studies of the ICP4 molecule have elucidated two broad transcriptional regulatory domains: a large C-terminal domain that is involved in transactivation and a second N-terminal domain that both activates and represses transcription (figure 5)(50, 256). The mutants used in this study were chosen to dissect the N-terminal regulatory domain with and without the C-terminal domain while leaving the other domains necessary for ICP4's function intact. In addition to the transcriptional regulatory domains between aa 30-210 and the C-terminus, ICP4 contains a DNA-binding domain between aa 263-487 and nuclear localization sequence at aa 723-732 (figure 5A)(208, 256). Two broad deletions that span the N-terminal regulatory domain were made, the 3-8 and 8-10 deletions (figure 5B). The 3-8 region spans amino acids 30-142 while the 8-10 spans 143-210. These deletions were made in the context of either full length (d3-8, d8-10) or a C-terminal truncation at amino acid 774 (nd3-8, nd8-10). The mutant virus n12 expresses a mutant of ICP4 truncated at aa 251 which lacks the DNA-binding domain making the peptide a non-functional negative control. KOS serves as the wildtype form of ICP4, exhibiting full regulatory function, and n208, a virus that expresses the C-terminal truncation at aa 774 acts as a positive control for the regulatory function of the N-terminal domain independent of the C-terminus. We studied the global viral gene expression

profile of these mutant viruses at the protein level as well as at the mRNA level of viral genes representative of IE, E, and L genes. Because DNA-binding is required for ICP4's regulatory function, the DNA-binding ability of the mutant ICP4 molecules was assessed as well as the ability to form tripartite complexes with TBP and TFIIB on DNA.

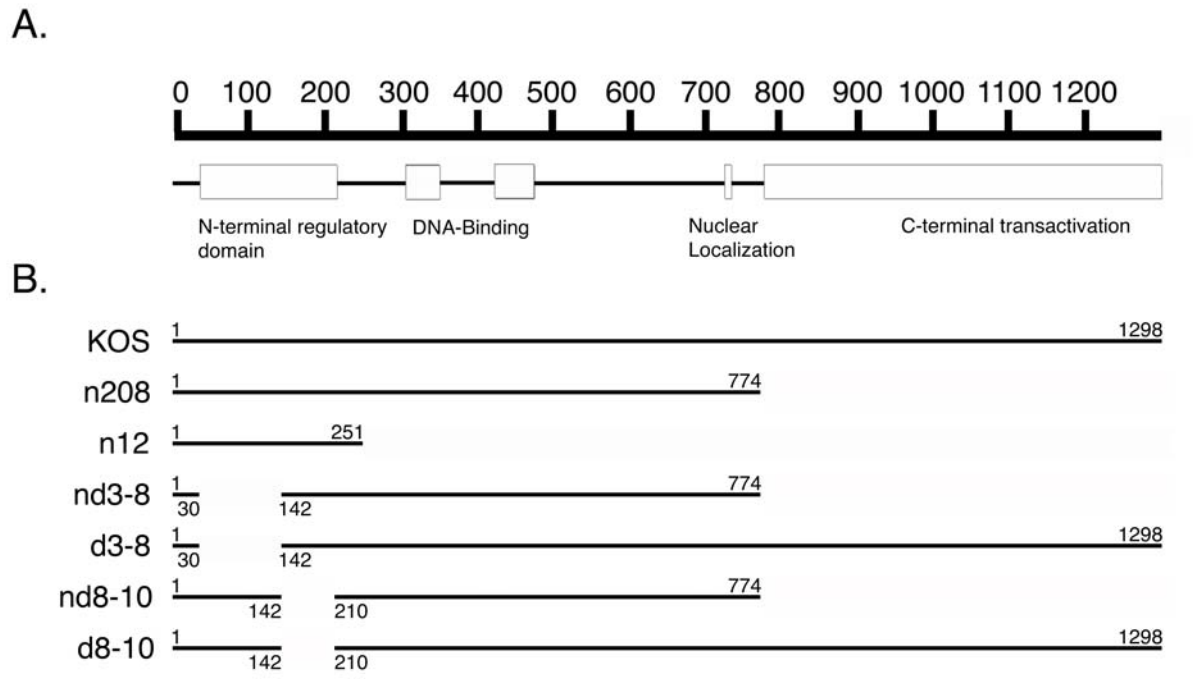

Figure 5: Structural schematic of the deletion and truncation mutants used in this study.
A) The primary sequence and various functional domains of ICP4 are shown. B) The expressed products of the deletion and nonsense mutants used in this study are shown. KOS and n12 were used as a positive control and negative control for ICP4 function. The n208 mutant was used to study the transcriptional regulatory activity of the N-terminus independently of the contributions of the C-terminus.

3.4.1 Characterization of mutant ICP4

The construction of the mutants used in this study has been described previously (49, 50, 256, 266). The approach taken to make the deletion mutants added a unique *Pst*I restriction site at the

site of the deletion (figure 6A). The KOS, n12, and n208 viruses do not contain deletions and thus contain the full-length 1.84 kb *Bam*HI Y fragment. The nd3-8 and d3-8 viruses share a common deletion of aa 30-142 resulting in a shortened 1.5 kb *Bam*HI Y fragment that contains a *Pst*I restriction site dividing it into two fragments of approximately 0.37 and 1.13 kb. The nd8-10 and d8-10 share a common deletion of aa 142-210 resulting in a 1.64 kb *Bam*HI Y fragment with a *Pst*I restriction site**,** resulting in two fragments of 0.70 and 0.94 kb. The presence and position of the novel *Pst*I site was confirmed via Southern blot (figure 6B). Total DNA was harvested from infected vero cells and digested with *Bam*H1 to isolate the *Bam*HI Y fragment as well as *Pst*I to test for the deletion. The digested DNA was separated on an agarose gel and visualized via Southern blot using a probe specific for the *Bam*HI Y fragment. All of the viruses exhibited the expected fragment sizes indicating the appropriate genetic structure of the respective ICP4 genes (figure 6B). In order to ensure proper expression and size of the ICP4 molecules produced, whole cell lysates of infected cells were prepared and submitted to western blotting. 5×10^5 vero cells were infected with the indicated virus at an MOI of 10. 6 hpi, the cells were scraped into 2x Laemmli sample buffer and analyzed via western blot using the n15 polyclonal antibody specific to the N-terminal 774 amino acids of ICP4. The KOS virus expresses full length ICP4 of 175 kDa, while the truncation mutants, n12 and n208, express ICP4 peptides of 41 and 96 kDa, respectively (figure 6C). The d3-8 virus has a deletion between aa 30-142 resulting in a predicted mass of approximately 160 kDa while nd3-8 has the same deletion but with the additional c-terminal truncation making an 81 kDa peptide. The d8-10 and nd8-10 share a deletion of aa 142-210 in the context of full length ICP4 or the c-terminal truncation resulting in a predicted mass of 160 kDa and 85 kDa, respectively. All the viruses

expressed a single band of the expected molecular weight indicating the proper expression and homologous nature of the mutants (figure 6C).

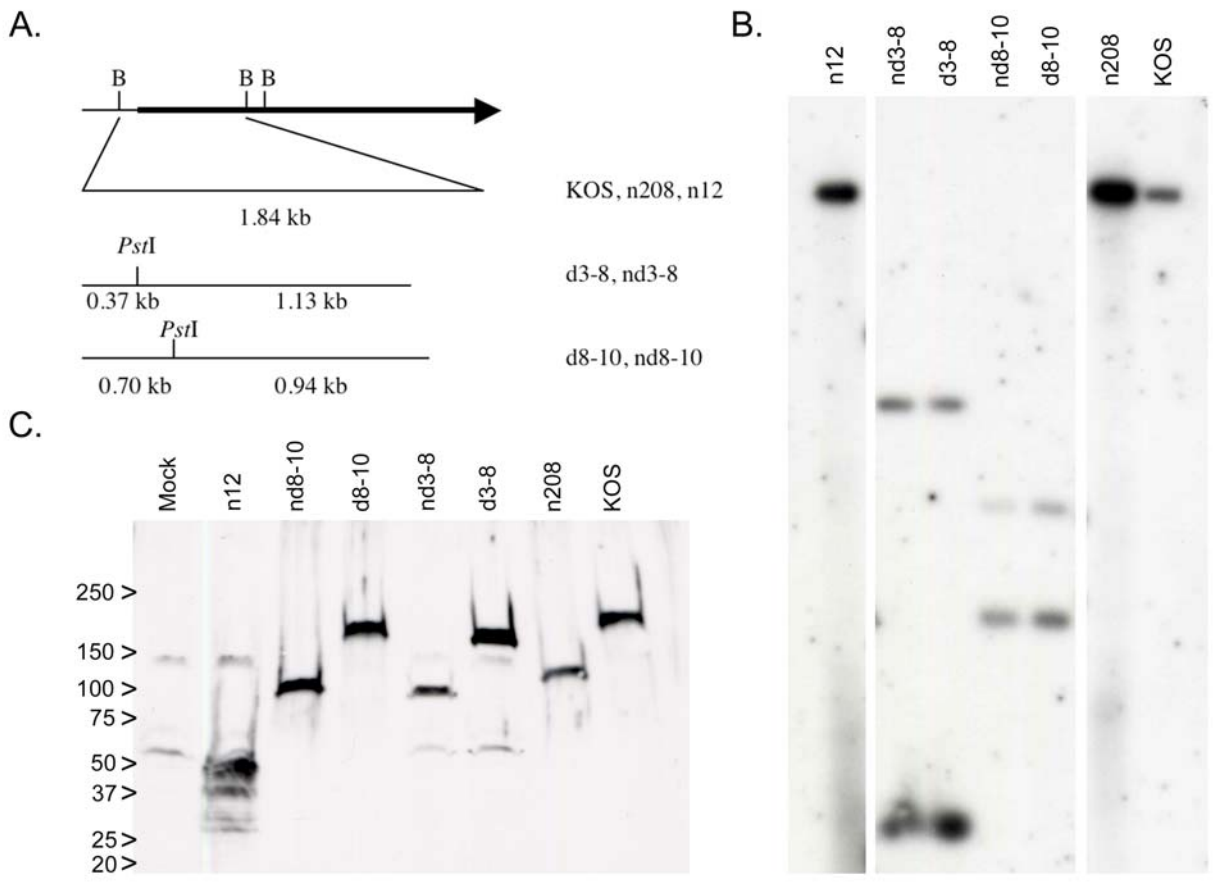

Figure 6: Characterization of the deletion and nonsense mutants used in this study.
A) The ICP4 ORF is described (bold arrow) in the context of the surrounding HSV-1 DNA. The BamHI Y fragment is shown in more detail, indicating the inserted Pst I site in the mutant viruses and the expected size of the BamHI-PstI digestion fragments. B) Viral genomic DNA was harvested from vero cells infected with either wildtype or mutant virus and digested with BamHI and PstI. The resulting fragments separated via electrophoresis and detected via Southern blot with a radiolabeled probe specific for the ICP4 orf. C) The expression and relative size of the ICP4 proteins expressed by the mutant viruses was analyzed via western blot. Vero cells were infected with the indicated virus at an MOI of 10. Total protein was harvested 6 hours post-infection and subjected to western blot analysis using a polyclonal antibody specific to the N-terminus of ICP4.

Because ICP4 is required for viral growth, mutant viruses that express functionally impaired ICP4 have distinct growth phenotypes. Viral growth curves were performed to assess the ability of the mutant viruses to replicate (figure 7). Vero cells were infected on ice with the

indicated virus at an MOI of 5. Virus was harvested 2, 4, 8, 12, 24, and 36 hours post-infection and titered on E5 cells. The wildtype virus, KOS, grew most efficiently with a virus yield at 24 hpi of 5.6×10^8 whereas the n12 did not grow above the levels of the initial inoculum and had a 24 hour yield approximately 5 orders of magnitude lower than wildtype. The d8-10 and d3-8 viruses showed both delayed kinetics and a reduction in growth with the d8-10 virus reduced by about a factor of 10 at 24 hours and d3-8 slightly more impaired at 26 times less than wildtype. The loss of the C-terminus in the n208 virus resulted in delayed kinetics and severely impaired growth with only a slight increase in virus yield over that of inoculum at 24 hpi. The mutant viruses nd8-10 and nd3-8 showed a dramatic reduction in growth similar to that of the nonfunctional n12, failing to reach the titer levels of the inoculum. The nd3-8 mutant was more impaired, however compared to nd8-10. The 3-8 deletion consistently shows a slightly greater reduction in viral growth than the 8-10 deletion, either in the context of full-length or lacking the C-terminus, suggesting the 3-8 mutation plays a more important role in viral growth. The 8-10 mutants are also impaired for growth, however, indicating that the entire N-terminal regulatory domain is needed for viral replication. In addition, mutants that lack both intact N- and C-terminal regulatory domains are the most impaired for growth, suggesting the presence of one of the domains can somewhat compensate for the loss of the other.

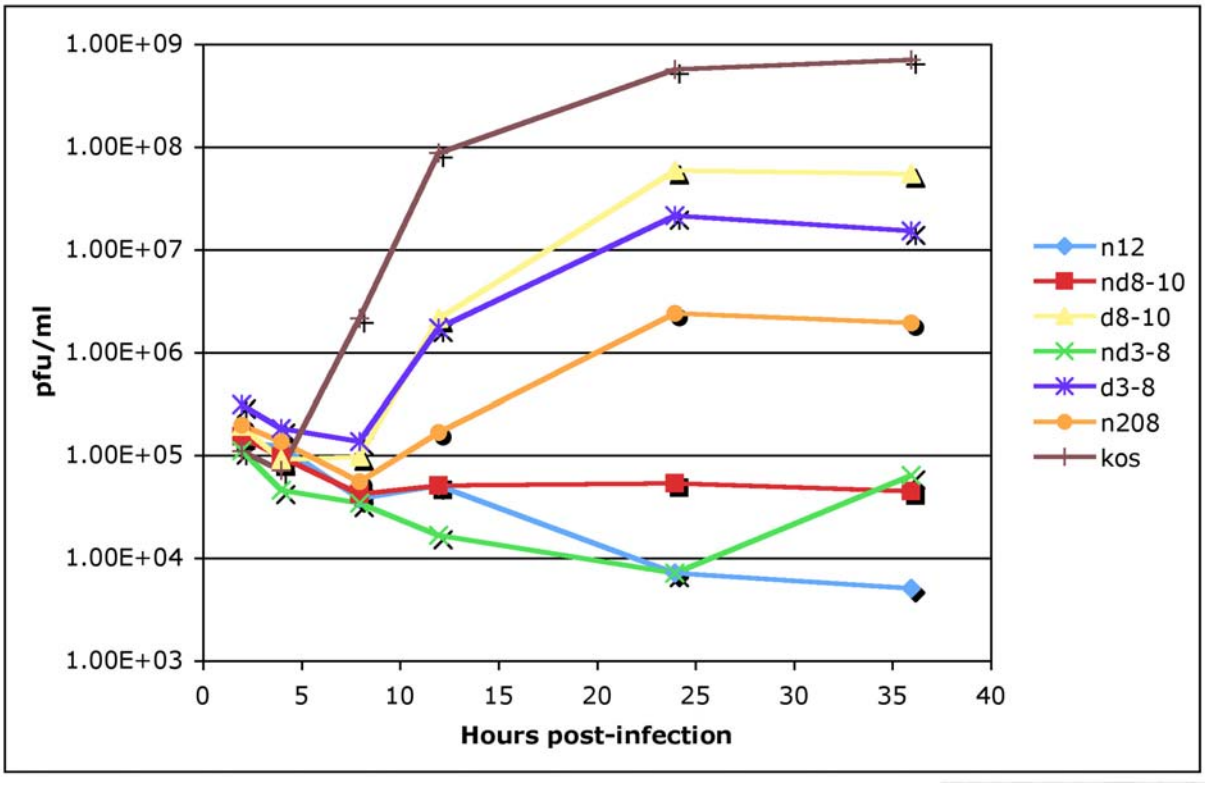

Figure 7: Effect of ICP4 mutations on viral growth.
Vero cells were infected at an MOI of 5 with either the TAP virus or kos. Virus was harvested 2, 4, 8, 12, 24, and 36 hours pi. Viral titers were determined via plaque assay on E5 cells.

3.4.2 Functional analysis of mutant ICP4

In order to first study the effects the mutations had on ICP4's ability to regulate gene expression, the protein expression profiles of the mutant viruses were examined. Vero cells were infected at an MOI of 10 with the indicated virus either in the presence or absence of PAA. The cells were treated with ^{35}S-methionine from 5-6 or 11-12 hpi to label newly synthesized peptides. Whole cell lysates were prepared and separated via SDS-PAGE and labeled protein was visualized via autoradiography. Three general protein expression profiles were seen based on wildtype KOS, the nonfunctional mutant n12, and the C-terminal truncation, n208. At 6 hpi in the absence of

PAA, KOS expresses representative proteins from all classes of genes (figure 8, lane 16). In the presence of PAA, however, KOS expresses increased levels of the early genes ICP6 and ICP8, reduced levels of the leaky late genes ICP5 and ICP25 and no true late genes due to the lack of DNA replication (figure 8, lane 8). At 12hpi, KOS expresses low levels of E proteins and an abundant amount of both the leaky late (ICP25, ICP5, and gB) and true late proteins (ICP1/2, ICP19, ICP20, and ICP48, figure 8, lane 24). Because n12 expresses a non-functional nonsense mutation of ICP4, it only expresses abundant IE without expressing early or late proteins. Thus, its expression profile is the same at either 6 or 12 hpi in the presence or absence of PAA. Significant levels of ICP0, ICP27, and ICP6, an early protein that is responsive to ICP0 and is expressed in the absence of ICP4 (47, 50, 242), can be seen, however (figure 8, compare lanes 2, 10, and 18). The n208 virus shows significant changes in its protein expression profile. The virus does not replicate its DNA to significant levels and thus does not express true late genes. As a consequence, E proteins accumulate at 6 hpi (figure 8, lanes 7 and 15). By 12 hpi, some leaky late, but not true late, proteins can be seen (figure 8, lane 23).

The deletion and truncation mutants all fit into the three general profiles (KOS-like, n12-like, and n208-like) with some small differences. Despite a reduction in replication efficiency, the d8-10 virus exhibited a KOS-like profile in all conditions with only a few exceptions. In the presence of PAA at 6 hpi, d8-10 did express gB and ICP25, but to lower levels compared to wildtype (figure 8, compare lanes 4 and 8). At 6 hpi in the absence of PAA, d8-10 expressed similar levels of the representative E proteins ICP6 and ICP8 as well as leaky-late proteins ICP5 and ICP25. It expressed similar levels of some true late proteins (L) as wildtype while expressing lower levels of the true late protein ICP19 and ICP20 and not expressing the true late proteins ICP1/2 and ICP48 (figure 8, compare lanes 12 and 16). By 12 hours, however,

expression of the leaky-late and true late proteins began approaching the levels of KOS while ICP6 expression of d8-10 was significantly increased compared to wildtype (figure 8, lanes 20 and 24) and was more similar to n12 (figure 8, lanes 20 and 18). Thus, the 8-10 deletion results in a partial delayed-expression phenotype with some additional, gene-specific defects in regulation.

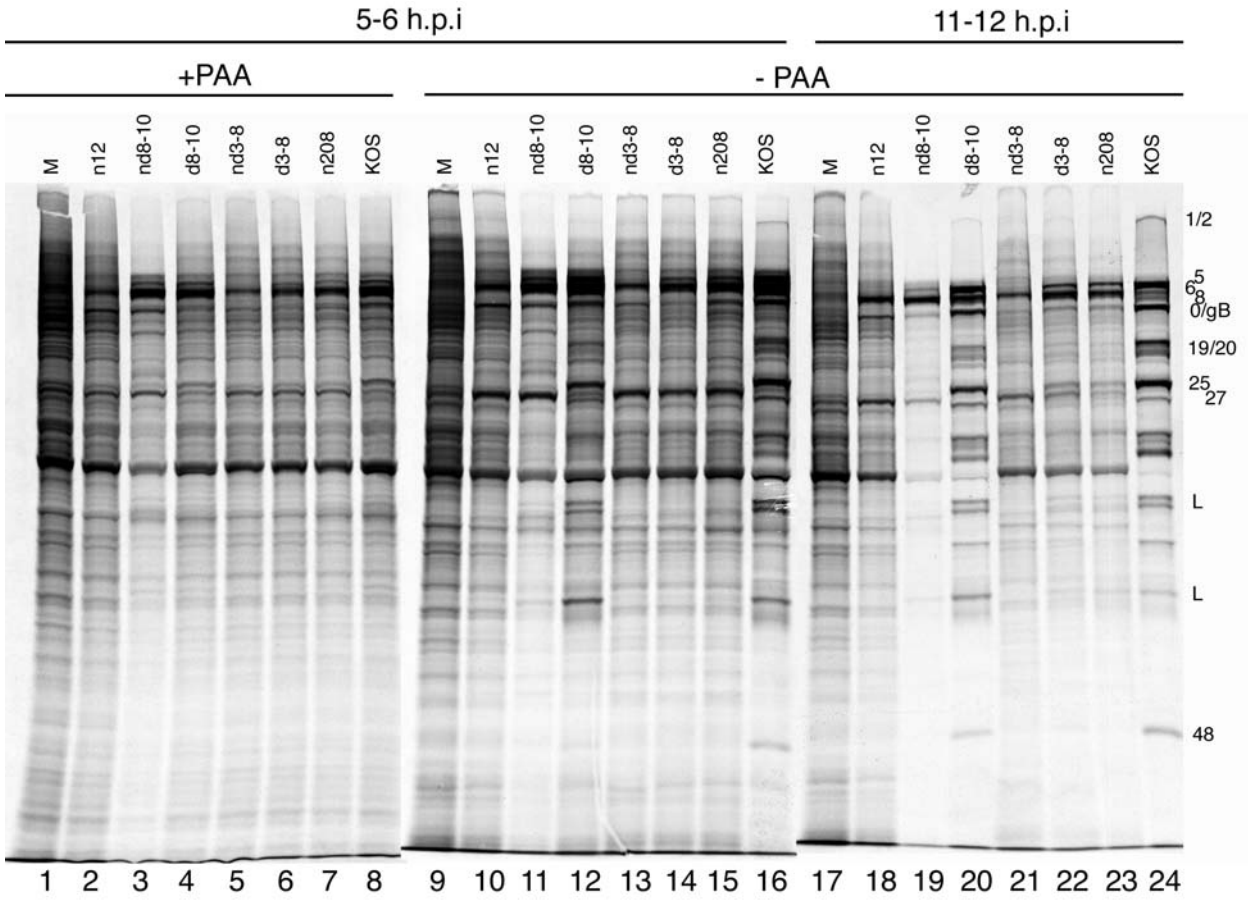

Figure 8: Protein expression profile of cells infected with the ICP4 deletion and nonsense mutants.
Vero cells were infected with wildtype or mutant ICP4 viruses at an MOI of 10. Either 6 or 12 hours post-infection (as indicated), the cells were treated with ^{35}S-methionine for one hour to radiolabel newly translated proteins. Total protein from cells infected with the indicated virus was harvested and resolved via SDS-PAGE. The ICP designations of several viral proteins are shown.

Members of the n208-like class include the nd8-10 and d3-8 viruses. At 12 hpi, nd8-10 accumulates lower levels of the leaky-late proteins ICP5 and ICP25 compared to n208 (figure 8, lanes 19 and 23). In contrast to the KOS-like d8-10, d3-8 shows a very similar phenotype to

n208 at all times and conditions studied (figure 8, lanes 6 and 7, 14 and 15, 22 and 23). Only the nd3-8 virus belongs to the n12-like class of protein profiles. nd3-8 seems to be slightly more deficient in IE protein expression than n12, however, showing a reduced level of ICP27 and the ICP0-activated ICP6. This study investigated the regulatory function of the mutant ICP4 indirectly through protein levels. Although changes in transcription rates do generally correlate directly with changes in protein levels, many other factors are involved in determining protein levels in the cell.

In order to more directly assess the transcriptional efficiency of the mutant ICP4, relative mRNA abundance of prototypic IE gene (for repression) and E and L genes (activation) was assessed via northern blot analysis. 5 million vero cells were infected in the presence or absence of PAA at an MOI of 10 with the indicated virus. 6 or 12 hpi, the cells were harvested and total RNA isolated and quantified. 20 ng of mRNA from each sample was loaded onto a formaldehyde-agarose gel and separated over-night. The mRNA was blotted onto a membrane and hybridized to probes specific for ICP4 (IE gene), tk (E gene), or gC (true late). Because the mRNA being probed for is identical in the case of tk and gC and only minimally changed in the case of ICP4, the mRNA processing and stability elements will be largely the same, thus making the rate of transcription the determining factor driving changes in message abundance. Comparing relative mRNA abundance between ICP4 mutants will provide insight into the effect of the mutations on regulatory function.

The ICP4 promoter contains a strong ICP4-binding site near the transcriptional initiation site and thus is strongly repressed by functional ICP4 (48, 71, 143, 191, 198, 232). As a result, KOS exhibits strong repression as shown by a low level of ICP4 mRNA in both PAA treated and untreated samples whereas the nonfunctional n12 exhibits an over-abundance of ICP4 mRNA

(figure 9A, top row, compare n12 and KOS). Because the truncation mutants are a result of an insertion of a stop codon into the coding sequence and not a deletion, the size of the respective mutant mRNAs are not significantly changed nor is their mobility on a gel. The ICP4 deletion mutants were constructed by actual deletion of the coding sequence, however, and as a result, run at a slightly higher mobility relative to wildtype and the truncation only mutants. Compared to the non-functional n12, none of the other mutations had a significant effect on ICP4's ability to repress its own transcription (figure 9A). In both the PAA treated and untreated samples, the d3-8 and n208 mutants show a slight increase in total ICP4 mRNA relative to wildtype (figure 9A, compare d3-8, n208, and KOS); however, these viruses clearly retain potent repressive function when compared to n12 (figure 9A, compare n12, d3-8, n208).

Thymidine kinase was chosen as a prototypic E gene to analyze ICP4-mediated transactivation of E genes. At 6 hpi, KOS accumulated an abundant amount of tk mRNA in both PAA treated and untreated samples (figure 9A), but by 12 hpi, less tk mRNA was present (figure 9B) indicative of the switch from E to L gene expression. In contrast, n12 accumulates undetectable or near undetectable levels in all times and conditions tested. The n208 virus, which lacks the C-terminal transactivation domain, retained partial transactivation, at 6 hpi accumulating slightly lower levels of tk compared to KOS but significantly higher than n12 (figure 9A, tk row). By 12 hours, however, n208 exhibited similar levels of tk as KOS (figure 9B, tk row). The d8-10 mutant accumulated similar amounts of tk mRNA compared to KOS at 6 hpi with a slight decrease in relative level in the PAA treated samples. This is in contrast to the d3-8 mutant, which showed a significant reduction, but not complete loss, of tk accumulation (figure 9A). The d3-8 level was comparable to the n208 mutant, which lacks the C–terminal transcriptional regulatory domain. At 12 hpi, the d8-10 virus showed an increased level of tk

mRNA compared to wildtype, possibly a result of the partial-delayed kinetics shown in the growth curve and protein expression profile for this virus. The d3-8 virus accumulated similar levels of tk message as both n208 and KOS (figure 9B). At 6 hpi, the nd8-10 mutant in the PAA (-) sample accumulated lower levels of tk mRNA compared to n208 and a significant reduction compared to KOS. This reduction was somewhat abrogated by PAA treatment. The nd3-8 mutant, however, was comparable to n12, exhibiting nearly undetectable levels of tk in both PAA treated and untreated samples (figure 9A). By 12 hpi, nd8-10 accumulated lower levels of tk than either n208 or KOS but higher levels than n12. nd3-8 again expressed levels similar to n12 (figure 9B).

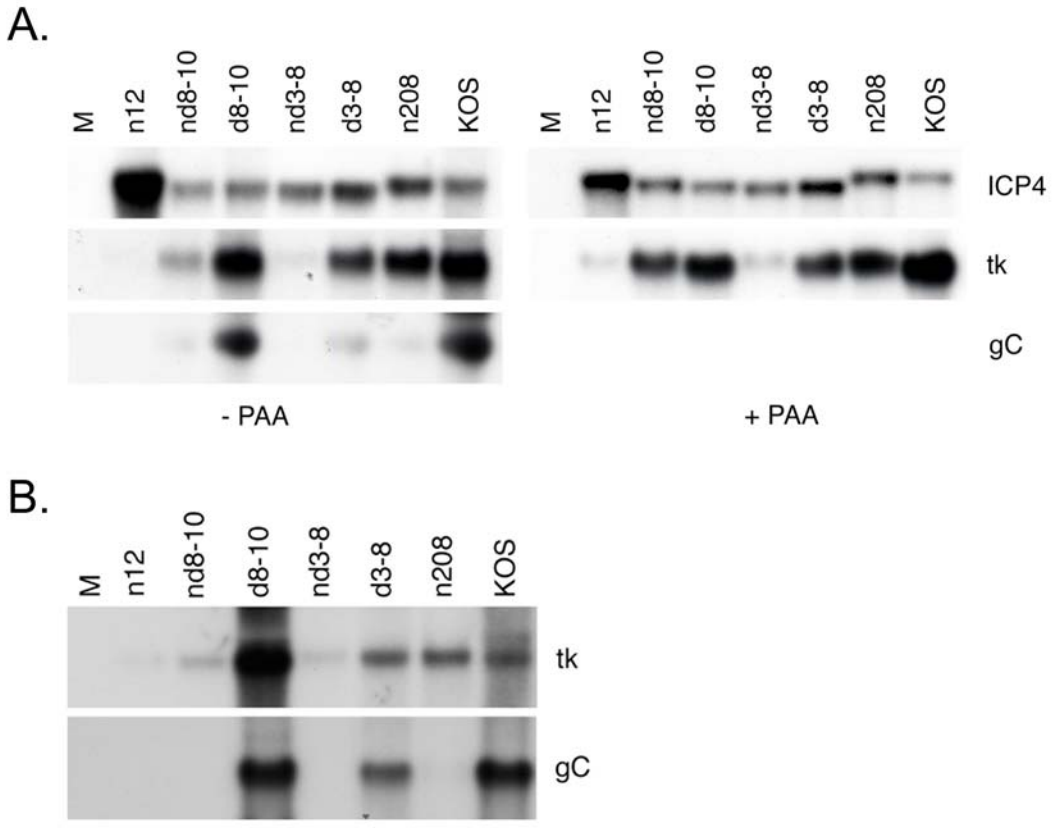

Figure 9: Accumulation of ICP4, tk, and gC transcripts in cells infected with deletion and nonsense mutants. A) Vero cells either in the presence or absence of PAA, as shown, were infected at an MOI of 10 with the indicated virus. Total RNA was harvested 6 (A) or 12 (B) hours post-infection and relative ICP4, tk, and gC mRNA abundance was determined via northern blot analysis. ICP4 normally represses the ICP4 gene and transactivates the tk and gC genes. Efficient late gene transactivation requires the C-terminus of ICP4, thus all of the truncation mutants exhibit reduced gC expression.

The expression of gC, a prototypic true late gene, is somewhat complicated by its dependence on viral DNA replication and thus early gene expression. An inability to express gC could be a result of either an indirect reduction in early gene expression or an inability to express gC directly. At 6 hpi, KOS accumulates abundant gC mRNA whereas no signal is detected for n12. The n208 virus exhibits a nearly undetectable level of gC message, despite robust early gene expression (figure 9A). This virus, however, does not efficiently replicate its DNA (50). By 12 hpi, gC message remains abundant in KOS infected cells, while n12 and n208 show undetectable levels (figure 9B). At 6 hpi, d8-10 exhibited similar levels of gC as KOS, while d3-8 accumulated a significantly reduced, yet still detectable, amount (figure 9A). This trend continued at 12 hpi with d8-10 comparable to KOS and d3-8 exhibiting an intermediate phenotype: reduced compared to KOS, but increased compared to n12 and n208 (figure 9B). The combination deletion and truncation mutants, nd8-10 and nd3-8, were strongly impaired in gC accumulation at both 6 and 12 hpi as they share the same truncation as n208 (figures 9A and B). However, at 6 hpi, nd3-8 exhibited undetectable levels of gC mRNA whereas n208 and nd8-10 both accumulated significantly reduced, yet still detectable, levels (figure 9A). In summary, the 8-10 deletion mutants retain significant transactivation function. The transactivation seen may be in part a result of the C-terminal transactivation domain compensating for the loss of the 8-10 region, considering the nd8-10 mutant exhibits less accumulation of tk mRNA compared to n208. This is in contrast to the 3-8 deletion; mutants lacking this region exhibit significantly reduced accumulation of both tk and gC mRNA. The nd3-8 mutant, which lacks the c-terminal transactivation domain in addition to the 3-8 region, accumulates undetectable, or nearly undetectable levels, of the tk and gC transcripts.

3.4.3 Deletion mutants do not affect ICP4 DNA-binding

ICP4's ability to bind DNA has been shown to be necessary for its ability to regulate transcription (50, 208, 256). In order to ensure functional changes seen in the mutants are a direct result of reduced regulatory function and not deficiencies in DNA-binding, electromobility shift assays were performed. Vero cells were infected at an MOI of 10 with the indicated virus. Six hpi, cells were harvested and solubilized using 2% NP-40 to generate a whole-cell extract. This extract was incubated with a γ^{32}P-ATP end-labeled probe consisting of the -108 to +27 of the ICP4 promoter, spanning the TATA box, transcription initiation site, and the ICP4 binding site. The bound probe was separated on a native 4% PAGE gel and visualized via autoradiography. The mock-infected cell extract had several gel-shifted bands indicative of cellular proteins binding to the ICP4 promoter (figure 10, M). All of the mutants studied, except n12 which expresses an ICP4 truncation that lacks the DNA-binding domain, retained the ability to bind DNA as shown by the gel-shifted band that is not found in the mock lane (figure 10). In addition, these bands correlate with the relative size of the deletion/truncation mutants of ICP4, indicating the shifted band is due specifically to ICP4 binding to the probe. The n208 truncation mutation increases the solubility of ICP4; thus, the mutants that contain this truncation, n208, nd8-10, and nd3-8, all bind more probe compared to their non-truncated counterparts, KOS, d8-10, and d3-8, respectively. The mutant that bound the least amount of probe, d8-10, retained near wildtype regulatory function (figures 8 and 9); thus, all the mutants studied (excluding n12) retained sufficient DNA-binding efficiency to preserve full regulatory function. Any deficiency in transactivation or repression would therefore be due not to reduced DNA-binding but to a direct inability to regulate gene expression.

Figure 10: DNA binding efficiency of the deletion and nonsense mutants.
Vero cells were infected at an MOI of 10 either with KOS or an ICP4 mutant virus as indicated. Total protein was harvested 6 hours post-infection in the presence of TLCK. The extracts were incubated with a radiolabeled ICP4 promoter probe and a nonspecific competitor for 30 min at room temperature and loaded onto a native polyacrylamide gel. The first lane consists of the radiolabeled probe with no protein extract. The rest of the lanes were incubated with extracts from cells infected with the indicated virus.

ICP4 has been shown to form a tripartite complex with TBP and TFIIB on promoters that contain consensus ICP4 DNA-binding sites (266). While the formation of TPCs has been associated with repression of transcription (11), it has also been shown that ICP4 interacts with TBP through TFIID in order to transactivate (26, 92). It is unclear whether ICP4 makes multiple different contacts with the GTFs from within different regions of the peptide in order to activate

and repress or whether the same interacting domain is involved in both functions. Because mutants containing the 3-8 mutation were significantly impaired in transactivation while retaining high levels of repression, they were tested for the ability to form TPCs. Purified ICP4 and TFIIB were incubated either in the presence or absence of recombinant TBP with the γ^{32}P-ATP end-labeled probe described above. The binding reactions were then resolved via native gel electrophoresis on a 4% acrylamide gel at 200 volts. Three distinct protein:DNA complexes were seen: TBP:TFIIB complex (DB) binding,ICP4 binding alone, and the TPC of ICP4:DB. The highest mobility complex, DB, was formed in all reactions TBP and TFIIB were co-incubated (figure 11, *). ICP4-binding could be seen both when incubated with TFIIB either in the presence or absence of TBP (figure 11, **). The relative sizes of the mutant ICP4 molecules are reflected in the mobility of the ICP4:DNA complex. The d3-8 ICP4 complex is not present in the TBP (-) reaction but is present in the TBP treated sample. The 3-8 deletion does not, however, significantly effect DNA-binding, because a clear ICP4:DNA complex is seen in the TBP (-) sample, in the nd3-8 samples, and in the d3-8 whole cell extract sample (figure 10). The largest complex is representative of the TPC and consists of ICP4, TBP, and TFIIB binding to the probe. Both nd3-8 and d3-8 as well as n208 and KOS efficiently form the TPC on DNA (figure 11, ***). As previously shown, the formation of the TPC is cooperative as seen by the marked increase in complex occupancy on the probe compared to both ICP4 and the DB complex alone. In the KOS sample, additional, larger complexes are seen that are a result of ICP4 oligomerizing on the probe, a function of the C-terminus of ICP4 (146). Thus, all of the mutants studied, except the nonfunctional n12, retain the ability to bind DNA. This is expected as none of the mutations were made in the DNA-binding region of the protein. In addition, the

3-8 deletion mutants retain the ability to form TPCs despite showing a reduced transactivation potential.

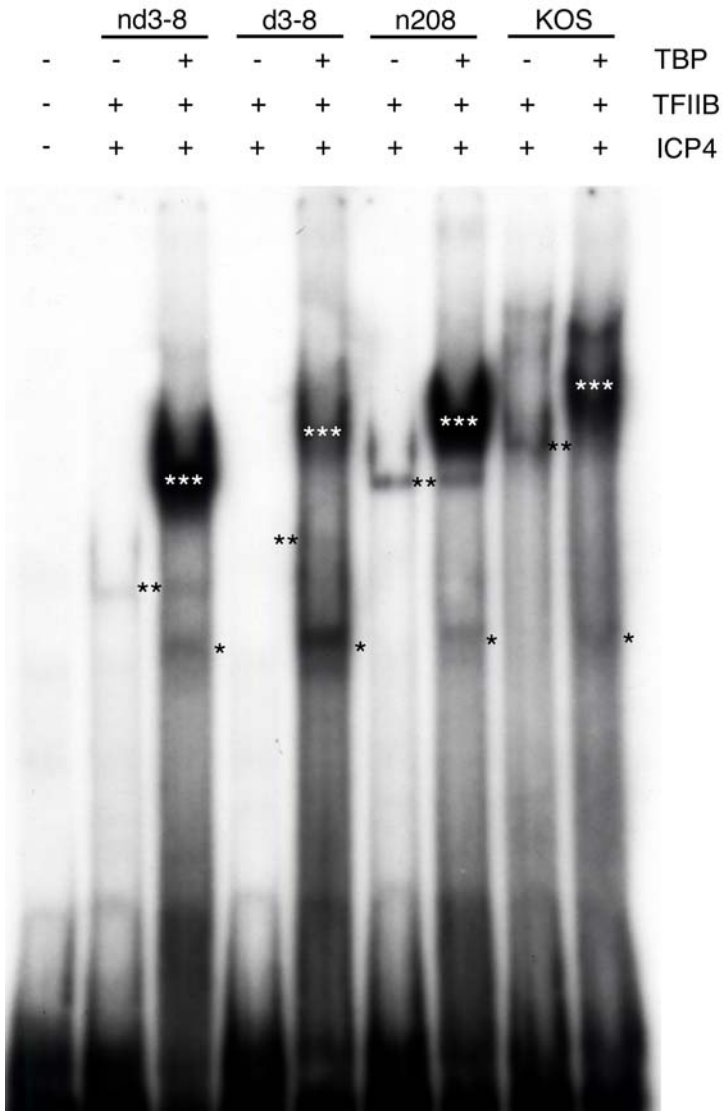

Figure 11: Tripartite formation on the ICP4 promoter by ICP4 mutants, TBP, and TFIIB.
5 ng of purified wt or mutant ICP4 was incubated for 30 min at room temperature with TFIIB and radiolabeled ICP4 promoter either in the presence or absence of TBP. The complexes were separated on a native polyacrylamide gel and visualized via autoradiography.

3.5 DISCUSSION

ICP4 is a critical regulator of viral gene expression. Its function is affected in part via interactions with the cellular general transcription machinery that are mediated through two regulatory domains, a C-terminal and an N-Terminal domain (49, 50, 207, 256). In this study, engineered viruses that express deletion and truncation mutants of ICP4 were used to dissect the contribution of different regions within the N-Terminal domain to the function of ICP4, either in the presence or absence of the C-terminal domain. As summarized in figure 12, we show that while the entire N-terminal regulatory region is necessary for efficient function, in the context of infection, the 3-8 mutation exhibited a slightly more deleterious effect on ICP4 activity than the 8-10 region. Consistently through viral growth curves, protein expression profiles, and mRNA abundance of specific viral genes, the 3-8 deletion mutants showed a greater reduction in transactivation relative to both the 8-10 deletion and to wildtype. No effect was seen in either deletion on the repressive ability of ICP4. The mutant phenotype was not due to a reduced DNA-binding ability, however, as all of the mutants bound DNA in EMSA studies. In addition, the 3-8 deletion mutants retained the ability to form TPCs on DNA with TBP and TFIIB, underscoring their role in repression, not transactivation.

ICP4 shares extensive sequence similarity with other human alphaherpesviruses, including VZV. These conserved regions correspond to regions of functional importance, including DNA-binding, nuclear localization, and transcription regulation. The region with the most extensive similarity is the C-terminal domain. The conserved region in the N-terminal regulatory domain is restricted to the polyserine tract, found in the 8-10 deletion. A sequence alignment comparing HSV-1, -2, and the herpes B virus however, revealed a second region of conversation, albeit limited to the most closely related herpesvirues to HSV-1 (figure 13). This

region, found in the 3-8 region in between amino acids 81-91, is most likely responsible for the function attributed to the 3-8 region. The fact that these regions are conserved amongst differing groups of viruses suggests they play distinct roles in ICP4's function, an observation borne out by this study.

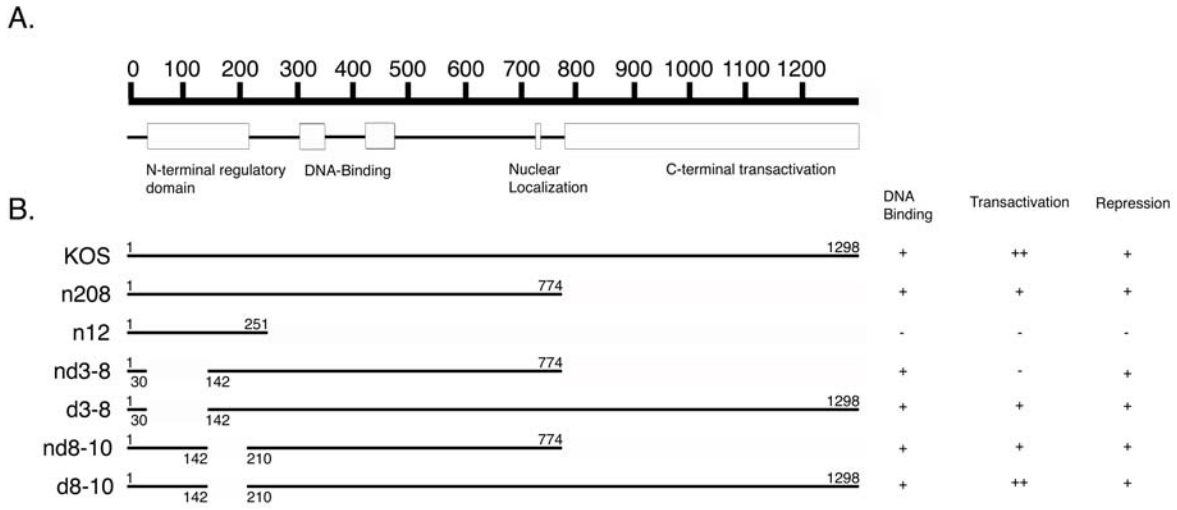

Figure 12: Summary of relative activities of mutant ICP4 used in this study.
A) The primary sequence of ICP4 is shown with the various functional domains indicated by white boxes. B) The peptides expressed by the mutants in this study are shown. Their relative DNA-binding, transactivation, and repression function are indicated.

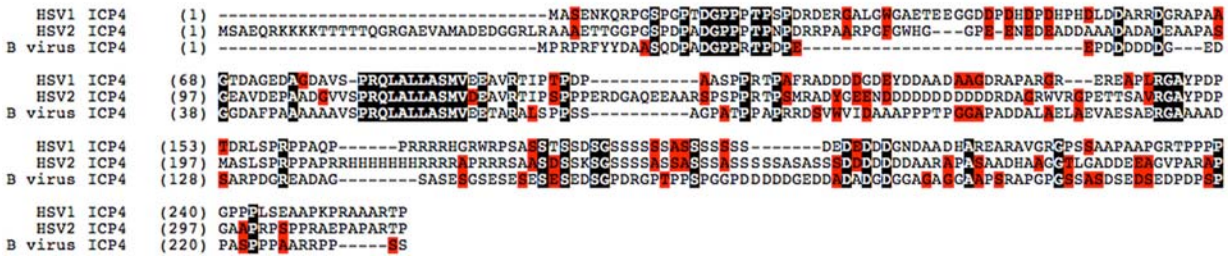

Figure 13: Regions of similarity between HSV-1, HSV-2, and herpes B virus ICP4 homologs in the N-terminal regulatory domain.
A sequence alignment was performed using the first 240 amino acids of HSV-1 ICP4, comparing the primary sequence of ICP4 in HSV-1, HSV-2, and the herpes B virus.

Due to ICP4's essential role in the viral life cycle, any impairment of viral function will have a direct effect on viral growth. Truncation of ICP4 at amino acid 774, which yields a peptide lacking the C-terminal transactivation domain but is capable of both repression and activation of some viral genes (49, 50), significantly impaired, but did not abolish viral growth. This is in contrast to the truncation at aa 251, which yields a non-functional peptide that contains the N-terminal regulatory domain, but lacks DNA-binding (49, 50), and resulted in a complete loss of viral replication, underscoring ICP4's dependence on DNA-binding for its function. Deletion of parts of the N-terminal regulatory domain with the DNA-binding and C-terminal domains intact resulted in a less severe reduction in viral growth as compared to n208. The d3-8 deletion virus showed a slightly larger reduction in growth as the d8-10 virus, however both regions, aa 30-142 and 143-210, are required for normal viral growth. The growth of n208, d3-8, and d8-10 indicate that although required for wildtype growth, neither the intact N- or C-terminal regulatory domains are necessary to maintain some low level of viral replication. Viruses that lack both intact transcriptional domains, however, do not grow. Interestingly, loss of the C-terminus alone results in a slightly greater than two orders of magnitude reduction in growth (n208 compared to KOS) while loss of only either the 3-8 or 8-10 region results in a reduction of 1 to 1.5 orders of magnitude, respectively, when compared with KOS. This suggests the C-terminus plays a larger role in viral growth, despite the observation that mutants lacking C-terminus retain both repressive and transactivation function. Deletion of both the entire N-terminal regulatory domain and the c-terminus after aa 774, however, yields a dominant negative mutant (257) making it difficult to study the contribution of the C-terminus in the absence of the entire N-terminal regulatory domain. Comparison of n208 to KOS, d3-8 to nd3-8, and d8-10 to nd8-10 allows the assessment of cooperativity between the N- and C-terminal

regions. Loss of the C-terminus with an intact N-terminal region results in a slightly larger than 2 orders of magnitude reduction in growth (figure 7, compare n208 to KOS) whereas the loss of the c-terminus while lacking either aa 30-142 or aa143-210 results in a drop of approximately 3 orders of magnitude (figure7, compare nd3-8 to d3-8, nd8-10 to d8-10). The loss of either the 3-8 or 8-10 region has the same impact in viral growth (1-1.5 orders of magnitude reduction) independent of the c-terminus (figure 7, compare KOS to d3-8 and d8-10, n208 to nd3-8 and nd8-10). This suggests that the C-terminal regulatory domain is uniquely sensitive to the lack of intact N-terminal domain whereas the N-terminal domain's function is less dependent on the C-terminus; the N-terminus may, therefore, play a role in the function of the C-terminus.

In addition to growth curve studies, global viral replication was assessed via protein expression profiles. In general, the deletion mutant viruses fit into three categories relative to the protein profiles of the control viruses: KOS-like, n208-like (exhibiting a slight reduction in Early genes, a significant impairment of leaky late genes, and no expression of late genes, and n12-like, no expression of early or late genes. Interestingly, the deletion mutants did not group with their control counterparts. While d8-10 did belong to the KOS-like group, d3-8's protein profile was more similar to n208's; nd8-10 behaved more like n208, but nd3-8 had more similarities to the nonfunctional n12. In addition, the protein expression profiles did not always correlate with the growth curves. While the d8-10 and d3-8 mutants have relatively similar growth kinetics (figure 7), their protein expression profiles are significantly dissimilar (figure 8), suggesting a different etiology for their similar replicative phenotypes.

The relative mRNA abundance of the tk and gC transcripts underscore the importance of the different transcriptional regulatory domains. The inability of C-terminal truncation mutants to accumulate gC mRNA agree with previously published data showing the importance of the C-

terminus to activate late genes (figure 9) (49, 50). The mRNA abundance data for the N-terminal deletion mutants agree with the growth curve and protein expression data showing the importance of both aa 30-141 and 142-210 to be necessary for normal transactivation (figure 9). Interestingly, our data showing the importance of the 3-8 region in ICP4-mediated transactivation is in direct contrast with previously published results showing the 8-10 region is required while the loss of the 3-8 region has no effect on transactivation (256). The previous work, however, was done using transient transfection of plasmids expressing the ICP4 deletion and a CAT reporter plasmid under the control of the tk promoter, whereas the studies shown here are in the context of viral infection and directly measuring tk mRNA abundance. The 8-10 region clearly plays an important role in ICP4-mediated transcription that is less relevant in the context of infection than in transfection, and conversely, the 3-8 region plays a critical role in transactivation in the context of infection but not transfection. Transfected plasmids are rapidly integrated into nucleosomal structures in mammalian cells (90, 225). These structures are largely repressive in nature, and this repression can be counter-acted by the viral promiscuous transactivator ICP0 at least partially through its disregulation of cellular HDACs (96, 163). In the absence of IE gene expression, viral genomes rapidly associate into a repressive nucleosomal state. The virus acts to counteract this both directly on IE promoters through VP16-mediated HAT activity and globally through ICP0's disregulation of cellular HDACs (96, 114, 163). In addition, HSV infection rapidly and dramatically alters the host cell, creating an environment that favors viral over cellular gene expression. ICP0 induces a general, pro-transcription state in the cell(67, 84, 197); ICP22 triggers an aberrantly phosphorylated form of RNA pol II (76, 77, 229); and HSV infection induces the phosphorylation of Sp1 (139), the loss of TFIIA expression (312), and the marginalization of the cellular DNA. The 8-10 region of ICP4 may be important

for ICP4 transactivation from a more closed, repressed chromatin state formed in the absence of viral factors while the 3-8 region may be be necessary for transactivation from a more open template and a transcription environment heared more for viral gene expression. The 8-10 region may therefore play a role in reactivation from latency when the viral genome is in a repressive chromatin state (53). The polyserine tract, which is located in the 8-10 region, has indeed been shown to be involved in viral growth and reactivation in neurons. Viral mutants that are lacking the polyserine tract replicate in the corneas of mice, albeit at a reduced level; efficiently infect the TG; but exhibit a dramatic reduction in growth and reactivation in neurons (12).

ICP4's DNA-binding is necessary but not sufficient for its regulatory function (50, 208, 256). All of the mutants in this study, except the non-functional n12, retain ICP4's DNA-binding domain. EMSA studies verify that these mutants all retain the ability to bind DNA, making any change in regulation a result of altered function and not DNA-binding. Interestingly, the role DNA-binding takes in ICP4's function differs between repression and activation. Despite no clear DNA-binding site, ICP4 acts, in a DNA-binding dependent manner, to recruit and stabilize pre-initiation complexes on promoters for transactivation (26, 92). In repression, ICP4 forms a TPC on the DNA through binding of a strong ICP4-binding site and interactions with TBP and TFIIB (94, 145, 266). The position of the ICP4 binding site relative to the TATA box directly correlates with strength of repression (145, 157). The ability of the transactivation defective but repression competent nd3-8 and d3-8 mutants to form TPCs *in vitro* (figure 11) underscores the role the TPC plays in repression, specifically. The role DNA-binding plays in ICP4's function is becoming increasingly more complex, however. ICP4 has been shown to oligomerize on DNA through its C-terminus, thus creating a relative high-affinity for the DNA

through an additive effect of multiple molecules binding at a lower affinity (146). ICP4's stabilization of PICs on viral promoters may also act to reciprocally stabilize ICP4 on the DNA. Indeed, ChIP analysis has shown the cooperative recruitment of TBP and ICP4 on L genes in a C-terminus and Inr dependent fashion, most likely through interactions with TAF1. The C-terminus truncated mutant n208 does not oligomerize on DNA, does not directly interact with TAF1, expresses some, but not all, early genes, inefficiently replicates its DNA, and does not express late genes. In repression, the already high affinity of ICP4 for its binding site is increased through the cooperative formation of the TPC (266). Thus, ICP4's DNA-binding is critical for regulation, functionally variable, and carefully modulated both through DNA sequence specificity and through stabilization of low-affinity binding through protein-protein interactions. Coarse, *in vitro* DNA-binding assays such as EMSA and DNAse footprinting may therefore not be sufficient to fully delineate between the subtle variations in DNA-binding *in vivo* that determine ICP4 transcriptional regulation.

ICP4 is a large, complex molecule with several domains that contribute to its function as a critical regulator of viral transcription. Extensive effort has been made to map the regions of the protein that affect its function. Studies of truncations of ICP4 have shown that the N-terminal 774 amino acids are sufficient for ICP4-meditated repression and transactivation (49, 50). This truncation mutant, while retaining transactivation function, showed a reduced expression of some early and leaky late genes, a severe impairment in DNA-replication, and no late gene expression indicating the importance of the C-terminus for some transactivation function. Interestingly, small, temperature sensitive mutations made in the C-terminus can result in a loss of ICP4 function at the non-permissive temperature that is retained by mutants lacking the C-terminus, suggesting a role in protein folding and stability (37, 46, 55, 209, 219). This

complexity coupled with the inability to isolate discreet regulatory domains in the N-terminus suggest ICP4's true regulatory domains are a result not of simple linear primary sequences as is common in most transcription factors, but a complex tertiary structure that may act as a platform to mediate multiple protein-protein contacts. The targeted mutagenesis of the discreet stretches of similariy between the closely related HSV-1, -2, and herpes B virus, and the polyserine tract that is conserved amongst the alphaherpesviruses will provide further insight into these functional domains and how they functionally and perhaps physically interact with each other and the C-terminus. The dependency of the C-terminus on an intact N-terminal regulatory domain presented here and the role the C-terminus plays in ICP4 protein folding raises the possibility of an intramolecular interaction between the N- and C-terminal regulatory domains. The GTF TFIIB contains a highly conserved charged cluster domain in the N-terminus that mediates a functionally relevant intramolecular interaction with the c-terminus, resulting in a dynamic equilibrium of open and closed confirmation TFIIB (109, 110, 233). In addition, the successive phosphorylation of Rb by a variety of cdks induces sequential, intramolecular interactions that incrementally regulate the function of Rb, including its dissociation with HDACs (104). Similarly, the phosphorylation of the polyserine tract in the N-terminal regulatory domain of ICP4 seen during infection (302, 303) may provide an effective way to dynamically alter the intramolecular interaction between the N- and C-terminus, thus modulating the structure of ICP4's tertiary activation domain and subsequently, its activity. This complexity would reflect the need to regulate transcription from multiple, divergent promoters through dynamic changes in the cellular transcriptional milieu induced as the viral life-cycle progresses.

4.0 PROTEINS INTERACTING WITH ICP4 DURING HSV-1 INFECTION

4.1 ABSTRACT

The infected cell polypeptide 4 (ICP4) is a critical regulator of viral gene expression and is required for productive infection. Because viral genes are transcribed by the cellular RNA polymerase II (RNA pol II), ICP4 must interact with the host machinery to regulate viral gene expression. It has been shown previously that ICP4 interacts in vitro with several members of the general transcription factors (GTFs), including TATA box-binding protein (TBP), TFIIB, and the TBP-associated factor 1 (TAF1). These interactions correlate with ICP4's ability to regulate transcription. In this study, the cellular transcription machinery contacted by ICP4 during infection was further characterized using tandem affinity purification (TAP). A virus that expresses TAP-tagged ICP4 under the control of its native promoter in a wild- type KOS background was engineered. The resulting virus grew with similar kinetics as wildtype and expressed comparable levels of TAP-ICP4. Nuclear extracts were prepared from isolated nuclei of infected HeLa cells via high salt extraction and submitted to TAP using IgG and calmodulin beads. The purified material was separated via SDS-PAGE, stained with colloidal blue, and gel slices were prepared. The gel slices were submitted to in-gel trypsin digestion, and the resulting peptides were eluted from the gel. The peptides were then analyzed via ESI-LC/MS, which identified 46 peptides with high confidence. One of the proteins identified was L22, a cellular

translation factor that was previously shown to interact with ICP4 via a two-hybrid screen. Proteins of particular interest found to copurify with ICP4 were TBP and several TAFs, TFII-I, and components of the Mediator complex. The role of Mediator in ICP4-mediated transactivation was further investigated using immunofluorescence and chromatin immunoprecipitaion. Mediator was found to colocalize with ICP4 starting at early and continuing into late times of infection. In addition, Mediator was recruited to viral promoters in an ICP4-dependent manner. Together, the data show that ICP4 interacts with components of the basal transcription machinery and coactivator complexes in the context of viral infection to regulate viral gene expression.

4.2 INTRODUCTION

During lytic infection, HSV-1 exhibits a strictly regulated temporal cascade of gene expression that is divided into three general stages, immediate early (IE) or α, early (E) or β, and late (L) or γ (120, 121). This expression program is a result of a complex interplay between viral and cellular factors at both the transcriptional and translational level that is poorly understood. The IE protein ICP4 is a critical regulator of viral gene expression and is required for productive infection (48, 198, 217). It activates E and L genes while repressing its own transcription, giving ICP4 a crucial role in establishing the gene expression cascade.

ICP4 regulates gene expression through interactions between its two broad regulatory domains, DNA, and various cellular factors. It exists as a large, 350 kDa homodimeric phosphoprotein in the cell and has N- and C-terminal transcriptional regulatory domains that are separated by a dimerization domain, nuclear localization sequence, and DNA-binding domain

(49, 50, 81, 207, 208, 256). ICP4 binds DNA to a degenerate consensus seqeunce, ATCGTCNNNNYCGRC where R = purine, Y = pyrimidine, and N = any base (72, 78). ICP4 has also been shown to bind DNA nonspecifically. DNA binding is required for ICP4-mediated transcriptional regulation, with specific binding sites associated with repression and non-specific binding important in transactivation (35, 61, 64, 102, 265).

Because viral genes are transcribed by the cellular RNA polymerase II (RNA pol II), the virus must recruit the host machinery to regulate viral gene expression (8, 41). Cellular transcription is traditionally thought to be initiated through a step-wise assembly of the pre-initation complex (PIC) consisting of the GTFs (TFIID, TFIIA, TFIIB, TFIIF, TFIIE, and TFIIH) and RNA pol II (21, 190, 202). The efficiency of formation of the PIC is a critical step in determining the rate of transcription and is thus a common site of both positive and negative transcriptional regulation (9, 31, 82, 159). TFIID, a large protein complex consisting of the TATA binding protein (TBP) and its associated factors (TAFs), is important for promoter recognition and acts as a scaffold on which the rest of the PIC nucleates (21, 193). ICP4 has been shown to stabilize the formation of PICs on viral promoters *in vitro* via enhancement of TFIID binding to the promoter through direct interactions *in vitro* with both TBP and TAF1 of the TFIID complex (26, 92). In addition, ICP4 cooperatively forms a stable, tripartite complex (TPC) on DNA with TBP and TFIIB at promoters containing the ICP4 binding site *in vitro* (145, 266). The formation of the TPC localizes to regions of ICP4 that are also required for efficient repression of activated transcription suggesting the TPC has a suppressive effect on promoters (94, 145). Formation of the TPC specifically inhibits activated transcription at a step prior to elongation while allowing basal transcription to proceed. This implies it does not simply preclude binding of the PIC to the promoter (95). Thus, ICP4 interferes with the action of

upstream activators through formation of TPCs to negatively regulate transcription from these promoters.

ICP4 mediates both positive and negative transcriptional regulation through complex interactions with DNA and several members of the cellular transcriptional machinery. It is unclear, however, what prevents robust activation of late genes until the onset of DNA replication and the decline in E gene expression after DNA replication despite the continued presence of ICP4 at these promoters. In this study, the cellular transcription machinery contacted by ICP4 is further characterized using tandem affinity purification (TAP). TAP is a general purification scheme that purifies native protein complexes from the cell through use of a bipartite tag consisting of two IgG binding domains from *Staphylococcus aureus* protein A and a calmodulin binding peptide (CBP) separated by a tobacco etch virus (TEV) protease cleavage site (220, 230). A virus that expresses an N-terminally TAP-tagged ICP4 under the control of its native promoter in a wild-type KOS background was engineered. Nuclear extracts were prepared from isolated nuclei of infected HeLa cells via the Dignam method (54) and submitted to TAP. The previous studies investigating ICP4 protein interactions were done *in vitro* with uninfected cellular extracts that do not reflect the conditions found in infected cells. HSV infection has been shown to significantly alter the host transcriptional environment (77, 139, 229, 312). Isolating ICP4 complexes in the context of infection allows for the characterization of protein interactions made in the natural protein stoichiometry that occurs during infection.

SDS-PAGE analysis of TAP-purified samples shows a multipart mixture of proteins complexing with ICP4. Mass spectrometry and western blot analysis identified several proteins that co-purify with ICP4, including TBP, several TAFs, TFII-I and subunits of the Mediator complex, among others. Colocalization studies using immunofluorescence show Mediator

subunits are recruited to viral replication compartments at early times of infection and persist into late infection. In addition, chromatin immunoprecipitation studies show the association of Mediator at promoters representative of all three major classes of genes, and this association is increased in the presence of ICP4, thus ICP4 recruits Mediator to viral promoters most likely to enhance their transcription. These data show the intricacies of viral gene regulation and suggest ICP4 may play a broader role in transcriptional regulation, acting to stimulate the formation of PICs both through recruitment of TFIID as well as mediator and potentially other cellular factors to the promoter. The dynamic nature of the cellular transcriptional environment during the course of infection, distinct promoter structures of each class, and a potential broader role for ICP4 in gene activation suggest the activation of the IE, E, and L gene classes may be through distinct mechanisms, thus allowing for the strictly regulated cascade of expression exhibited during lytic infection.

4.3 MATERIALS AND METHODS

4.3.1 Cells and viruses

HeLa, vero, HEL, and E5 cell lines were all maintained by standard procedures. E5 cells were derived from vero cells and express complementing levels of ICP4 (47, 49). The HeLa, Vero, and HEL cell lines were all obtained from ATCC. Both the HSV-1 wildtype strain KOS and TAP-ICP4 viruses were propagated on Vero cells while the nonfunctional ICP4 nonsense mutant n12 (50) was grown on E5 cells. Viral growth curves were performed on vero cells. 5×10^5 cells were infected on ice with KOS or TAP-4 HSV-1 at an MOI of 5. Virus was allowed to adsorb

for 1 hour on ice, then cells were washed twice with cold Tris-buffered saline (TBS) containing 0.25 mM Tricine, 137 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, and 0.68 mM $CaCl_2$, pH 7.35. The infection was allowed to proceed at 37^{o}C for 2, 4, 8, 12, 24, or 36 hours as indicated. Cells were then scraped into the media, freeze-thawed twice, and sonicated. Viral titers were determined on E5 cells via plaque assay.

4.3.2 Construction of the TAP-ICP4 virus

A virus expressing an N-terminally TAP-tagged ICP4 was constructed. The plasmid pcDNA Zome-N containing the N-terminal TAP tag construct was digested with *Eco*RI. The overhanging ends were filled in using Klenow enzyme (NEB) and ligated together with T4 DNA ligase (NEB). The resulting plasmid was then digested with *Hind*III, the overhanging ends were filled in, and a *Sal*I linker (NEB) was ligated to the blunt ends. The construct was then digested with *Pst*I and *Sal*I to free the tag construct from the plasmid. The TAP-construct was then inserted into the ICP4-containing pi2 plasmid (256) digested with *Pst*I and *Sal*I to form the NTAP-pi2 plasmid. The NTAP-pi2 plasmid was then used to rescue the ICP4 nonsense mutant n12, as described previously (12). Plaque isolates were screened by Southern blot, and successful recombinants were submitted to several rounds of plaque purification. The resulting TAP-ICP4 virus expresses ICP4 from its native promoter with the TAP tag replacing the first 17 amino acids of ICP4.

4.3.3 TAP procedure

The TAP protocol was performed as described previously with several modifications (220). 1.5 x 10^9 HeLa cells were infected with either KOS or TAP virus in DMEM 5% FBS at an MOI of 10. The infection was allowed to proceed for six hours at 37^{o}C, after which the cells were scraped into the media and pelleted via centrifugation at 5000 RPM for 5 minutes at 4^{o}C. All remaining steps were done at 4^{o}C. The cells were washed twice in a total volume of 40 ml TBS plus 0.2mM PMSF, 0.2mM *Nα-p*-tosyl-L-lysine chloromethyl ketone (TLCK), and 0.5mM DTT added just prior to use. The cells were then pelleted at 2000 RPM for five minutes, and nuclear pellets were then prepared following the Dignam method with some modifications (54). The cell pellet was first resuspended in hypotonic buffer (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) containing 0.2 mM PMSF, 0.2 mM TLCK, and 0.5 mM DTT using approximately five times the pellet volume. The cells were pelleted at 2000 RPM for five minutes and then then resuspended again in hyptonic buffer containing 0.2 mM PMSF, 0.2 mM TLCK, and 0.5 mM DTT (approximately three times initial pellet volume) and incubated for ten minutes on ice. The cell suspension was then transferred to a pre-chilled small wheaton dounce homogenizer to disrupt the cellular membranes (about 15 strokes). The efficiency of douncing was assessed via staining with trypan blue. The nuclei were the pelleted by centrifugation at 3000 rpm for 10 minutes. The nuclei were gently resuspended in half the total pellet volume using low salt buffer (20 mM Hepes pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM ethylenediaminetetraacetic acid (EDTA)) containing 0.2 mM PMSF, 0.2 mM TLCK, and 0.5 mM DTT added just prior to use. High salt buffer (20 mM Hepes pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 1.6 M KCl, 0.2 mM EDTA) with 0.2 mM PMSF, 0.2 mM TLCK, and 0.5 mM DTT using one third total volume of nuclear suspension was then added to the suspension dropwise

for a final KCl concentration of 400 mM. Samples were incubated for 30 minutes at 4°C with gentle mixing. The extract was then clarified via centrifugation at 14,500 rpm for 30 minutes at 4°C. 250 μl (~125 μl packed volume) IgG-Sepharose 6 Fast Flow bead suspension (Amersham Biosciences) was added to four Poly-prep chromatography columns (BioRad). The beads were equilibrated by washing in five ml equilibration buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, and 0.05% Tween-20) followed by 5 ml 0.5 M acetic acid, pH 3.4, 5 ml equilibration buffer, and 10 ml IgG binding buffer (15 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 200 mM KCL, 0.1% NP40, and 0.5 mM EDTA) with 0.2 mM PMSF, 0.2 mM TLCK, and 0.5 mM DTT. The nuclear extracts were diluted approximately two-fold in IgG binding buffer with 0.2 mM PMSF, 0.2 mM TLCK, and 0.5 mM DTT and added in equal amounts to the four columns. Extracts were incubated at 4°C for four hours with gentle end-over-end rotation. Unbound extract was allowed to drain from the column. The column was then washed with 5 ml of IgG binding buffer with 0.2 mM PMSF, 0.2 mM TLCK, and 0.5 mM DTT, 5 ml without PMSF or TLCK but with 0.5 mM DTT, and 5 ml TEV protease buffer (10 mM Tris pH 8.0, 150 mM NaCl, 0.1% NP40, 0.5 mM EDTA) containing 1 mM DTT added just prior to use. The columns were then incubated overnight at 4°C in 1 ml TEV protease buffer containing 10 μl (100 U) AcTEV protease (Invitrogen) and 10 μl (250 U) Benzonase nuclease (Novagen) per column to release the bound proteins from the column and to digest any contaminating DNA in the sample. The eluates from the columns were drained into a new tube. Three ml calmodulin binding buffer (10 mM Tris-HCl pH 8.0, 10 mM β-mercaptoethanol, 150 mM NaCl 1 mM MgAcetate, 1 mM imidazole, 2 mM $CaCl_2$, and 0.1% NP-40) with an additional 3 μl 1 M $CaCl_2$ (1 μl/ml) to ensure saturation of residual EDTA was passed through the column and combined with the eluate from above for efficient collection of eluted protein complexes for a total of approximately 16 ml eluate in

calmodulin binding buffer. 500 µl (~250 µl packed volume) calmodulin affinity resin (Stratagene) was added to two poly prep columns and equilibrated 3x with 5 ml calmodulin binding buffer. 8 ml of eluate was then added to each calmodulin column and incubated with rotation for 4 hours at 4°C. The columns were then washed in 10 ml calmodulin wash buffer (10 mM Tris-HCl pH 8.0, 10 mM β-mercaptoethanol, 150 mM NaCl 1 mM MgAcetate, 1 mM imidazole, and 2 mM $CaCl_2$). The bound complexes were then eluted in 10 ml calmodulin elution buffer (10 mM Tris-HCl pH 8.0, 10 mM β-mercaptoethanol, 150 mM NaCl 1 mM MgAcetate, 1 mM imidazole, and 2 mM ethylene glycol tetraacetic acid (EGTA)), combined, and concentrated using iCON concentrators, 7ml/9k (Pierce).

4.3.4 Mass spectrometry

Purified TAP samples were separated via SDS-PAGE and visualized using a colloidal blue staining kit (Invitrogen) following manufacturers protocol. Gel slices were excised for the length of the gel and submitted to in-gel trypsin digestion as described previously (258). The resulting peptide mixture was separated by capillary C18 HPLC using a ThermoElectron Surveyor liquid chromatograph, and the effluent was directly analyzed on a ThermoElectron LCQ Deca XP Plus quadrupolar ion trap mass spectrometer using a nanospray ionization source. The instrument collected both MS and MS/MS spectra. The data was analyzed and searched against both human and HSV-1 Uniprot/Swissprot databases using the SEQUEST search engine in the BioWorks Browser, version 3.3.1 SP1 (Thermo Fisher Scientific, Inc.). The MS analysis was performed by the Genomics and Proteomics Core Laboratories at the University of Pittsburgh.

4.3.5 Western blot analysis

TAP-purified samples were separated via SDS-PAGE and analyzed via western blot as previously described (255). The antibodies used were diluted in 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween-20 (TBS-T) and 1% milk. They were as follows: 1:5000 dil of N15, a rabbit polyclonal specific to the N-terminus of ICP4 as previously characterized (255); 1:500 TBP (233R, Covance); 1:100 TAF1 (Santa Cruz); 1:100 TAF4 (Santa cruz, sc-736), 1:2500 TAF4 (152); 1:1000 ICP0 1112 (Rumbaugh-Goodwin Institute); 1:1000 ICP27 1113 (Rumbaugh-Goodwin Institute); 1:250 CRSP77 (Santa cruz); 1:100 Med7 (Santa cruz); and 1:1000 TRAP220 (Santa cruz). The secondary antibodies used were 1:5000 dilutions of an anti-rabbit, anti-mouse, and anti-goat polyclonal IgG-HRP conjugates (Promega) in TBS-T. Bound antibody was visualized using the ECL Plus Western Blotting Detection System (Amersham) per manufacturer's protocol.

4.3.6 Immunofluorescence

One day prior to infection, HEL cells were plated onto coverslips. 1 x 10^6 HEL cells were plated in 60 mm dishes with four round coverslips per dish. The next day, the cells were infected with KOS at an MOI of 10 on ice. The cells were chilled on ice and treated with ice-cold inoculum in TBS. The virus was allowed to adhere for one hour on ice with periodic gentle rocking after which the inoculum was removed and replaced with warm media. The infections were allowed to proceed for two, four, and eights hours. Subsequently, the media was removed, and the cells were washed once in warm phosphate buffered saline (PBS). The cells were fixed with 1% paraformaldehyde (PFA) in PBS for 11 min at 37^{o}C. The PFA was removed, and the cells were

incubated at room temperature in PBS containing 100 mM glycine to stop the reaction. The cells were washed for five minutes in room temperature PBS three times and then incubated in 50 mM NH_4Cl in PBS for 10 minutes. The cells were permeabilized with 0.5% triton X-100 in PBS with 1% bovine serum albumen (BSA) for 15 minutes and then washed in PBS-T (PBS with 0.1% triton X-100) and 1% BSA three times for 10 minutes each. The primary antibodies were diluted in PBS-T and 1% BSA. The antibodies used were: 1:500 dilution of N15 (rabbit polyclonal) for ICP4 and 1:1000 dilution for TRAP220 (Goat polyclonal, Santa cruz). The cells were incubated for two hours with 100 µl antibody dilution per slide. The coverslips were washed with PBS-T containing 15 BSA six times 10 minutes each. The appropriate secondary antibodies (donkey anti-goat Alexa Fluor488 or donkey anti-rabbit Alexa Fluor595 both from Invitrogen) were diluted 1:500 in 100 µl per coverslip PBS-T and 1% BSA and incubated on the slides for one hour in the dark. The coverslips were washed in PBS-T eight times seven minutes each in the dark. The coverslips were then mounted on glass slides using Immu-mount (Thermo Scientific). The slides were analyzed and images captured using a Leica DMI4000 B microscope.

4.3.7 Chromatin immunoprecipitation

ChIP assays were performed as described previously with some exceptions (247). One day prior to infection, 5×10^6 vero cells were plated onto 100 mm dishes. The following day, the cells were infected on ice in cold TBS at an MOI of 10 with the indicated viruses. The virus was allowed to adsorb for one hour on ice with gentle, periodic rocking, after which the inoculum was removed and replaced with warm media. The infections were allowed to proceed for four hours prior to formaldehyde fixation. Sonicated chromatin was harvested as described

previously (247). The samples were pre-cleared using protein A and G agarose beads with salmon sperm DNA (Millipore 16) as described. The primary antibodies (3 µl purified n15 for ICP4, 1 µl 8WG16 (Covance) for RNA pol II, and 1.5 µl each of TRAP220 (Santa Cruz) and CRSP77 (Santa cruz) for Mediator were pre-treated and bound to the appropriate protein A and G beads for four hours at 4^{o}C with gentle mixing. Unbound antibody was washed away using 1 ml ChIP dilution buffer 3 times 5 minutes each. Pre-cleared sample was then incubated with the antibody:bead mixture overnight at 4^{o}C with gentle mixing. The bound protein complexes were washed, eluted, uncross-linked, and digested using proteinase K as described previously (247). The released DNA was harvested via phenol:chloroform extraction followed by ethanol precipitation and resuspended in 150 µl H_2O. The DNA was then analyzed via RT-PCR using SYBR GreenER qPCR SuperMix (Invitrogen) as described previously (247). The primers used for the PCR reaction are shown in table 1.

Table 1: Primers used for PCR

Primer	Sequence	Position
ICP0prom	ATAAGTTAGCCCTGGCCCGA	-24 to +36
	GCTGCGTCTCGCTCCG	
tkprom	CAGCTGCTTCATCCCCGTGG	-200 to +56
	AGATCTGCGGCACGCTGTTG	
gCprom	CGCCGGTGTGTGATGATTT	-90 to +80
	TTTATACCCGGGCCCCAT	

4.4 RESULTS

Transcription of viral genes follows a strictly regulated temporal cascade and is mediated through the cellular general transcription factors and RNA pol II. Several lines of evidence have shown that the cellular machinery is altered during the course of infection, resulting in a changing pool of available factors, which may contribute to the implementation of the cascade. ICP4, however, is required for the proper activation of both E and L gene classes. ICP4 has been shown to interact *in vitro* with both TBP and TAF1 of TFIID, and these interactions are required for ICP4 to function in reconstituted transcription reactions (26, 145, 266). In addition, the C-terminus of ICP4 is required for activation of L but not E genes (49, 50). The evidence that transcription of the classes of viral genes is mediated through distinct cellular complexes and different regions of ICP4 suggests ICP4 activates transcription through the recruitment of different cellular complexes to distinct promoter classes. In this study, we used tandem affinity purification (TAP) to determine the protein interactions made by ICP4 during infection. We also investigated the relevance of a subset of these interactions *in vivo* through immunofluorescence and chromatin immunoprecipitation studies.

4.4.1 Characterization of TAP-ICP4

Tandem affinity purification (TAP) was used to investigate the protein interactions made by ICP4 during infection. TAP uses dual purification steps to gently and specifically purify large complexes while reducing contaminating false-positives. The TAP-tag, consisting of two IgG binding domains from *Staphylococcus aureus* protein A and a calmodulin binding peptide (CBP) separated by a tobacco etch virus (TEV) protease cleavage site (220, 230), was inserted into the

N-terminal ICP4 coding region and then recombined into the viral locus via homologous recombination as described in the materials and methods. The tag was added to the N-terminus because it has previously been shown to tolerate addition of other epitopes (26). Successful recombinants were plaque purified and confirmed via Southern blot. The TAP-tag was inserted at amino acid 17 of ICP4, resulting in the loss of the first 17 amino acids and is under control of the native ICP4 promoter (figure 14A). Proper protein expression and size were confirmed via western blot. Vero cells were infected with the non-functional nonsense mutant n12, TAP-ICP4, or wildtype KOS. Whole cell lysates were prepared 6 hours post-infection and submitted to western blotting, probing with n15, a polyclonal antibody to the N-terminal 774 amino acids of ICP4. Wildtype ICP4 has an apparent molecular weight of 175 kDa; whereas, TAP-ICP4 exhibits a slightly lower mobility when compared to wildtype, corresponding to the additional 184 amino acids (approximately 21 kDa) of the TAP-tag (figure 14B). The mutant n12 expresses a severely truncated protein of approximately 41 kDa. Both wildtype and TAP-ICP4 were expressed to similar levels showing the addition of the tag did not disrupt ICP4 regulation or protein stability. The n12 mutant has dramatically lower levels of the truncated ICP4. This is most likely due to inefficient recognition of the truncated protein by the n15 antibody as the n12 mRNA is greatly overexpressed due to loss of ICP4's repressive function (see Chapter 3).

Viral growth curves were performed to assess the effects of the addition of the tag on viral growth. Because ICP4 is required for replication, any effect on ICP4 function will result in altered viral growth. Vero cells were infected on ice with either KOS or TAP-ICP4 at an MOI of 5. Virus was harvested 2, 4, 8, 12, 24, and 36 hours post-infection and titered on E5 cells. Both the KOS and TAP-ICP4 viruses exhibited similar growth, showing the addition of the TAP-tag does not significantly impact viral replication or ICP4 function (figure 14C).

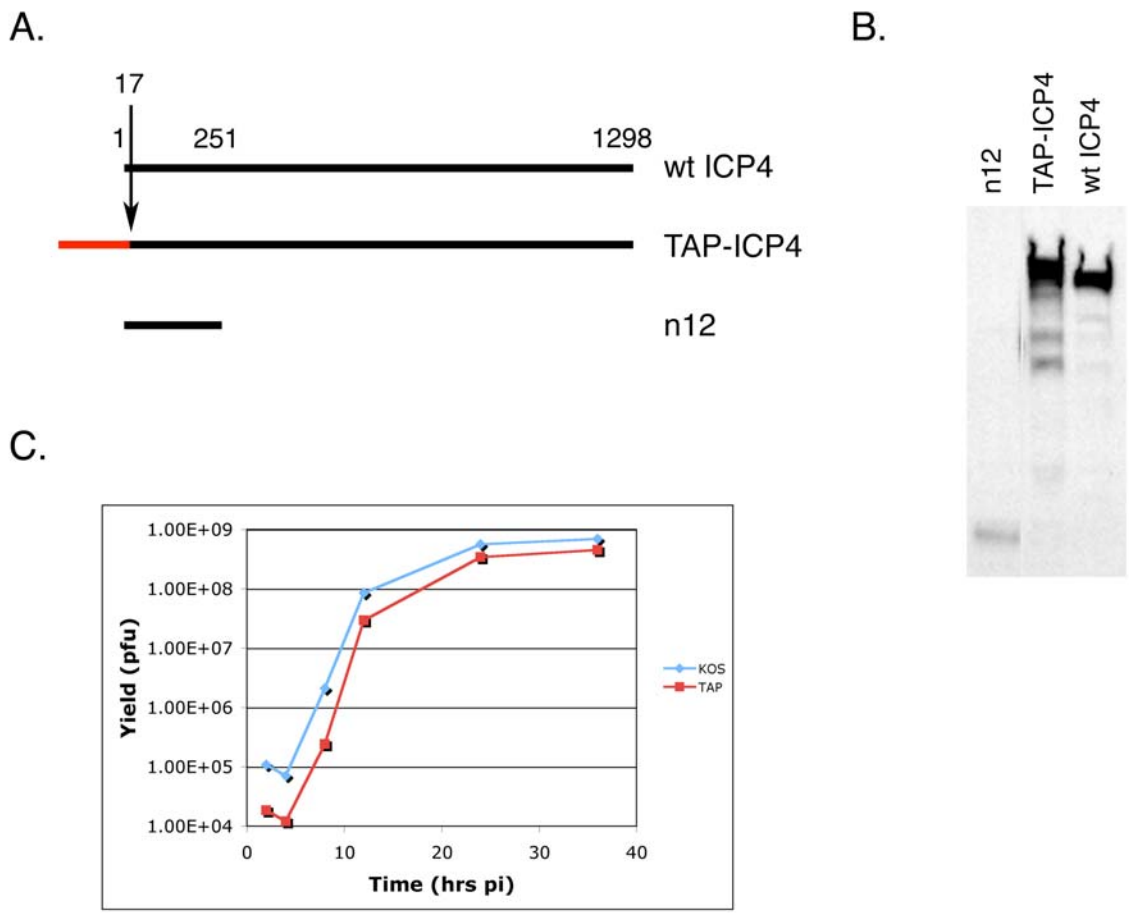

Figure 14: Generation and characterization of TAP-ICP4.
A) Schematic comparing the primary sequence of the ICP4 peptides expressed by the viruses used in this study. The TAP-tag is shown in red. B) Expression of the ICP4 peptides from cells infected with the indicated virus was assessed via western blot of whole cell extracts from vero cells infected with the indicated virus. The proteins were visualized with the ICP4-specific antibody n15. C) The effect of the addition of the TAP-tag on viral growth was analyzed via growth curve. Vero cells were infected at an MOI of 5 with either the TAP virus or KOS. Virus was harvested 2, 4, 8, 12, 24, and 36 hours pi. Viral titers were determined via plaque assay.

4.4.2 Optimization of TAP purification of ICP4

The TAP method purifies protein complexes using two affinity steps, an irreversible prot A:IgG interaction and the reversible CBP:calmodulin interaction. This allows for a gentle, but specific isolation of complexes from cells. The preparation of the protein extracts prior to purification is a critical step in any purification scheme. It is crucial to efficiently isolate the target protein

while retaining the integrity of any complexes to which it participates. We chose to prepare protein extracts using the Dignam high salt extraction method; a method that is commonly used to prepare transcriptionally active nuclear extracts (54). Both the nuclear extraction and TAP procedure were optimized for maximal ICP4 purification. Nuclei were isolated from TAP-ICP4 infected HeLa cells 6 hours post-infection as described in the materials and methods. The isolated nuclei were divided into 4 treatments and extracted in buffer containing 150, 200, 300, or 500 mM KCl. The resulting nuclear extracts were submitted to the TAP procedure. Both unconcentrated (-) or concentrated (+) TAP-purified samples were separated via SDS-PAGE and visualized by silver staining (figure 15A). ICP4, the dark band near the top of the gel, was most efficiently isolated using 200 and 300 mM KCl. There was a marked reduction in ICP4 at 500 mM KCl, due, at least in part, to the lysing of the nuclei resulting in significant DNA contamination and reduction in the extract yield. Despite only a slight difference in total ICP4 isolated at 200 and 300 mM KCl, there is an increase in proteins copurifying with ICP4 at higher salt, as shown by the increased banding complexity at 300 mM KCl. Indeed, the 500 mM KCl sample had less ICP4 than the 200 mM salt, but had more copurifying proteins. Thus ICP4 extraction exhibits a slight salt dependence, but the proteins with which it interacts have a greater dependence, with the most efficient copurification at higher salt concentrations.

The experiment outlined above measured the final, TAP-purified samples. It is possible that any change in protein complex purification could be attributed to the efficiency by which the individual proteins are initially extracted from the nucleus. To test this, the concentrated TAP-purified samples from above along with aliquots from the corresponding nuclear extracts were separated via SDS-PAGE and submitted to western blotting (figure 15B). The blots were probed with antibodies for ICP4 and TBP (figure 15B, upper left and lower left, respectively). TBP was

chosen because it has been shown to interact with ICP4 in several studies. There was a slight increase in the amount of ICP4 purified at higher KCl concentrations, as seen by silver staining. There was a corresponding slight increase in total ICP4 initially extracted from the nucleus, suggesting the efficiency of TAP-ICP4-pull down is primarily dependent on the initial nuclear extraction (figure 15B, upper left). In contrast, extraction of the 37 kDa TBP was not affected by the salt concentration used, but, in accordance with the pattern seen in the silver stained gel, it only copurified with ICP4 at higher KCl concentrations (figure 15B, lower left). The high molecular weight bands seen in the nuclear extracts of the TBP blot correspond to the Prot A domain of the TAP-tag binding to the antibody used in the western blot. This band is not seen in the final, purified lanes because the Prot A domain is cleaved from the tag as part of the purification procedure. This shows that the TAP procedure is capable of successfully isolating ICP4-containing protein complexes, and that at least a subset of these complexes is only efficiently extracted from the nucleus at high salt concentrations.

In order to confirm the salt dependency of complex extraction and purification, nuclei were again isolated but were extracted with only the 300 mM or 500 mM KCl buffers. The nuclear extracts were purified via TAP, and the concentrated TAP-purified samples and their corresponding nuclear extracts were submitted to western blotting. The resulting blots were probed with TBP and TAF1 (figure 15B, lower right and upper right, respectively), both of which have been shown to interact with ICP4. Again, there was no difference in the amount of either TBP or TAF1 in the 300 and 500 mM KCl nuclear extracts. There was a slight increase in the total amount of TBP that copurified with ICP4 in the 500 mM KCl sample. TAF1, however, showed a dramatic increase in copurification from the 300 to 500 mM KCl extracted samples. Thus, ICP4 protein complexes containing TBP and TAF1 are tightly retained in the nucleus and

require high salt to efficiently extract them, whereas TBP and TAF1 not interacting with ICP4 do not require high salt. In addition, at least a portion of ICP4:TBP complexes appear to not include TAF1, as shown by the TBP copurifying with ICP4 at 300 mM salt compared to the relative little amount of TAF1 interacting. These experiments indicate that the highest KCl concentration that could reliably be employed to prepare the nuclear extracts should be used. Unfortunately, the 500 mM KCl buffer often resulted in nuclear lysis during purification; as a result, 400 mM KCl was used in all subsequent TAP experiments.

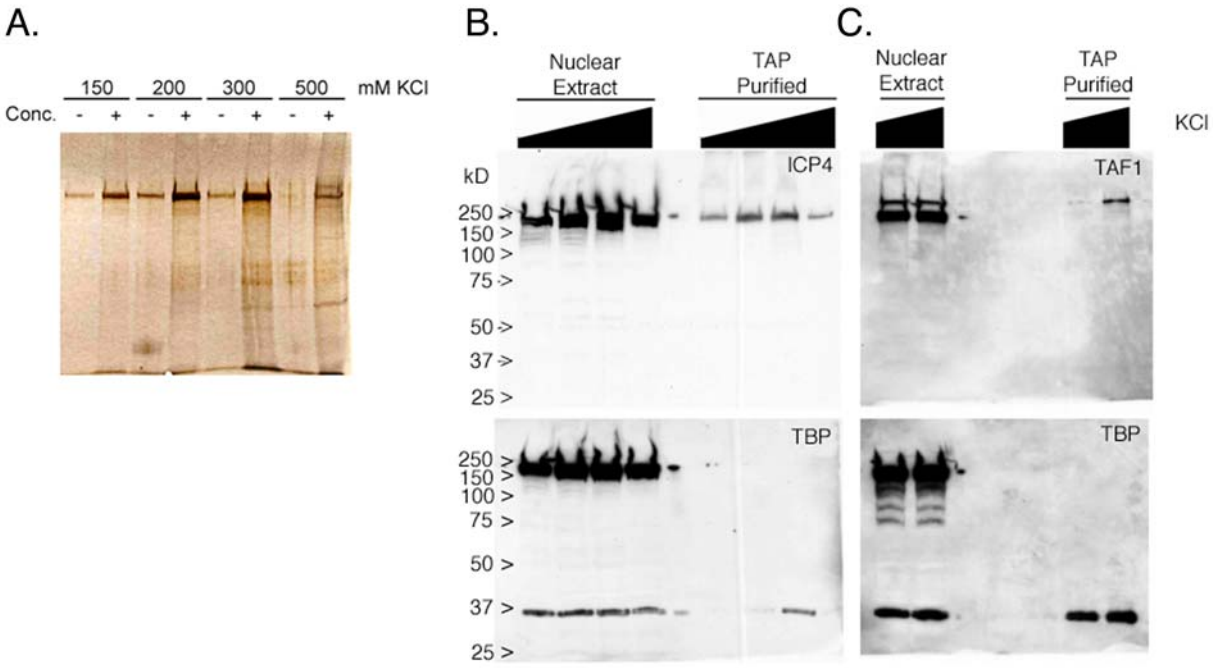

Figure 15: Effect of KCl concentration on protein extraction and complex isolation via TAP.
A) Isolated nuclei from infected HeLa cells were divided into 4 samples and submitted to TAP using increasing KCl concentrations of 150, 200, 300, and 500 mM for protein extraction. The resulting TAP-purified samples were separated via SDS-PAGE before and after concentration and protein bands visualized via silver staining. B) Nuclear extracts and concentrated TAP samples from A were probed for ICP4 and TBP via western blot. The high molecular weight band in the nuclear extract is the protein A subunit of the TAP-tag binding to the secondary antibody during western blotting. This band is absent in the TAP purified lane due to the cleavage of the protein A domain during purification. C) The experiment in A was repeated with the 300 and 500 mM KCl concentrations and probed via western blot for TBP and TAF1.

4.4.3 Characterization of proteins copurifying with ICP4

The goal of this study was to characterize those proteins with which ICP4 forms complexes during the course of infection. TAP was used to isolate those complexes and mass spectrometry was used to characterize them. 1.5 x 10^9 HeLa cells were infected with either KOS or TAP-ICP4. Six hours post-infection, the cells were harvested and ICP4 protein complexes were isolated using the TAP procedure as described in the materials and methods. The purified protein complexes were separated via SDS-PAGE and visualized via silver and colloidal blue staining (figure 16). Many more proteins were isolated by TAP from the TAP-ICP4 compared to KOS infected nuclear extracts; however, some proteins were purified from the KOS nuclear extracts, suggesting a low frequency of non-TAG specific purification. As expected, ICP4 was the major component of the purified samples (figure 16, TAP-ICP4 major 175 kDa band) due to both its being the target protein for purification and its higher abundance compared to most cellular proteins.

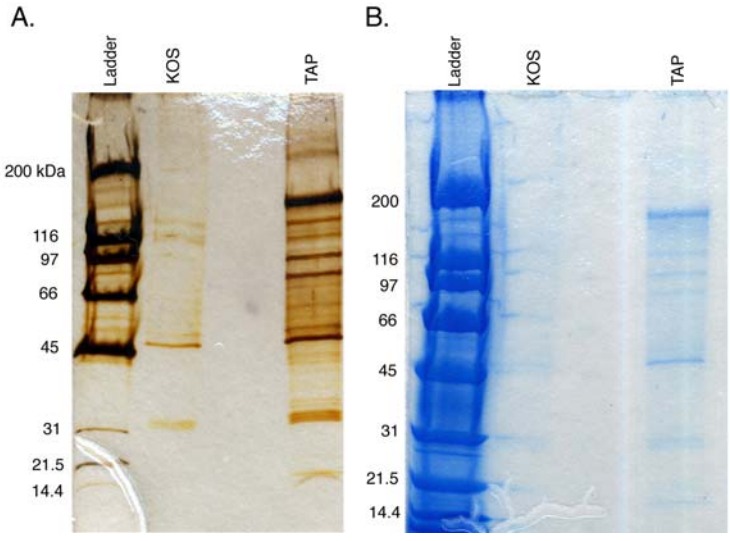

Figure 16: Visualization of total proteins interacting with ICP4.
TAP-purified samples from nuclear extracts of TAP-ICP4 and KOS infected cells were prepared. Samples were separated via SDS-PAGE, and total protein was visualized by A) silver stain and B) colloidal blue stain in preparation for MS analysis.

In preparation for the mass spectroscopic analysis, the purified samples were separated via SDS-PAGE and visualized with colloidal blue (figure 16). To ensure characterization of both high- and low- abundance constituents, gel slices were excised from the length of the TAP-ICP4 lane with an emphasis on clearly visible bands. The proteins were digested *in situ* with trypsin and the resulting peptides were eluted from the gel and characterized via LC-MS/MS. The analysis identified with high confidence 46 proteins listed in table 2. The Uniprot, protein name, probability score (Ppro) assigned by the SEQUEST software, and molecular weight of the protein are indicated. The Ppro score is the probability that the protein listed is a random match to the spectral data. Only those proteins with a Ppro score of less than 10^{-3} are listed. The proteins were initially categorized on whether they were identified from gel slices of the appropriate molecular weight. Of the 46, 14 were identified from gel slices corresponding to incorrect weights. This does put the identity of these proteins in question; however, the inherent inefficiency in protein separation using SDS-PAGE and the possibility of degradation products in the gel may explain this discrepancy.

The remaining 32 proteins were further divided into four categories: viral proteins, transcription factors, calmodulin binding proteins, and other, miscellaneous proteins. Two proteins that have previously been shown to interact with ICP4, TBP and L22, were identified. The L22 and ICP4 interaction was shown through a two-hybrid screen (158). The isolation of L22 via TAP and the identification via MS shows again that TAP and MS are valid methods of isolating and characterizing ICP4 protein complexes, respectively. Of particular interest for this study are the 11 transcription factors found to copurify with ICP4. TFIID has previously been shown to interact with ICP4, and the identification of TBP and 8 TAFs verifies the *in vitro* findings. Interestingly, TAF1 was not identified in the MS analysis, despite being present as

shown by western blot (figure 15). Whether this is due to inefficient separation vis SDS-page, inefficient trypsin digestion, inefficient elution from the gel, or general recalcitrance to MS identification is unclear. In addition to components of TFIID, MS analysis also identified Med8, a component of the Mediator complex, and TFII-I, a transcription factor important for Inr-driven transcription were all identified to copurify with ICP4.

Several proteins found in the TAP-purified samples are likely not interacting with ICP4 but are present as a result of the purification system used. IgG kappa chain C is most likely a contaminant from the IgG column, and 7 proteins have previously been shown to interact with calmodulin (108, 268, 280, 282, 285). These contaminating false positives are most likely responsible for the proteins found in the KOS-infected lane in the silver stained gel (figure 16). Indeed, the strong, approximately 50 kDa band found in both the TAP-ICP4 and KOS infected lanes corresponds to the calmodulin-binding elongation factor 1-α1 and 2.

The MS analysis identified only two viral proteins: ICP4 and, interesting, the U_S3 kinase. ICP4 has previously been shown to interact *in vitro* with both ICP0 and ICP27 *(204, 309)* but neither was identified in the MS analysis. To confirm the MS results, western blot analysis was performed on TAP-purified samples from both KOS and TAP-ICP4 infected cells. Samples were harvested as submitted to western blotting using antibodies against ICP4, ICP0, and ICP27 as described previously. Significant amounts of ICP4, ICP0, and ICP27 were all detected in the nuclear extracts lane derived from TAP-ICP4 infected cells (figure 17). Significant levels of ICP4 were detected in the TAP-ICP4 purified lane, but none were detected from the KOS lane, thus ICP4 purification via TAP is dependent on the presence of the TAP-tag. No ICP0 or ICP27 was detected in either the KOS or TAP-ICP4 purified lanes showing in these conditions, ICP4 does not interact with ICP0 or ICP27.

Table 2: Proteins identified by MS analysis.

	Uniprot desig	Protein	P(pro)	MW
Viral proteins	ICP4_HHV11	Trans-acting transcriptional protein ICP4- Human herpesvirus 1	5.57E-11	132843.3
	KR1_HHV11	Serine/threonine-protein kinase - Human herpesvirus 1	3.32E-04	52834.3
Transcription	TBP_HUMAN	TATA-box-binding protein (TATA-box factor)	1.50E-04	37697.9
	TAF9B_HUMAN	Transcription initiation factor TFIID subunit 9B	4.55E-07	27621.7
	TAF6_HUMAN	Transcription initiation factor TFIID subunit 6	6.58E-09	72668.2
	TAF5_HUMAN	Transcription initiation factor TFIID subunit 5	3.38E-07	86829.6
	TAF4B_HUMAN	Transcription initiation factor TFIID subunit 4B	3.54E-07	91090.0
	TAF4_HUMAN	Transcription initiation factor TFIID subunit 4	6.77E-12	110113.7
	TAF3_HUMAN	Transcription initiation factor TFIID subunit 3	8.16E-04	103581.1
	TAF13_HUMAN	Transcription initiation factor TFIID subunit 13	2.24E-06	14287.0
	TAF10_HUMAN	Transcription initiation factor TFIID subunit 10	7.50E-05	21711.2
	MED8_HUMAN	Mediator of RNA polymerase II transcription subunit 8 homolog	9.78E-04	29079.7
	GTF2I_HUMAN	General transcription factor II-I (GTFII-I) (TFII-I)	1.15E-05	112415.6
Miscellaneous	WDTC1_HUMAN	WD and tetratricopeptide repeats protein 1	8.07E-04	75919.3
	TTC17_HUMAN	Tetratricopeptide repeat protein 17	3.07E-04	129557.5
	TMOD1_HUMAN	Tropomodulin-1	7.84E-04	40568.9
	ROA1_HUMAN	Heterogeneous nuclear ribonucleoprotein A1 (Helix-destabilizing protein)	1.84E-05	38714.3
	RL22_HUMAN	60S ribosomal protein L22	3.22E-07	14655.7
	PAWR_HUMAN	PRKC apoptosis WT1 regulator protein	2.90E-05	36567.2
	NUD10_HUMAN	Diphosphoinositol polyphosphate phosphohydrolase 3 alpha	9.09E-04	10499.0
	K1967_HUMAN	Protein KIAA1967 (Deleted in breast cancer gene 1 protein)	2.03E-05	102900.9
	HS70L_HUMAN	Heat shock 70 kDa protein 1L (Heat shock 70 kDa protein 1-like)	4.03E-05	70374.6
	GFAP_HUMAN	Glial fibrillary acidic protein, astrocyte (GFAP)	3.09E-06	49879.9
	CSK21_HUMAN	Casein kinase II subunit alpha	3.20E-07	45143.3
	CALR_HUMAN	Calreticulin precursor (CRP55)	9.05E-04	48141.1
Calmodulin binding	MYLK2_HUMAN	Myosin light chain kinase 2, skeletal/cardiac muscle	4.75E-04	64553.3
	MATR3_HUMAN	Matrin-3 - Homo sapiens	1.11E-05	94622.6
	EF1A2_HUMAN	Elongation factor 1-alpha 2 (EF-1-alpha-2)	9.24E-04	50469.8
	EF1A1_HUMAN	Elongation factor 1-alpha 1 (EF-1-alpha-1)	3.05E-07	50140.5
	DCAK1_HUMAN	Serine/threonine-protein kinase DCAMKL1	3.03E-04	82223.5
	CXB3_HUMAN	Gap junction beta-3 protein (Connexin-31)	4.18E-04	30817.8
	CALM_HUMAN	Calmodulin (CaM)	2.83E-05	16706.3
Incorrect MW	S10A7_HUMAN	Protein S100-A7 (S100 calcium-binding protein A7)	8.60E-08	11325.7
	RYR1_HUMAN	Ryanodine receptor 1 (Skeletal muscle-type ryanodine receptor)	5.38E-01	565179.0
	RCOR3_HUMAN	REST corepressor 3	6.65E-04	55581.1
	RBCC1_HUMAN	RB1-inducible coiled-coil protein 1	6.65E-04	183059.0
	PRD15_HUMAN	PR domain zinc finger protein 15	2.64E-04	169267.5
	PI4KA_HUMAN	Phosphatidylinositol 4-kinase alpha (PI4-kinase alpha)	7.84E-04	231289.6
	NSN5B_HUMAN	NOL1/NOP2/Sun domain family member 5B	1.37E-04	17679.2
	MAS1L_HUMAN	Mas-related G-protein coupled receptor MRG	9.85E-04	42410.4
	KAC_HUMAN	Ig kappa chain C region	6.33E-14	11608.8
	ITPR2_HUMAN	Inositol 1,4,5-trisphosphate receptor type 2	7.70E-06	308075.1
	GRB10_HUMAN	Growth factor receptor-bound protein 10	1.20E-05	67231.1
	F109A_HUMAN	Protein FAM109A	4.75E-04	27215.1
	CO032_HUMAN	Uncharacterized protein C15orf32	3.36E-04	20262.4
	APTX_HUMAN	Aprataxin	5.30E-04	40739.8

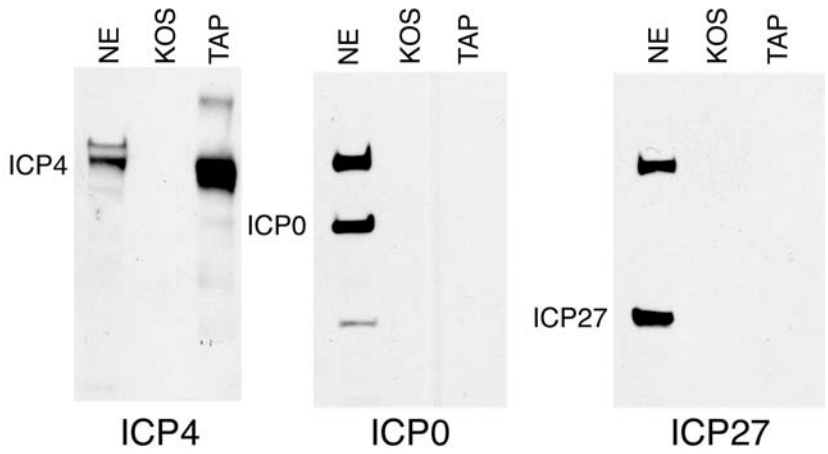

Figure 17: Interaction of ICP4 with ICP0 and ICP27.
TAP-purified samples from nuclear extracts of TAP-ICP4 and KOS infected cells were prepared. Samples were separated via SDS-PAGE and submitting to western blotting using antibodies against ICP4, ICP0, and ICP27.

4.4.4 ICP4 interaction with TFIID

As mentioned previously, ICP4 has been shown to interact with both TBP and TAF1, both components of TFIID, through a variety of *in vitro* methods, and through these interactions, stimulate PIC formation and transactivation (26, 92). It has been extrapolated from these data that ICP4 interacts with TFIID, a large cellular complex made up of 14 subunits. TBP and several other TAF components of TFIID were identified in ICP4-containing complexes isolated via TAP (table 2). In order to confirm those results and to ensure their association is specific to the TAP-tagged ICP4, western blot analysis was performed on purified samples from KOS and TAP-ICP4 nuclear extracts as described above. The resulting blots were probed for specific subunits of TFIID: TAF1, TAF4, TAF7, and TBP (figure 18), two of which (TAF4 and TBP) were identified via MS. All four proteins were detected both in the TAP-ICP4 nuclear extract and the TAP-ICP4 lane. None were detected in the KOS infected lane, showing that isolation via TAP required TAP-ICP4. Thus, ICP4 interacts with several subunits of TFIID, including TBP,

TAF1, -3, -4, -4b, -5, -6, -7,-9b, -10, and -13 as shown either by MS or western blot of TAP-ICP4 complexes.

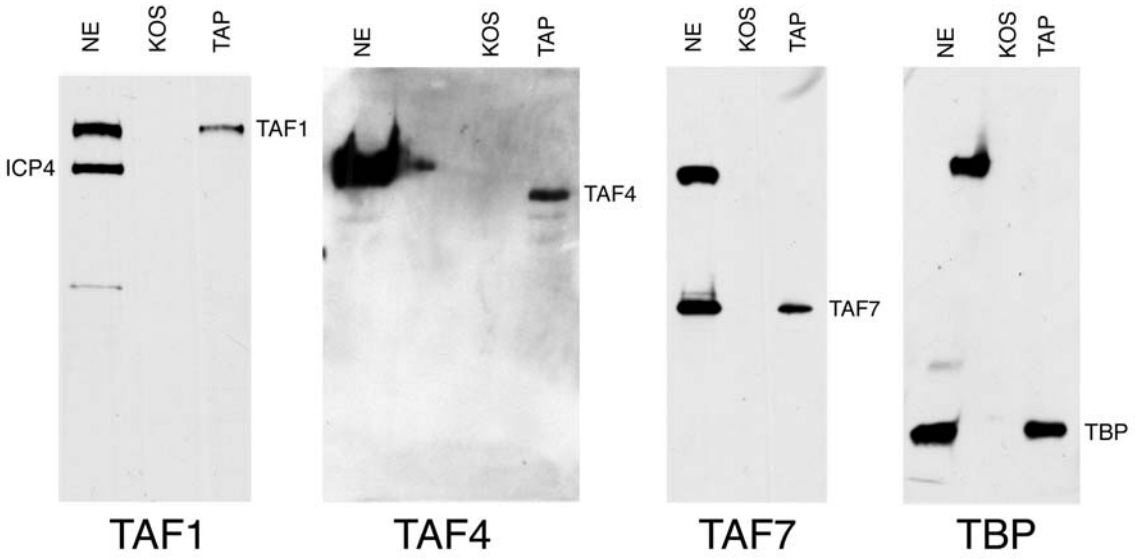

Figure 18: Interaction between ICP4 and TFIID.
TAP-purified samples from nuclear extracts of TAP-ICP4 and KOS-infected cells were prepared. Samples were separated via SDS-PAGE and submitted to western blotting using antibodies against TAF1, TAF4, TAF7, and TBP.

4.4.5 ICP4 interacts with Mediator and recruits it to viral promoters.

The Mediator component Med8 was identified via MS in ICP4-complexes purified via TAP. Mediator is a large cellular protein complex made up of over 30 subunits. It functions primarily as a bridge between upstream activators and the GTFs and RNA pol II. Western blot analysis was used to confirm the presence of Mediator subunits in TAP-purified ICP4 complexes, as described previously. Western blots were probed for three distinct subunits of Mediator: TRAP220, CRSP77, and Med7 (figure 19A). Antibodies to Med8, the subunit identified in the mass spectroscopic analysis, were unavailable. TRAP220, CRSP77, and Med7 were all detected

specifically in the TAP-ICP4 lane, showing again that their copurification required the presence of TAP-tagged ICP4.

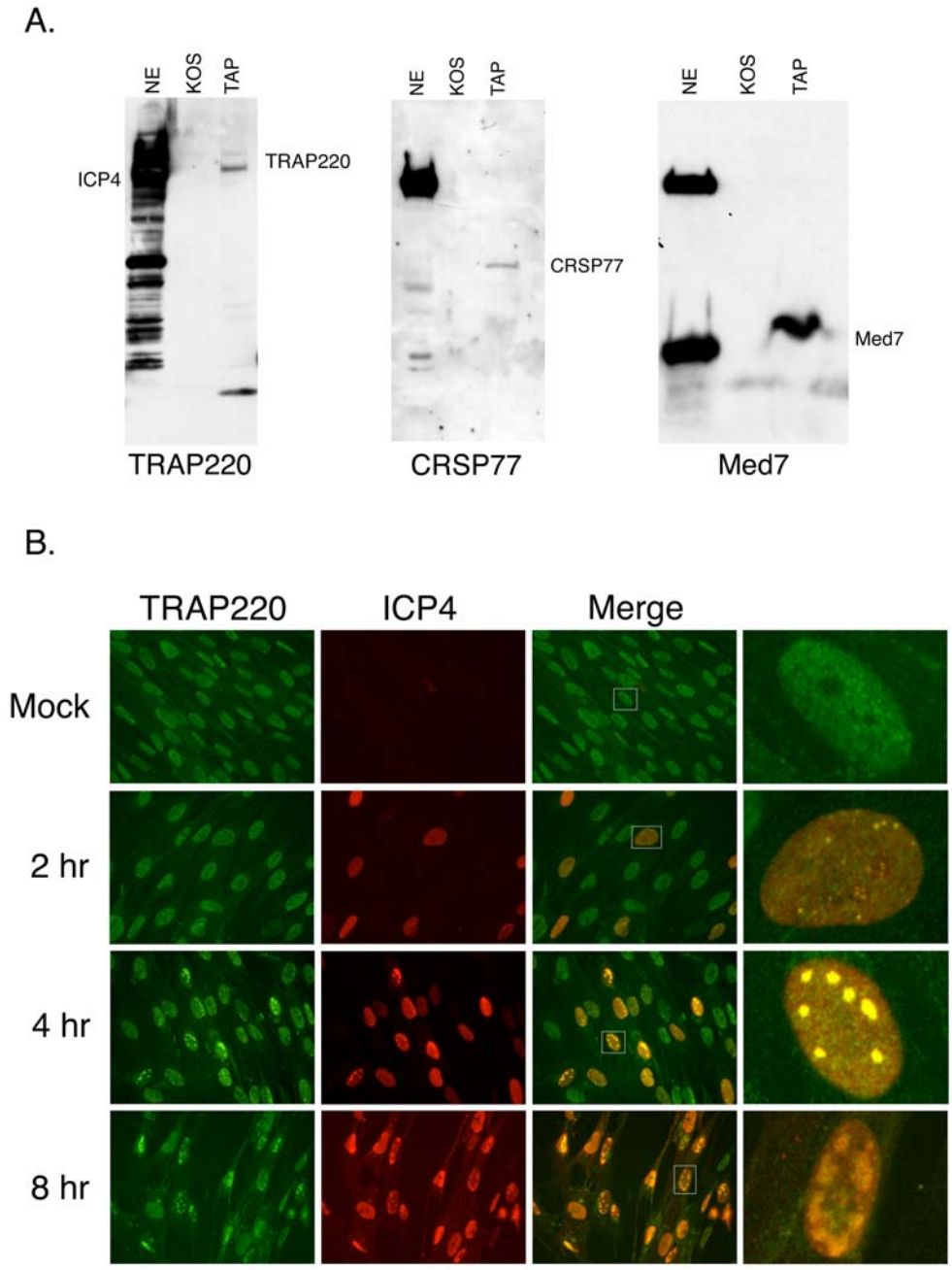

Figure 19: Interaction and colocalization of ICP4 and Mediator.
A) TAP-purified samples from KOS and TAP-ICP4 infected cells were prepared. Samples were separated via SDS-PAGE and western blotted for TRAP220, CRSP77, and Med7. B) HEL cells were infected at an MOI of 10. The infections were allowed to proceed for 2, 4, or 8 hpi pror to fixation and treatment for immunfluorescence. The cells were stained for ICP4 (red) and TRAP220 (green). An enlarged view of a representative cell from the merged panel is also shown.

VP16, the viral transactivator integral to IE gene activation, has previously been shown to interact with Mediator subunits (306). Mediator has not, however, been implicated in ICP4-mediated expression of either E or L genes. In order to investigate the kinetics of ICP4-Mediator association, immunofluorescence of infected HEL cells was used. HEL cells were infected at an MOI of 10 with KOS and fixed at 2, 4, or 8 hours post-infection (figure 19B). The cells were then stained with antibodies directed against the Mediator component TRAP220 in green and ICP4 in red. A digitally magnified view of a single, representative HEL cell as shown by the white box is also shown. As expected, no ICP4 signal was seen in the Mock infected cells. By 2 hpi, a subset of infected cells are expressing ICP4 as seen by a diffuse, nuclear stain with several small ICP4 foci. At least a subset of these foci contain viral genomes and are sites of viral transcription and, later, DNA replication (269). By 4 hpi, ICP4 can be seen localizing into small replication compartments, which by 8 hpi, are fully matured. TRAP220 exhibits a diffuse, primarily nuclear staining. HSV infection, however, induces a dramatic reorganization of TRAP220. As early as 2 hpi, those cells expressing ICP4 show recruitment of TRAP220 into the small, ICP4-containing foci. As infection proceeds and the ICP4 foci enlarge and mature, TRAP220 recruitment to and colocalization with ICP4 continues. Thus, TRAP220, and by extension Mediator, is recruited to ICP4 foci early in infection and continues through late times of infection.

The previous experiments suggest Mediator plays a direct role in ICP4-mediated viral transcription. In order to test this, Mediator recruitment by ICP4 to promoters representative of the three classes of viral genes was assessed via chromatin immunoprecipitation. Vero cells were infected at an MOI of 10 with n12 or KOS, and ChIP analysis was performed 4 hpi as described in the materials and methods. The association of ICP4, RNA pol II, and Mediator to

the ICP0, tk, and gC promoters was analyzed as a function of wildtype (KOS) or nonfunctional (n12) ICP4. The data is represented as the total number of genomes precipitated determined by qRT-PCR as described previously (figure 20). The primers used to amplify the ICP0, tk, or gC promoters are described in table 1. As expected, wildtype ICP4 was found at high levels at all of the promoters tested; whereas the n12 ICP4, which lacks a DNA-binding domain, was not detected (figure 20, left panel). Thus, comparison of protein association to promoters in KOS and n12 infected cells allows for analysis of the role functional ICP4 plays in recruitment of these proteins.

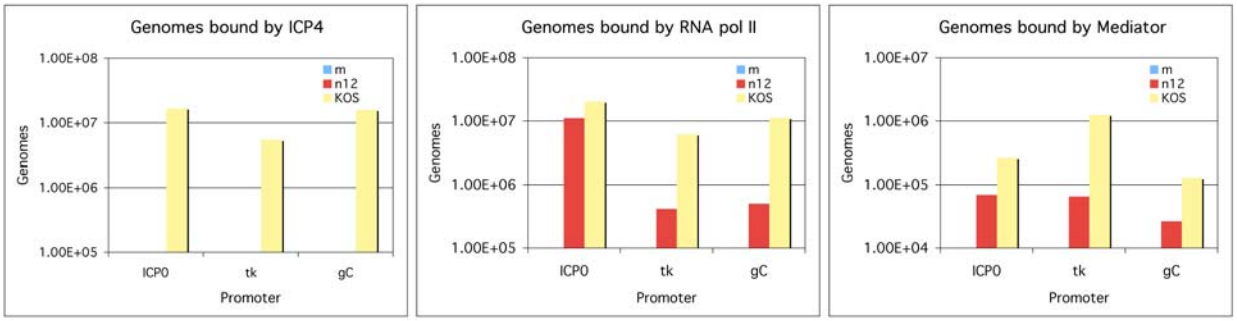

Figure 20: Binding of ICP4, RNA pol II, and Mediator to the ICP0, tk, and gC promoters in KOS and n12 infected cells.
ChIP analysis was peformed 4 hpi to assess the binding of Mediator and RNA pol II to the ICP0, tk, and gC promoters as a function of ICP4 and DNA replication. The total genomes bound and the percent genomes relative to the input are shown.

IE genes reach peak expression between two and four hours, after which their expression declines. Thus, at four hours, there should be robust expression of ICP0, an IE gene representative. In addition, gene expression profiles of KOS and n12 show that n12 overexpresses IE genes compared to KOS showing that IE gene expression is independent of

ICP4 function. Indeed, previous ChIP studies have shown RNA pol II is efficiently recruited to ICP0 promoters independently of ICP4 (247). As expected, the data in figure 20 show that RNA pol II associates with the ICP0 promoter both in the presence (KOS) and absence (n12) of functional ICP4 (figure 20, middle panel). By analogy, Mediator recruitment should also be independent of ICP4. Interestingly, there was a higher association of Mediator at ICP0 promoters in KOS compared to n12 infected cells (figure 20, right panel). This may be explained in part by an increase in total viral genomes due to low levels of viral DNA replication at 4 hpi seen in KOS but not n12, which does not replicate its DNA. It may also be due to a more efficient recruitment of Mediator by ICP4 compared to VP16 or an artifact of the experimental system. It is possible that the transcription complexes assembled by VP16 on ICP0 may partially block the antibody binding sites on TRAP220 and CRSP77 used for the assay, thus reducing the efficiency of antibody pulldown.

E gene expression reach maximal levels between 4 and 6 hour post-infection and are dependent on functional ICP4. As expected, RNA pol II is associated to the tk promoter 4 hpi in an ICP4 dependent manner, with over 10 fold less associated during n12 infection compared to KOS (figure 20, middle panel). Similarly, Mediator recruitment to the tk promoter also required functional ICP4 with a similar, over 10-fold increase in Mediator association during KOS infection compared to n12.

Late gene expression reaches peak levels after E genes and the onset of DNA replication and requires functional ICP4 for expression. Thus, at 4 hpi, robust Late gene expression would not be expected. Previous ChIP studies have shown, however, that TBP and RNA pol II associate to gC in the absence of DNA replication and Late gene expression in an ICP4 dependent manner. Indeed, RNA pol II associates maximally with the gC promoter only in the

presence of ICP4 (figure 20, middle panel). Likewise, efficient Mediator association also required functional ICP4 with just under a 10-fold increase in KOS infected cells. In summary, figure 20 shows that Mediator is found associated to promoters of representatives of all three classes of viral genes. In addition, the association of Mediator was increased in the presence of functional ICP4. This, combined with the data showing Mediator is a component of purified ICP4 protein complexes, suggests that ICP4 recruits Mediator to promoters.

4.5 DISCUSSION

ICP4 is the major viral transcriptional regulator, and is necessary for the proper expression of all three major classes of viral genes (48, 198, 217). It represses its own transcription and transactivates both E and L genes. Several studies have shown that ICP4 interacts with both TBP and TAF1 of TFIID through N- and C-terminal regulatory domains to recruit and stabilize PICs on the promoter (26, 92). These interactions are critical for it to regulate transcription. ICP4's large size, complicated transcription regulatory domains, and ability to activate a diverse set of promoters, however, suggest ICP4 may play a broader role in stimulating transcription than recruitment of TFIID. To investigate this possibility, ICP4 containing complexes were isolated from infected cells using TAP. The proteins contained in these complexes were characterized by MS and western blot analysis. The data show that in addition to TFIID, ICP4 interacts with other cellular factors, including Mediator and TFII-I. The role of Mediator in ICP4 transcription was further analysed using IF and ChIP assays, showing that Mediator associates at representative promoters of the three classes of viral genes. Additionally, efficient Mediator recruitment required ICP4. Taken together, the data show that ICP4 plays a broader

role in viral transcription than recruitment of TFIID to the promoter and provide further insight into the regulation of the viral gene expression cascade.

4.5.1 Complex isolation and characteriztion

The goal of this study is to isolate and characterize protein complexes formed by ICP4 during infection *in vivo*. Because we were particularly interested in ICP4's transcription regulation, the ICP4 complexes were extracted from the nucleus of infected cells prior to purification using the Dingham method of purifying transcriptionally active nuclear extracts (54). The copurification of TBP and TAF1 with ICP4 confirm the previous *in vitro* data (26, 92). In addition, the cellular ribosomal protein L22 was identified by the MS analysis. L22 has previously been shown to interact with ICP4 via a two-hybrid screen (158). The characterization of proteins previously shown to interact with ICP4 validate the TAP method as an efficient method of isolating ICP4 protein complexes as well as the capacity of MS and western blot analysis to characterize them. The MS analysis was unable to identify all the proteins in the TAP-purified complexes, however, as shown by the western blot identification of several proteins, including TAF1, TAF7, TRAP220, Med7, and CRSP77, not identified by MS. Whether this was due to insufficient amounts of these proteins in the sample or their general incompatibility with MS identification is unclear. Thus, although the MS analysis identified several components of ICP4 complexes, it is not comprehensive.

The MS analysis identified several novel proteins. Many of these proteins can be discounted, however, due either to known interactions with calmodulin used during the purification procedure or due to purification from gel slices of inappropriate molecular weight. Those proteins that bind to calmodulin are isolated not based on interactions with ICP4 but with

interactions with the affinity resin used during purification. As a result, they can be seen in samples prepared from TAP-ICP4 and KOS infected extracts (figure 16, table 2).

In addition to the calmodulin-binding proteins, several identified proteins were found in gel slices corresponding to the incorrect molecular weight. This does not necessarily mean the identification is false, however, as protein degradation and inefficient separation by SDS-PAGE could explain the discrepancy. The identification of these proteins should be treated with some skepticism, however, and most of these proteins' relationship with HSV infection is unclear. One notable exception is CoREST, which is part of a complex that is thought to be disrupted during HSV infection (96), and hence a potential interaction between it and ICP4 may warrant further investigation.

4.5.2 ICP4 copurification with protein kinases

The MS analysis also identified two kinases of interest, the HSV U_s3 kinase and casein kinase II (CKII). ICP4 is heavily phosphorylated during the course of infection, and the poly-serine tract, a major site of ICP4 phosphorylation, has consensus sites for the cellular kinases protein kinase A (PKA), PKC, and CKII. ICP4 is phosphorylated *in vitro* by PKA and PKC (302, 303). The serine tract and its phosphorylation by PKA are important for viral growth, particularly in neurons (12). An interaction between CKII and ICP4 and the presence of CKII consensus sites on ICP4 suggest ICP4 may also be phosphorylated *in vivo* by CKII. This analysis is complicated, however, by the observation that CKII also phosphorylates calmodulin (240). Thus, presence in the purified sample may be due to an interaction with calmodulin, not ICP4.

In addition to CKII, the U_s3 kinase was also found in the TAP-purified ICP4 complexes. The U_s3 kinase has multiple roles during HSV infection, including the phosphorylation of

histone deacetylases 1 and 2, CoREST, and U_L34, among other viral and cellular proteins (136, 216, 222). It plays an important role in preventing apoptosis in infected cells, promotes capsid egress from the nucleus, and may play a role an indirect role in the induction of the aberrantly phosphorylated form of RNA pol II during infection (15, 58, 189). Although it has not been shown to directly phosphorylate ICP4, studies of the VZV homologs of U_s3 (Orf66) and ICP4 (IE62) have shown that Orf66 phosphorylates IE62 near its NLS to regulate its nuclear localization during infection (307). ICP4 and IE62 do exhibit similar localization patterns during infection, being predominantly nuclear early in infection and then becoming cytoplasmic late in infection. The change in IE62 localization is more dramatic than ICP4, however, making it unclear if there is a similar mechanism in regulating ICP4's localization.

4.5.3 ICP4 interaction with TFII-I

The only transcriptionally related protein that copurified with ICP4 and does not take part in large protein complexes is TFII-I. TFII-I is a cellular protein involved in both basal and activated transcription through its recognition of the Inr element of the core promoter (238). It is required, along with TFIID, for activation of $TATA^-$ Inr^+ but not $TATA^+$ promoters (168). In addition, TFII-I initiates the formation of PICs that are distinct from those formed by TFIIA, as shown by differential mobility in EMSA studies (237). Taken together, this suggests that TFII-I may play a role specifically in L gene expression, as L gene promoters are the only viral promoters that contain an Inr (298), and L gene expression does not require TFIIA (312). The Inr element is necessary for the L gene's TFIIA independent expression. Thus, TFII-I fits very well with what is known of HSV biology. The ICP4-TFII-I interaction was unable to be confirmed, however, by either western blot or IF (data not shown). This does not necessarily

refute a potential ICP4:TFII-I interaction, as both western blot and IF analysis are dependent on proper antibody:antigen recognition, whereas MS is not. ICP4 may interact with a form of TFII-I that is not recognized by the monoclonal antibody used in these assays. Further study needs to be performed to clarify the role TFII-I plays in ICP4 mediated transactivation of viral genes.

4.5.4 TAF-containing complexes and ICP4 function

Previous *in vitro* studies have shown that ICP4 regulates transcription at least in part through direct interactions with both TBP and TAF1 of TFIID (26, 92). In addition, immunfluorescence studies have shown that components of TFIID, including TBP, TAF1, and TAF4 are reorganized during HSV infection and colocalize with ICP4 (223). Finally, ChIP assays have shown that TBP recruitment to representative E and L promoters during infection is dependent on ICP4 (247). The copurification of both TBP and TAF1 with ICP4 shown here (figure 18, table 2) confirms the previous studies. The additional identification of TAF3, -4, -4B, -5, -6, -7, -9B, -10, -13 (figure 18, table 2) shows that ICP4 stably associates with the entire TFIID complex. Interestingly, an ICP4:TBP complex lacking TAF1 can also be isolated (figure 15). This suggests ICP4 forms at least two distinct complexes with TBP: one with TAF1 and one without. It is reasonable to assume that the complex containing both TBP and TAF1 corresponds to TFIID. The complex lacking TAF1, however, is less clear. ICP4 has previously been shown to participate in a tripartite complex with TBP and TFIIB on an ICP4 DNA binding site that is associated with ICP4-mediated repression (94, 145, 266). The ICP4:TBP complex lacking TAF1 may be reflective of this tripartite complex.

In addition to TFIID, several other TAF-containing complexes have been described. These complexes, including TFTC, STAGA, and PCAF, all play a role in activator-dependent

transcription, adopt structures similar to that of TFIID, and share many of the same enzymatic activities, including HAT, and may play a role in recruiting TBP to promoters (192, 252). It has recently been proposed that a subset of TAFs (TAF4, -5, -6, -9, and -12) form a stable a scaffold on which TFIID and the other TAF-containing complexes are formed (300). Interestingly, TAF4, -5, -6, and -9, were all shown to copurify with ICP4 (figure 18, table 2). TAF12 was also identified to copurify with ICP4 in a second MS experiment (data not shown). Thus, all of the components of the TAF subcomplex copurify with ICP4. It is possible that, in addition to TFIID, ICP4 may activate transcription through interactions with other TAF-containing complexes via this core TAF subcomplex.

4.5.5 Role of Mediator in ICP4-mediated transactivation

In addition to TFIID, components of the Mediator associate with ICP4 during infection (figure 19, table 2). Mediator is a large, multisubunit complex that acts as a general cofactor, bridging activators and the general transcription machinery, primarily through facilitating the entry of pol II into the PIC (129, 154, 276, 279). It also plays a role in activator-dependent stabilization of the scaffold complex that enhances transcription reinitiation from the same promoter (311). The complex can be divided into three domains, the head, middle and tail. The head domain primarily interacts with pol II while the middle and tail domains interact with upstream activators, thus executing its bridging function (18, 27, 38). The mediator subunits identified in this study are found in different regions of the mediator complex; both Med8 and CRSP77 localize to the head region of mediator while Med7 and TRAP220 are found in the middle region. The copurification of components from two distinct regions of Mediator suggests ICP4

associates with the entire complex. Although Mediator has been shown to interact with other viral activators, including HSV VP16, this is the first evidence for an interaction with ICP4.

ICP4 is involved in the proper expression of all three phases of viral gene expression. As this is the first evidence for an ICP4:Mediator interaction, it is unclear what role Mediator plays in ICP4-mediated transcription regulation. The ICP4 complexes used for TAP that showed an ICP4-Mediator interaction were isolated at 6 hpi, at which there is both E and L gene expression. In order to try and determine the temporal role an ICP4:Mediator interaction may play during infection, immunofluorescence studies were performed.

There was a significant reorganization of TRAP220 during infection, with strong co-localization of ICP4 and TRAP220 starting at early and proceeding through late times of infection (figure 19). The characterization of a VP16:Mediator interaction suggests Mediator is involved in IE gene expression (306). The strong colocalization between ICP4 and Mediator suggests an additional role in E and L gene expression.

The role Mediator plays in E and L gene expression was confirmed via ChIP assay. Figure 20 shows that Mediator is present at all three classes of genes, confirming the IF data, and that Mediator association is greatly increased in the presence of ICP4. Because ICP4 is required for the expression of both E and L genes, it is possible that the Mediator recruitment to these promoters is due to their active transcription rather than recruitment by ICP4. Several lines of evidence suggest, however, that Mediator is recruited to the promoter by ICP4. First, the copurification of Mediator with ICP4 suggests a direct interaction between them, as expected in the case of ICP4-mediated recruitment. In addition, ICP0 expression is independent of ICP4, and is efficiently transcribed both in n12 and KOS infections. Mediator association on the ICP0 promoter was also increased in the presence of ICP4, suggesting Mediator recruitment is not

simply due to active transcription. Finally, at 4hpi, late genes are not robustly expressed, thus recruitment of Mediator to these promoters is most likely not due simply to active transcription.

As mentioned previously, this is the first evidence of an ICP4:Mediator interaction. IE62, the VZV homolog of ICP4, has recently been shown to interact with Mediator, however. The N-terminal acidic activation domain of IE62 was shown to directly interact with Med25 of Mediator and IE62 recruits Mediator to an IE62 responsive promoter (307). Although IE62 and ICP4 share significant sequence similarity, ICP4 does not contain an acidic transcription activation domain. IE62's interaction with Mediator is more similar to VP16's interaction with Mediator. VP16 also interacts with Med25 through an acidic activation domain (306), suggesting a common Mediator-interaction motif. ICP4, which lacks an acidic activation domain, must interact with Mediator through a different mechanism. Thus, the interaction with Mediator appears to be conserved amongst the alpha-herpesvirus major transactivators, but appears to have arisen through distinct evolutionary pathways. The implications of this are unclear but present an intriguing distinction between ICP4 and IE62 and their function.

The goal of this study was to isolate and characterize protein complexes ICP4 participates in during infection. The use of TAP allowed for the gentle, yet specific isolation of ICP4 and associated proteins. It is possible, however, that these complexes were not representative of those formed *in vivo*, but of those form *in vitro* during the purification steps. The high salt-dependent copurification but not nuclear extraction of TBP and TAF1 with ICP4 (figure 15) suggests these complexes are indeed formed in the nucleus and not during later purification steps. The copurification of several members of large cellular complexes show that the TAP method was able to isolate large, ICP4 containing complexes; however, it makes it difficult to

draw any conclusions about direct protein:protein interactions made by ICP4. It does, however provide general insight into what protein complexes are involved in ICP4-mediated transcription.

Viral gene expression follows a highly regulated cascade of IE, E, and L genes. Specifically how this cascade is attained is not completely understood, but several lines of evidence show that E and L genes are differentially activated both from the cellular and the viral standpoint. Efficient activation of E genes is dependent on the action of upstream cellular activators, such as Sp1, and the GTF TFIIA, whereas L gene activation is not (35, 178). Proper L gene expression requires DNA replication, the Inr element, and the C-terminal transactivation domain of ICP4, whereas E gene expression does not (93, 140). Indeed, loss of the C-terminus of ICP4, and thus the ICP4-TAF1 interaction, disrupts L but not E gene expression, as well as the recruitment of TBP to L but not E promoters (247). This suggests ICP4-mediated recruitment of TBP to the different gene classes is through distinct mechanisms and that this difference may specifically be the context in which TBP is recruited. The observation that ICP4 forms two distinct complexes with TBP, one with and without TAF1 (figure 15), further supports this hypothesis.

That TFIIA expression is impaired at late times of infection has been well established (312). Additionally, TFIIA is dispensable for L but not E gene expression in an Inr element dependent manner (312). Interestingly, TFII-I-induced PICs are distinct from those formed by TFIIA, further supporting the role of TFII-I specifically in L gene expression (237). TFIIA has a wide role in transcription which includes stabilization of free TBP monomer interacting at the promoter (36). TFIIA also plays a role in recruiting TBP to promoters mediated by the yeast SAGA complex (291), a homolog of the TAF containing complex STAGA. This and the copurification of the TAF stable-subcomplex thought to act as a scaffold for the formation of

both TFIID and the other TAF-containing complexes suggest that ICP4-mediated recruitment of TBP to E genes may not be through TFIID but through the other TAF-containing complexes.

The studies described here provide great insight into the mechanism of ICP4 transactivation of viral genes. They show that ICP4 has a broad role in transcription, acting at the promoter through interactions with the GTFs through TBP and TAFs as well as TFII-I and through interactions with upstream cofactors such as the mediator complex. The investigation into the specific role of some of these interactions on activation at specific promoter classes will further understanding both of the global regulation of viral genes as well as cellular transcription in general. HSV provides a unique system in which to study the role of upstream activators, core promoter elements such as Inr sequences, and formation of distinct PICs in transcription. It is clear ICP4 activates transcription through interactions with a variety of cellular factors. It is likely the mechanism of ICP4 transactivation of E and L genes is through recruitment of distinct cellular complexes. Direct analysis of occupancy related to ICP4 at E and L promoters is necessary to fully understand the local function of ICP4 and the global regulation of the viral gene cascade.

5.0 SUMMARY AND GENERAL DISCUSSION

HSV gene expression follows a strictly regulated temporal cascade that can be divided into three broad phases: the IE, E, and L genes. This cascade is regulated by a complex physical and functional interaction between viral and cellular factors. ICP4, the major viral transcription regulator, is required for the proper expression of all three classes. Through its repression of its own IE expression and transactivation of E and L genes, ICP4 plays a crucial role in the viral lifecycle (48, 67, 84, 86, 197, 198, 224). In addition, HSV infection dramatically alters the makeup of the cellular transcription machinery during the course of infection (77, 139, 229, 312). Thus, ICP4 must be able to regulate transcription from a diverse set of promoters in a changing cellular transcription mileu. ICP4 has previously been shown to have N- and C-terminal transcriptional regulatory domains that interact with TBP and TAF1 of TFIID to both activate and repress transcription (26, 92, 145, 256, 266). While the N- and C-terminal regulatory domains are separated by a large stretch of amino acids in the primary sequence, temperature-sensitive mutations in the C-terminus can impact the function of the N-terminus (37). ICP4's large size, complex structure, and ability to act at diverse promoters and in changing environments, led to the hypothesis that ICP4's transcriptional regulatory domains come together to form a tertiary structure, providing large surfaces for interactions with a variety of cellular and possibly viral proteins to orchestrate the gene expression cascade. The goals of the study were to

characterize the N-terminal regulatory domain and the role of the C-terminus in its function and to investigate the various protein interactions made by ICP4, *in vivo*.

To characterize the N-terminal regulatory domain, deletion mutants dissecting the region were made. These deletions were made both with and without an intact C-terminal domain in order to to determine the role of the C-terminus in the function of the N-terminal domain. In this study, we show that the entire N-terminal regulatory domain is necessary for viral replication and ICP4 function, since viruses expressing ICP4 with either the 3-8 (aa 30-142) or 8-10 (aa 142-210) deletions exhibited reduced viral replication (figure 7), altered protein expression profiles (figure 8), and reduced transactivation of E and L genes (figure 9). Loss of either the 3-8 or 8-10 region did not affect ICP4's ability to repress its own transcription (figure 9) or to bind DNA (figure 10). The mutants lacking the 3-8 region showed a slightly greater reduction in viral growth compared to the 8-10 deletion (figure 7) but a more dramatic reduction in the transactivation function, as exhibited by both protein expression (figure 8) and abundance of tk and gC mRNA (figure 9). These data suggest that while both the 3-8 and 8-10 region play an important role in ICP4 function, the 3-8 region may be more directly responsible for ICP4 transactivation.

ICP4 exhibits several regions of similarity with other alpha-herpesviruses that correspond to functional domains (30, 174). Interestingly, the N-terminal regulatory domain shares the least similarity with IE62. The polyserine tract found in the N-terminus is the only region with significant similarity to IE62 and is contained in the 8-10 region. The 3-8 deletion, which is shown to be important for ICP4 function by this study, does not contain any significant similarity to IE62. Sequence alignments of the ICP4 sequences from HSV-1, HSV-2, and herpes B virus, however, revealed some discreet regions of similarity, including one between aa 81-90 within the

3-8 deletion (figure 13). The observation that both regions shown to be important for ICP4 function include regions of similary conserved amongst closely related viruses suggest that these conserved regions may be responsible for the function attributed to 3-8 and 8-10 deletions. The fact that these regions are conserved amongst differing groups of viruses suggests they play distinct roles in ICP4's function, an observation borne out by this study. Further studies using targeted deletions of these conserved regions will provide further insight into ICP4's function.

The second goal of this study was to isolate and characterize the proteins with which ICP4 interacts during infection. Tandem affinity purification was used to isolate ICP4 complexes from infected cells. These complexes were subsequently characterized through a combination of mass spectroscopic and western blot analyses. Using MS, we were able to identify, with high confidence, a number of proteins that interact with ICP4 (table 2). The identification of proteins previously shown to interact with ICP4, such as TBP, TAF1, and L22, verify the efficacy both of the TAP isolation and the methods used to identify potential interactors (figure 18, table 2). Of particular interest were TBP and the TAFs, Med8, and TFII-I. The mass spec analysis identified TBP and TAFs 3, 4, 4B, 5, 6, 9B, 10, and 13. Western blot analysis verifies the TBP and TAF4 analysis and also identified TAFs 1 and 7 (figure 18). ICP4 has previously been shown to stabilize TFIID binding to promoters *in vitro* (26, 92). The association with TBP and the TAFs in this study, both in the MS and western blot data, reinforces these findings. There are other TAF containing complexes that exist in the cell in addition to TFIID, however, and these complexes include several of the TAFs that co-purify with ICP4, specifically, TAF4, TAF5, TAF6, TAF9b, and TAF10. These complexes, including TFTC, STAGA, and PCAF, all play a role in activator-dependent transcription, adopt structures similar to that of TFIID, and share many of the same enzymatic activities, including HAT, as

TFIID (reviewed in (192)). Recently, a core TAF-subcomplex consisting of TAF4, TAF5, TAF6, TAF9, and TAF12 has been proposed. This subcomplex acts as a scaffold on which all of the TAF-containing complexes are formed (300). The entire TAF subcomplex was identified by MS or western blot in the ICP4 complexes, suggesting ICP4 may associate with this core subcomplex. The copurification of the TFIID-specific TAF1, TAF3, TAF4b, TAF7, and TAF13 confirms the presence of TFIID and shows ICP4 activates transcription from at least some viral promoters through the stabilization of TFIID on these promoters. ICP4's association with the proposed TAF-subcomplex raises the possibility of an interaction with one or more of the other TAF-containing complexes, in addition to TFIID.

The Mediator complex was also shown to associate with ICP4. The mediator acts as a general cofactor, bridging activators and the general transcription machinery, primarily through facilitating the entry of pol II into the PIC (129, 154, 276, 279). It also plays a role in activator-dependent stabilization of the scaffold complex that enhances transcription reinitiation from the same promoter (311). Several different forms of mediator have been isolated, dependent on the technique used for isolation, suggesting the mediator complex consists of a core complex that associates with other proteins depending on the transcriptional environment (reviewed in (278)). The complex can be divided into three domains, the head, middle and tail. The head domain primarily interacts with pol II while the middle and tail domains interact with upstream activators, thus executing its bridging function (18, 27, 38). The presence of Mediator in the TAP sample was shown both by MS (Med8) and western blot (Med7, TRAP220, and CRSP77). In addition, immunofluorescence studies of infected cells showed a significant reorganization of TRAP220 during infection, with strong co-localization of ICP4 and TRAP220 starting at early and proceeding through late times of infection (figure 19). Thus, we were able to show an ICP4-

mediator interaction through western blot (figure 19), mass spectrometry (table 2), and through the strong co-localization of ICP4 and TRAP220 during infection (figure 19). The mediator subunits identified in this study are found in different regions of the mediator complex; both Med8 and CRSP77 localize to the head region of mediator while Med7 and TRAP220 are found in the middle. The copurification of components of two distinct regions of mediator suggests the involvement of the entire mediator complex in viral transcription. These data confirm a role for Mediator in viral growth starting early in infection and continuing into late in infection. In addition, ChIP analysis shows that Mediator is indeed recruited to viral promoters in an ICP4-dependent manner, showing a role for Mediator specifically in ICP4-mediated activation. The characterization of Med7, Med8, TRAP220, and CRSP77 is the first evidence showing a role for mediator in ICP4-mediated activation and suggests ICP4 plays a larger role than solely direct TFIID stabilization onto the promoter.

The final protein of interest is the general transcription factor TFII-I. TFII-I is involved in both basal and activated transcription through its recognition of the Inr element of the core promoter (238). It is required, along with TFIID, for activation of $TATA^{-}$ Inr^{+} but not $TATA^{+}$ promoters (168). In addition, TFII-I initiates the formation of PICs that are distinct from those formed by TFIIA, as shown by differential mobility in EMSA studies (237). This suggests that ICP4-mediated activation of L genes through recruitment of TFIID may also involve TFII-I. Although TFII-I was identified with high confidence in the TAP sample by MS, its presence could not be confirmed by western blot or immunofluorescence. This does not necessarily refute a potential ICP4-TFII-I interaction, as ICP4 may interact with a form of TFII-I that is not recognized by the monoclonal antibody used in these assays. Further study needs to be performed to clarify the role TFII-I plays in ICP4 mediated transactivation of genes.

It is clear from these studies that ICP4 does indeed interact with a variety of cellular factors to regulate transcription. These interactions are facilitated through the functional domains outline above. The N-terminal regulatory domain interacts with TBP while the C-terminus interacts with TAF1 (26, 266). Is unclear what regions mediate ICP4's interaction with Mediator or TFII-I, however. ICP4 and IE62 share significant regions of homology, especially in the C-terminus, and both interact with Mediator. IE62 interacts with Mediator through an acidic activation domain found in its N-terminus (307). ICP4, however, does not encode an acidic activation domain making it unclear which region or regions of ICP4 are necessary for interaction with Mediator. The regions of ICP4 that interact with TFII-I are also not clear. As described earlier however, its probable role in L gene expression implicates the C-terminus. Studies combining the deletion mutants described above and TAP purification and characterization of ICP4-containing protein complexes would provide insight into which region or regions of ICP4 are necessary for interactions with the cellular machinery. This in turn will lead to a better understanding of ICP4 function and the regulation of viral gene expression.

The data obtained through TAP provide general insight into ICP4-mediated transcription and the regulation of the viral cascade of transcription. Viral gene expression follows a highly regulated cascade of IE, E, and L genes. Specifically how this cascade is attained is not completely understood, but several lines of evidence show that E and L genes are differentially activated both from the cellular and the viral standpoint. E gene promoters contain binding sites for several cellular upstream activators, such as Sp1, which serve to recruit and stabilize the PIC at the promoter (132). L genes, in contrast, are very simple and consist only of a TATA box and an Inr element (97, 98, 273). The Inr element is a common cellular promoter element recognized by TAF1 of TFIID that is required specifically for L gene expression (28, 93, 140). In addition

to TFIID, TFII-I recognizes the Inr element and both TFII-I and TFIID have been shown to be necessary for activation of Inr driven promoters (168, 238). This strongly implicates both TFII-I and TFIID specifically in L gene expression. Loss of the C-terminus of ICP4, and thus the ICP4-TAF1 interaction, disrupts L but not E gene expression, as well as the recruitment of TBP to L but not E promoters (247). This suggests ICP4-mediated recruitment of TBP to the different gene classes is through distinct mechanisms and that this difference may specifically be the context in which TBP is recruited.

The final difference in PIC formation at E and L promoters is the involvement of the GTF, TFIIA. Previous studies have shown that TFIIA expression is impaired at late times of infection. In addition, TFIIA is dispensable for L but not E gene expression in an Inr element dependent manner (312). TFIIA has a broad role in transcription, including stabilization of free TBP monomer binding to the promoter (36). In addition, TFIIA has been implicated in the recruitment of TBP to promoters mediated by the yeast SAGA complex (291), a homolog of the TAF containing complex STAGA. It is possible, in light of several lines of evidence outlined above, that ICP4-mediated recruitment of TBP to E genes may not be through TFIID but through the other TAF-containing complexes. There is no direct evidence either for or against this, however it is an intriguing hypothesis that fits the known data.

The further investigation into the specific role these proteins interactions have on ICP4-mediated activation at specific promoter classes speculated on here will provide great insight both into the global regulation of viral genes as well as cellular transcription in general. HSV provides a unique system in which to study the role of upstream activators, core promoter elements such as Inr sequences, and the involvement of TFIIA in transcription. Unfortunately, the traditional methods of separating E and L gene expression via use of viral DNA replication

inhibitors and time post-infection are confounded by the finding that ICP4, TBP, and pol II can all be found on L promoters early in infection in the presence of PAA (247). Thus, direct analysis of viral promoter occupancy via ChIP may be necessary to answer several of the questions raised here.

BIBLIOGRAPHY

1. 2004. Finishing the euchromatic sequence of the human genome. Nature **431:**931-45.
2. **Ackermann, M., D. K. Braun, L. Pereira, and B. Roizman.** 1984. Characterization of herpes simplex virus 1 alpha proteins 0, 4, and 27 with monoclonal antibodies. J Virol **52:**108-18.
3. **Adelman, K., B. Salmon, and J. D. Baines.** 2001. Herpes simplex virus DNA packaging sequences adopt novel structures that are specifically recognized by a component of the cleavage and packaging machinery. Proc Natl Acad Sci U S A **98:**3086-91.
4. **Advani, S. J., R. Brandimarti, R. R. Weichselbaum, and B. Roizman.** 2000. The disappearance of cyclins A and B and the increase in activity of the G(2)/M-phase cellular kinase cdc2 in herpes simplex virus 1-infected cells require expression of the alpha22/U(S)1.5 and U(L)13 viral genes. J Virol **74:**8-15.
5. **Advani, S. J., R. R. Weichselbaum, and B. Roizman.** 2001. cdc2 cyclin-dependent kinase binds and phosphorylates herpes simplex virus 1 U(L)42 DNA synthesis processivity factor. J Virol **75:**10326-33.
6. **Advani, S. J., R. R. Weichselbaum, and B. Roizman.** 2003. Herpes simplex virus 1 activates cdc2 to recruit topoisomerase II alpha for post-DNA synthesis expression of late genes. Proc Natl Acad Sci U S A **100:**4825-30.
7. **Albright, S. R., and R. Tjian.** 2000. TAFs revisited: more data reveal new twists and confirm old ideas. Gene **242:**1-13.
8. **Alwine, J. C., W. L. Steinhart, and C. W. Hill.** 1974. Transcription of herpes simplex type 1 DNA in nuclei isolated from infected HEp-2 and KB cells. Virology **60:**302-7.
9. **Arnosti, D. N., A. Merino, D. Reinberg, and W. Schaffner.** 1993. Oct-2 facilitates functional preinitiation complex assembly and is continuously required at the promoter for multiple rounds of transcription. Embo J **12:**157-66.
10. **Bachenheimer, S. L., and B. Roizman.** 1972. Ribonucleic acid synthesis in cells infected with herpes simplex virus. VI. Polyadenylic acid sequences in viral messenger ribonucleic acid. J Virol **10:**875-9.
11. **Baohua, G., R. Kuddus, and N. A. DeLUca.** 1995. Repression of activator-mediated transcription by herpes simplex virus ICP4 via a mechanism involving interactions with the basal transcription factors TATA-binding protein and TFIIB. Molecular Cellular Biology **15:**3618-3626.
12. **Bates, P. A., and N. A. DeLuca.** 1998. The polyserine tract of herpes simplex virus ICP4 is required for normal viral gene expression and growth in murine trigeminal ganglia. J Virol **72:**7115-24.
13. **Batterson, W., and B. Roizman.** 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of alpha genes. J Virol **46:**371-7.

14. **Beard, P. M., N. S. Taus, and J. D. Baines.** 2002. DNA cleavage and packaging proteins encoded by genes U(L)28, U(L)15, and U(L)33 of herpes simplex virus type 1 form a complex in infected cells. J Virol **76:**4785-91.
15. **Benetti, L., J. Munger, and B. Roizman.** 2003. The herpes simplex virus 1 US3 protein kinase blocks caspase-dependent double cleavage and activation of the proapoptotic protein BAD. J Virol **77:**6567-73.
16. **Blaho, J. A., N. Michael, V. Kang, and e. al.** 1992. Differences in the poly(ADP-ribosyl)ation patterns of ICP4, the herpes simplex virus major regulatory protein, in infected cells and in isolated nuclei. Journal of Virology **66:**6398-6407.
17. **Blaho, J. A., and B. Roizman.** 1991. ICP4, the major regulatory protein of herpes simplex virus, shares features common to GTP-binding proteins and is adenylated and guanylated. Journal of Virology **65:**3759-3769.
18. **Blazek, E., G. Mittler, and M. Meisterernst.** 2005. The mediator of RNA polymerase II. Chromosoma **113:**399-408.
19. **Boehmer, P. E., and I. R. Lehman.** 1997. Herpes simplex virus DNA replication. Annu Rev Biochem **66:**347-84.
20. **Bryant, H. E., S. E. Wadd, A. I. Lamond, S. J. Silverstein, and J. B. Clements.** 2001. Herpes simplex virus IE63 (ICP27) protein interacts with spliceosome-associated protein 145 and inhibits splicing prior to the first catalytic step. J Virol **75:**4376-85.
21. **Buratowski, S., S. Hahn, L. Guarente, and P. A. Sharp.** 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. Cell **56:**549-61.
22. **Buratowski, S., S. Hahn, P. A. Sharp, and L. Guarente.** 1988. Function of a yeast TATA element-binding protein in a mammalian transcription system. Nature **334:**37-42.
23. **Bushnell, D. A., K. D. Westover, R. E. Davis, and R. D. Kornberg.** 2004. Structural basis of transcription: an RNA polymerase II-TFIIB cocrystal at 4.5 Angstroms. Science **303:**983-8.
24. **Cai, W., T. L. Astor, L. M. Liptak, C. Cho, D. M. Coen, and P. A. Schaffer.** 1993. The herpes simplex virus type 1 regulatory protein ICP0 enhances virus replication during acute infection and reactivation from latency. J Virol **67:**7501-12.
25. **Cai, W., and P. A. Schaffer.** 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J Virol **66:**2904-15.
26. **Carrozza, M. J., and N. A. DeLuca.** 1996. Interaction of the viral activator protein ICP4 with TFIID through TAF250. Mol Cell Biol **16:**3085-93.
27. **Chadick, J. Z., and F. J. Asturias.** 2005. Structure of eukaryotic Mediator complexes. Trends Biochem Sci **30:**264-71.
28. **Chalkley, G. E., and C. P. Verrijzer.** 1999. DNA binding site selection by RNA polymerase II TAFs: a TAF(II)250-TAF(II)150 complex recognizes the initiator. Embo J **18:**4835-45.
29. **Chen, H. T., and S. Hahn.** 2003. Binding of TFIIB to RNA polymerase II: Mapping the binding site for the TFIIB zinc ribbon domain within the preinitiation complex. Mol Cell **12:**437-47.
30. **Cheung, A. K.** 1989. DNA nucleotide sequence analysis of the immediate-early gene of pseudorabies virus. Nucleic Acids Res **17:**4637-46.
31. **Choy, B., and M. R. Green.** 1993. Eukaryotic activators function during multiple steps of preinitiation complex assembly. Nature **366:**531-6.

32. **Clements, G. B., and N. D. Stow.** 1989. A herpes simplex virus type 1 mutant containing a deletion within immediate early gene 1 is latency-competent in mice. J Gen Virol **70 (Pt 9):**2501-6.
33. **Clements, J. B., R. J. Watson, and N. M. Wilkie.** 1977. Temporal regulation of herpes simplex virus type 1 transcription: location of transcripts on the viral genome. Cell **12:**275-85.
34. **Cocchi, F., L. Menotti, P. Dubreuil, M. Lopez, and G. Campadelli-Fiume.** 2000. Cell-to-cell spread of wild-type herpes simplex virus type 1, but not of syncytial strains, is mediated by the immunoglobulin-like receptors that mediate virion entry, nectin1 (PRR1/HveC/HIgR) and nectin2 (PRR2/HveB). J Virol **74:**3909-17.
35. **Coen, D. M., S. P. Weinheimer, and S. L. McKnight.** 1986. A genetic approach to promoter recognition during trans induction of viral gene expression. Science **234:**53-9.
36. **Coleman, R. A., A. K. Taggart, S. Burma, J. J. Chicca, 2nd, and B. F. Pugh.** 1999. TFIIA regulates TBP and TFIID dimers. Mol Cell **4:**451-7.
37. **Compel, P., and N. A. DeLuca.** 2003. Temperature-dependent conformational changes in herpes simplex virus ICP4 that affect transcription activation. J Virol **77:**3257-68.
38. **Conaway, R. C., S. Sato, C. Tomomori-Sato, T. Yao, and J. W. Conaway.** 2005. The mammalian Mediator complex and its role in transcriptional regulation. Trends Biochem Sci **30:**250-5.
39. **Conley, A. J., D. M. Knipe, P. C. Jones, and B. Roizman.** 1981. Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of gamma polypeptides. J Virol **37:**191-206.
40. **Cook, M. L., and J. G. Stevens.** 1973. Pathogenesis of herpetic neuritis and ganglionitis in mice: evidence for intra-axonal transport of infection. Infect Immun **7:**272-88.
41. **Costanzo, F., G. Campadelli-Fiume, L. Foa-Tomasi, and E. Cassai.** 1977. Evidence that herpes simplex virus DNA is transcribed by cellular RNA polymerase B. J Virol **21:**996-1001.
42. **Courtney, R. J., and M. Benyesh-Melnick.** 1974. Isolation and characterization of a large molecular-weight polypeptide of herpes simplex virus type 1. Virology **62:**539-51.
43. **Cramer, P., D. A. Bushnell, and R. D. Kornberg.** 2001. Structural basis of transcription: RNA polymerase II at 2.8 angstrom resolution. Science **292:**1863-76.
44. **Decman, V., M. L. Freeman, P. R. Kinchington, and R. L. Hendricks.** 2005. Immune control of HSV-1 latency. Viral Immunol **18:**466-73.
45. **Delius, H., and J. B. Clements.** 1976. A partial denaturation map of herpes simplex virus type 1 DNA: evidence for inversions of the unique DNA regions. J Gen Virol **33:**125-33.
46. **DeLuca, N. A., M. A. Courtney, and P. A. Schaffer.** 1984. Temperature-sensitive mutants in herpes simplex virus type 1 ICP4 permissive for early gene expression. J Virol **52:**767-76.
47. **DeLuca, N. A., A. M. McCarthy, and P. A. Schaffer.** 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J Virol **56:**558-70.
48. **DeLuca, N. A., and P. A. Schaffer.** 1985. Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. Molecular Cellular Biology **5:**1997-2008.

49. **DeLuca, N. A., and P. A. Schaffer.** 1987. Activities of herpes simplex virus type 1 (HSV-1) ICP4 genes specifying nonsense peptides. Nucleic Acids Res **15:**4491-511.
50. **DeLuca, N. A., and P. A. Schaffer.** 1988. Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4. J Virol **62:**732-43.
51. **Desai, P., G. L. Sexton, J. M. McCaffery, and S. Person.** 2001. A null mutation in the gene encoding the herpes simplex virus type 1 UL37 polypeptide abrogates virus maturation. J Virol **75:**10259-71.
52. **Desai, P. J.** 2000. A null mutation in the UL36 gene of herpes simplex virus type 1 results in accumulation of unenveloped DNA-filled capsids in the cytoplasm of infected cells. J Virol **74:**11608-18.
53. **Deshmane, S. L., and N. W. Fraser.** 1989. During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. J Virol **63:**943-7.
54. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res **11:**1475-89.
55. **Dixon, R. A., and P. A. Schaffer.** 1980. Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. J Virol **36:**189-203.
56. **Dohner, K., K. Radtke, S. Schmidt, and B. Sodeik.** 2006. Eclipse phase of herpes simplex virus type 1 infection: Efficient dynein-mediated capsid transport without the small capsid protein VP26. J Virol **80:**8211-24.
57. **Dohner, K., A. Wolfstein, U. Prank, C. Echeverri, D. Dujardin, R. Vallee, and B. Sodeik.** 2002. Function of dynein and dynactin in herpes simplex virus capsid transport. Mol Biol Cell **13:**2795-809.
58. **Durand, L. O., S. J. Advani, A. P. Poon, and B. Roizman.** 2005. The carboxyl-terminal domain of RNA polymerase II is phosphorylated by a complex containing cdk9 and infected-cell protein 22 of herpes simplex virus 1. J Virol **79:**6757-62.
59. **Egloff, S., and S. Murphy.** 2008. Cracking the RNA polymerase II CTD code. Trends Genet **24:**280-8.
60. **Eidson, K. M., W. E. Hobbs, B. J. Manning, P. Carlson, and N. A. DeLuca.** 2002. Expression of herpes simplex virus ICP0 inhibits the induction of interferon-stimulated genes by viral infection. J Virol **76:**2180-91.
61. **Eisenberg, S. P., D. M. Coen, and S. L. McKnight.** 1985. Promoter domains required for expression of plasmid-borne copies of the herpes simplex virus thymidine kinase gene in virus-infected mouse fibroblasts and microinjected frog oocytes. Mol Cell Biol **5:**1940-7.
62. **Eisfeld, A. J., S. E. Turse, S. A. Jackson, E. C. Lerner, and P. R. Kinchington.** 2006. Phosphorylation of the varicella-zoster virus (VZV) major transcriptional regulatory protein IE62 by the VZV open reading frame 66 protein kinase. J Virol **80:**1710-23.
63. **Emami, K. H., A. Jain, and S. T. Smale.** 1997. Mechanism of synergy between TATA and initiator: synergistic binding of TFIID following a putative TFIIA-induced isomerization. Genes Dev **11:**3007-19.
64. **Everett, R. D.** 1984. A detailed analysis of an HSV-1 early promoter: sequences involved in *trans*-activation by viral immediate-early gene products are not early-gene specific. Nucleic Acids Research **12:**3037-3056.

65. **Everett, R. D.** 1987. A detailed mutational analysis of Vmw110, a trans-acting transcriptional activator encoded by herpes simplex virus type 1. Embo J **6:**2069-76.
66. **Everett, R. D.** 1988. Analysis of the functional domains of herpes simplex virus type 1 immediate-early polypeptide Vmw110. J Mol Biol **202:**87-96.
67. **Everett, R. D.** 1984. Trans activation of transcription by herpes virus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. Embo J **3:**3135-41.
68. **Everett, R. D., P. Freemont, H. Saitoh, M. Dasso, A. Orr, M. Kathoria, and J. Parkinson.** 1998. The disruption of ND10 during herpes simplex virus infection correlates with the Vmw110- and proteasome-dependent loss of several PML isoforms. J Virol **72:**6581-91.
69. **Everett, R. D., G. Sourvinos, C. Leiper, J. B. Clements, and A. Orr.** 2004. Formation of nuclear foci of the herpes simplex virus type 1 regulatory protein ICP4 at early times of infection: localization, dynamics, recruitment of ICP27, and evidence for the de novo induction of ND10-like complexes. J Virol **78:**1903-17.
70. **Everett, R. D., G. Sourvinos, and A. Orr.** 2003. Recruitment of herpes simplex virus type 1 transcriptional regulatory protein ICP4 into foci juxtaposed to ND10 in live, infected cells. J Virol **77:**3680-9.
71. **Faber, S. W., and K. W. Wilcox.** 1988. Association of herpes simplex virus regulatory protein ICP4 with sequences spanning the ICP4 gene transcription initiation site. Nucleic Acids Res **16:**555-70.
72. **Faber, S. W., and K. W. Wilcox.** 1986. Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. Nucleic Acids Res **14:**6067-83.
73. **Faber, S. W., and K. W. Wilcox.** 1986. Characterization of a herpes simplex virus regulatory protein: aggregation and phosphorylation of a temperature-sensitive variant of ICP 4. Arch Virol **91:**297-312.
74. **Flores, O., E. Maldonado, and D. Reinberg.** 1989. Factors involved in specific transcription by mammalian RNA polymerase II. Factors IIE and IIF independently interact with RNA polymerase II. J Biol Chem **264:**8913-21.
75. **Forget, D., M. F. Langelier, C. Therien, V. Trinh, and B. Coulombe.** 2004. Photo-cross-linking of a purified preinitiation complex reveals central roles for the RNA polymerase II mobile clamp and TFIIE in initiation mechanisms. Mol Cell Biol **24:**1122-31.
76. **Fraser, K. A., and S. A. Rice.** 2007. Herpes simplex virus immediate-early protein ICP22 triggers loss of serine 2-phosphorylated RNA polymerase II. J Virol **81:**5091-101.
77. **Fraser, K. A., and S. A. Rice.** 2005. Herpes simplex virus type 1 infection leads to loss of serine-2 phosphorylation on the carboxyl-terminal domain of RNA polymerase II. J Virol **79:**11323-34.
78. **Freeman, M. J., and K. L. Powell.** 1982. DNA-binding properties of a herpes simplex virus immediate early protein. J Virol **44:**1084-7.
79. **Freeman, M. L., B. S. Sheridan, R. H. Bonneau, and R. L. Hendricks.** 2007. Psychological stress compromises CD8+ T cell control of latent herpes simplex virus type 1 infections. J Immunol **179:**322-8.

80. **Frenkel, N., and B. Roizman.** 1972. Separation of the herpesvirus deoxyribonucleic acid duplex into unique fragments and intact strand on sedimentation in alkaline gradients. J Virol **10:**565-72.
81. **Gallinari, P., K. Wiebauer, M. C. Nardi, and J. Jiricny.** 1994. Localization of a 34-amino-acid segment implicated in dimerization of the herpes simplex virus type 1 ICP4 polypeptide by a dimerization trap. J Virol **68:**3809-20.
82. **Geisberg, J. V., J. L. Chen, and R. P. Ricciardi.** 1995. Subregions of the adenovirus E1A transactivation domain target multiple components of the TFIID complex. Mol Cell Biol **15:**6283-90.
83. **Gelman, I. H., and S. Silverstein.** 1987. Dissection of immediate-early gene promoters from herpes simplex virus: sequences that respond to the virus transcriptional activators. Journal of Virology **61:**3167-3172.
84. **Gelman, I. H., and S. Silverstein.** 1985. Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc Natl Acad Sci U S A **82:**5265-9.
85. **Geraghty, R. J., C. Krummenacher, G. H. Cohen, R. J. Eisenberg, and P. G. Spear.** 1998. Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. Science **280:**1618-20.
86. **Godowski, P. J., and D. M. Knipe.** 1986. Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation. Proc Natl Acad Sci U S A **83:**256-60.
87. **Goodrich, J. A., T. Hoey, C. J. Thut, A. Admon, and R. Tjian.** 1993. Drosophila TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. Cell **75:**519-30.
88. **Gorlich, D., S. Kostka, R. Kraft, C. Dingwall, R. A. Laskey, E. Hartmann, and S. Prehn.** 1995. Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. Curr Biol **5:**383-92.
89. **Gorlich, D., S. Prehn, R. A. Laskey, and E. Hartmann.** 1994. Isolation of a protein that is essential for the first step of nuclear protein import. Cell **79:**767-78.
90. **Gorman, C. M., B. H. Howard, and R. Reeves.** 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. Nucleic Acids Res **11:**7631-48.
91. **Greaves, R., and P. O'Hare.** 1989. Separation of requirements for protein-DNA complex assembly from those for functional activity in the herpes simplex virus regulatory protein Vmw65. J Virol **63:**1641-50.
92. **Grondin, B., and N. DeLuca.** 2000. Herpes simplex virus type 1 ICP4 promotes transcription preinitiation complex formation by enhancing the binding of TFIID to DNA. J Virol **74:**11504-10.
93. **Gu, B., and N. DeLuca.** 1994. Requirements for activation of the herpes simplex virus glycoprotein C promoter in vitro by the viral regulatory protein ICP4. J Virol **68:**7953-65.
94. **Gu, B., R. Kuddus, and N. A. DeLuca.** 1995. Repression of activator-mediated transcription by herpes simplex virus ICP4 via a mechanism involving interactions with the basal transcription factors TATA-binding protein and TFIIB. Mol Cell Biol **15:**3618-26.

95. **Gu, B., R. Rivera-Gonzalez, C. A. Smith, and N. A. DeLuca.** 1993. Herpes simplex virus infected cell polypeptide 4 preferentially represses Sp1-activated over basal transcription from its own promoter. Proc Natl Acad Sci U S A **90:**9528-32.
96. **Gu, H., Y. Liang, G. Mandel, and B. Roizman.** 2005. Components of the REST/CoREST/histone deacetylase repressor complex are disrupted, modified, and translocated in HSV-1-infected cells. Proc Natl Acad Sci U S A **102:**7571-6.
97. **Guzowski, J. F., J. Singh, and E. K. Wagner.** 1994. Transcriptional activation of the herpes simplex virus type 1 UL38 promoter conferred by the cis-acting downstream activation sequence is mediated by a cellular transcription factor. J Virol **68:**7774-89.
98. **Guzowski, J. F., and E. K. Wagner.** 1993. Mutational analysis of the herpes simplex virus type 1 strict late UL38 promoter/leader reveals two regions critical in transcriptional regulation. J Virol **67:**5098-108.
99. **Ha, I., W. S. Lane, and D. Reinberg.** 1991. Cloning of a human gene encoding the general transcription initiation factor IIB. Nature **352:**689-95.
100. **Ha, I., S. Roberts, E. Maldonado, X. Sun, L. U. Kim, M. Green, and D. Reinberg.** 1993. Multiple functional domains of human transcription factor IIB: distinct interactions with two general transcription factors and RNA polymerase II. Genes Dev **7:**1021-32.
101. **Hahn, S.** 1998. The role of TAFs in RNA polymerase II transcription. Cell **95:**579-82.
102. **Halpern, M. E., and J. R. Smiley.** 1984. Effects of deletions on expression of the herpes simplex virus thymidine kinase gene from the intact viral genome: the amino terminus of the enzyme is dispensable for catalytic activity. J Virol **50:**733-8.
103. **Hann, L. E., W. J. Cook, S. L. Uprichard, D. M. Knipe, and D. M. Coen.** 1998. The role of herpes simplex virus ICP27 in the regulation of UL24 gene expression by differential polyadenylation. J Virol **72:**7709-14.
104. **Harbour, J. W., R. X. Luo, A. Dei Santi, A. A. Postigo, and D. C. Dean.** 1999. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell **98:**859-69.
105. **Hardwicke, M. A., and R. M. Sandri-Goldin.** 1994. The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection. J Virol **68:**4797-810.
106. **Hardy, W. R., and R. M. Sandri-Goldin.** 1994. Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. J Virol **68:**7790-9.
107. **Harris, R. A., R. D. Everett, X. X. Zhu, S. Silverstein, and C. M. Preston.** 1989. Herpes simplex virus type 1 immediate-early protein Vmw110 reactivates latent herpes simplex virus type 2 in an in vitro latency system. J Virol **63:**3513-5.
108. **Hathaway, D. R., R. S. Adelstein, and C. B. Klee.** 1981. Interaction of calmodulin with myosin light chain kinase and cAMP-dependent protein kinase in bovine brain. J Biol Chem **256:**8183-9.
109. **Hawkes, N. A., R. Evans, and S. G. Roberts.** 2000. The conformation of the transcription factor TFIIB modulates the response to transcriptional activators in vivo. Curr Biol **10:**273-6.
110. **Hayashi, F., R. Ishima, D. Liu, K. I. Tong, S. Kim, D. Reinberg, S. Bagby, and M. Ikura.** 1998. Human general transcription factor TFIIB: conformational variability and interaction with VP16 activation domain. Biochemistry **37:**7941-51.
111. **Hayward, G. S., R. J. Jacob, S. C. Wadsworth, and B. Roizman.** 1975. Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the

relative orientations of their long and short components. Proc Natl Acad Sci U S A **72:**4243-7.

112. **Heine, J. W., R. W. Honess, E. Cassai, and B. Roizman.** 1974. Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. J Virol **14:**640-51.
113. **Herold, B. C., D. WuDunn, N. Soltys, and P. G. Spear.** 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. J Virol **65:**1090-8.
114. **Herrera, F. J., and S. J. Triezenberg.** 2004. VP16-dependent association of chromatin-modifying coactivators and underrepresentation of histones at immediate-early gene promoters during herpes simplex virus infection. J Virol **78:**9689-96.
115. **Hoey, T., R. O. Weinzierl, G. Gill, J. L. Chen, B. D. Dynlacht, and R. Tjian.** 1993. Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. Cell **72:**247-60.
116. **Holstege, F. C., P. C. van der Vliet, and H. T. Timmers.** 1996. Opening of an RNA polymerase II promoter occurs in two distinct steps and requires the basal transcription factors IIE and IIH. Embo J **15:**1666-77.
117. **Homa, F. L., and J. C. Brown.** 1997. Capsid assembly and DNA packaging in herpes simplex virus. Rev Med Virol **7:**107-122.
118. **Homa, F. L., J. C. Glorioso, and M. Levine.** 1988. A specific 15-bp TATA box promoter element is required for expression of a herpes simplex virus type 1 late gene. Genes Dev **2:**40-53.
119. **Homa, F. L., T. M. Otal, J. C. Glorioso, and M. Levine.** 1986. Transcriptional control signals of a herpes simplex virus type 1 late (gamma 2) gene lie within bases -34 to +124 relative to the 5' terminus of the mRNA. Mol Cell Biol **6:**3652-66.
120. **Hones, R. W., and B. Roizman.** 1975. Regulation of herpes virus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proceedings of the National Academy of Sciences, USA **72:**1276-1280.
121. **Hones, R. W., and B. Roizman.** 1974. Regulation of herpes virus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. Journal of Virology **14:**8-19.
122. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc Natl Acad Sci U S A **72:**1276-80.
123. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J Virol **14:**8-19.
124. **Honess, R. W., and D. H. Watson.** 1974. Herpes simplex virus-specific polypeptides studied by polyacrylamide gel electrophoresis of immune precipitates. J Gen Virol **22:**171-85.
125. **Igarashi, K., R. Fawl, R. J. Roller, and B. Roizman.** 1993. Construction and properties of a recombinant herpes simplex virus 1 lacking both S-component origins of DNA synthesis. J Virol **67:**2123-32.
126. **Imbalzano, A. N., D. M. Coen, and N. A. DeLuca.** 1991. Herpes simplex virus transactivator ICP4 operationally substitutes for the cellular transcription factor Sp1 for efficient expression of the viral thymidine kinase gene. J Virol **65:**565-74.

127. **Imbalzano, A. N., and N. A. DeLuca.** 1992. Substitution of a TATA box from a herpes simplex virus late gene in the viral thymidine kinase promoter alters ICP4 inducibility but not temporal expression. J Virol **66:**5453-63.
128. **Imbalzano, A. N., A. A. Shepard, and N. A. DeLuca.** 1990. Functional relevance of specific interactions between herpes simplex virus type 1 ICP4 and sequences from the promoter-regulatory domain of the viral thymidine kinase gene. J Virol **64:**2620-31.
129. **Ito, M., C. X. Yuan, S. Malik, W. Gu, J. D. Fondell, S. Yamamura, Z. Y. Fu, X. Zhang, J. Qin, and R. G. Roeder.** 1999. Identity between TRAP and SMCC complexes indicates novel pathways for the function of nuclear receptors and diverse mammalian activators. Mol Cell **3:**361-70.
130. **Jackson, S. A., and N. A. DeLuca.** 2003. Relationship of herpes simplex virus genome configuration to productive and persistent infections. Proc Natl Acad Sci U S A **100:**7871-6.
131. **Jean, S., K. M. LeVan, B. Song, M. Levine, and D. M. Knipe.** 2001. Herpes simplex virus 1 ICP27 is required for transcription of two viral late (gamma 2) genes in infected cells. Virology **283:**273-84.
132. **Jones, K. A., K. R. Yamamoto, and R. Tjian.** 1985. Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. Cell **42:**559-72.
133. **Jordan, R., and P. A. Schaffer.** 1997. Activation of gene expression by herpes simplex virus type 1 ICP0 occurs at the level of mRNA synthesis. J Virol **71:**6850-62.
134. **Kalderon, D., W. D. Richardson, A. F. Markham, and A. E. Smith.** 1984. Sequence requirements for nuclear location of simian virus 40 large-T antigen. Nature **311:**33-8.
135. **Kao, C. C., P. M. Lieberman, M. C. Schmidt, Q. Zhou, R. Pei, and A. J. Berk.** 1990. Cloning of a transcriptionally active human TATA binding factor. Science **248:**1646-50.
136. **Kato, A., M. Yamamoto, T. Ohno, H. Kodaira, Y. Nishiyama, and Y. Kawaguchi.** 2005. Identification of proteins phosphorylated directly by the Us3 protein kinase encoded by herpes simplex virus 1. J Virol **79:**9325-31.
137. **Khanna, K. M., A. J. Lepisto, V. Decman, and R. L. Hendricks.** 2004. Immune control of herpes simplex virus during latency. Curr Opin Immunol **16:**463-9.
138. **Kieff, E. D., S. L. Bachenheimer, and B. Roizman.** 1971. Size, composition, and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. J Virol **8:**125-32.
139. **Kim, D.-B., and N. A. DeLuca.** 2002. Phosphorylation of transcription factor Sp1 during herpes simplex virus type 1 infection. Journal of Virology **76:**6473-6479.
140. **Kim, D. B., S. Zabierowski, and N. A. DeLuca.** 2002. The initiator element in a herpes simplex virus type 1 late-gene promoter enhances activation by ICP4, resulting in abundant late-gene expression. J Virol **76:**1548-58.
141. **Knickelbein, J. E., K. M. Khanna, M. B. Yee, C. J. Baty, P. R. Kinchington, and R. L. Hendricks.** 2008. Noncytotoxic lytic granule-mediated CD8+ T cell inhibition of HSV-1 reactivation from neuronal latency. Science **322:**268-71.
142. **Kristie, T. M., and B. Roizman.** 1988. Differentiation and DNA contact points of host proteins binding at the cis site for virion-mediated induction of alpha genes of herpes simplex virus 1. J Virol **62:**1145-57.
143. **Kristie, T. M., and B. Roizman.** 1986. DNA-binding site of major regulatory protein alpha 4 specifically associated with promoter-regulatory domains of alpha genes of herpes simplex virus type 1. Proc Natl Acad Sci U S A **83:**4700-4.

144. **Kristie, T. M., and B. Roizman.** 1987. Host cell proteins bind to the cis-acting site required for virion-mediated induction of herpes simplex virus 1 alpha genes. Proc Natl Acad Sci U S A **84:**71-5.
145. **Kuddus, R., B. Gu, and N. A. DeLuca.** 1995. Relationship between TATA-binding protein and herpes simplex virus type 1 ICP4 DNA-binding sites in complex formation and repression of transcription. J Virol **69:**5568-75.
146. **Kuddus, R. H., and N. A. DeLuca.** 2007. DNA-dependent oligomerization of herpes simplex virus type 1 regulatory protein ICP4. J Virol **81:**9230-7.
147. **Kwong, A. D., and N. Frenkel.** 1987. Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs. Proc Natl Acad Sci U S A **84:**1926-30.
148. **Kwong, A. D., and N. Frenkel.** 1989. The herpes simplex virus virion host shutoff function. J Virol **63:**4834-9.
149. **Kwong, A. D., J. A. Kruper, and N. Frenkel.** 1988. Herpes simplex virus virion host shutoff function. J Virol **62:**912-21.
150. **La Boissiere, S., T. Hughes, and P. O'Hare.** 1999. HCF-dependent nuclear import of VP16. Embo J **18:**480-9.
151. **LaBoissiere, S., and P. O'Hare.** 2000. Analysis of HCF, the cellular cofactor of VP16, in herpes simplex virus-infected cells. J Virol **74:**99-109.
152. **Lavigne, A. C., G. Mengus, M. May, V. Dubrovskaya, L. Tora, P. Chambon, and I. Davidson.** 1996. Multiple interactions between hTAFII55 and other TFIID subunits. Requirements for the formation of stable ternary complexes between hTAFII55 and the TATA-binding protein. J Biol Chem **271:**19774-80.
153. **Lee, T. I., and R. A. Young.** 2000. Transcription of eukaryotic protein-coding genes. Annu Rev Genet **34:**77-137.
154. **Lee, Y. C., J. M. Park, S. Min, S. J. Han, and Y. J. Kim.** 1999. An activator binding module of yeast RNA polymerase II holoenzyme. Mol Cell Biol **19:**2967-76.
155. **Lees-Miller, S. P., M. C. Long, M. A. Kilvert, V. Lam, S. A. Rice, and C. A. Spencer.** 1996. Attenuation of DNA-dependent protein kinase activity and its catalytic subunit by the herpes simplex virus type 1 transactivator ICP0. J Virol **70:**7471-7.
156. **Lehtinen, M. O., T. K. Lehtinen, and P. O. Leinikki.** 1984. Enrichment of early HSV-induced proteins in phosphonoformate-treated cells. Acta Virol **28:**11-8.
157. **Leopardi, R., N. Michael, and B. Roizman.** 1995. Repression of the herpes simplex virus 1 alpha 4 gene by its gene product (ICP4) within the context of the viral genome is conditioned by the distance and stereoaxial alignment of the ICP4 DNA binding site relative to the TATA box. J Virol **69:**3042-8.
158. **Leopardi, R., and B. Roizman.** 1996. Functional interaction and colocalization of the herpes simplex virus 1 major regulatory protein ICP4 with EAP, a nucleolar-ribosomal protein. Proc Natl Acad Sci U S A **93:**4572-6.
159. **Lin, Y. S., and M. R. Green.** 1991. Mechanism of action of an acidic transcriptional activator in vitro. Cell **64:**971-81.
160. **Lin, Y. S., I. Ha, E. Maldonado, D. Reinberg, and M. R. Green.** 1991. Binding of general transcription factor TFIIB to an acidic activating region. Nature **353:**569-71.
161. **Lo, K., and S. T. Smale.** 1996. Generality of a functional initiator consensus sequence. Gene **182:**13-22.

162. **Locker, H., and N. Frenkel.** 1979. BamI, KpnI, and SalI restriction enzyme maps of the DNAs of herpes simplex virus strains Justin and F: occurrence of heterogeneities in defined regions of the viral DNA. J Virol **32:**429-41.
163. **Lomonte, P., J. Thomas, P. Texier, C. Caron, S. Khochbin, and A. L. Epstein.** 2004. Functional interaction between class II histone deacetylases and ICP0 of herpes simplex virus type 1. J Virol **78:**6744-57.
164. **Long, M. C., V. Leong, P. A. Schaffer, C. A. Spencer, and S. A. Rice.** 1999. ICP22 and the UL13 protein kinase are both required for herpes simplex virus-induced modification of the large subunit of RNA polymerase II. J Virol **73:**5593-604.
165. **Lukonis, C. J., J. Burkham, and S. K. Weller.** 1997. Herpes simplex virus type 1 prereplicative sites are a heterogeneous population: only a subset are likely to be precursors to replication compartments. J Virol **71:**4771-81.
166. **Mackem, S., and B. Roizman.** 1980. Regulation of herpesvirus macromolecular synthesis: transcription-initiation sites and domains of alpha genes. Proc Natl Acad Sci U S A **77:**7122-6.
167. **Mackem, S., and B. Roizman.** 1982. Structural features of the herpes simplex virus alpha gene 4, 0, and 27 promoter-regulatory sequences which confer alpha regulation on chimeric thymidine kinase genes. J Virol **44:**939-49.
168. **Manzano-Winkler, B., C. D. Novina, and A. L. Roy.** 1996. TFII is required for transcription of the naturally TATA-less but initiator-containing Vbeta promoter. J Biol Chem **271:**12076-81.
169. **Maul, G. G., H. H. Guldner, and J. G. Spivack.** 1993. Modification of discrete nuclear domains induced by herpes simplex virus type 1 immediate early gene 1 product (ICP0). J Gen Virol **74 (Pt 12):**2679-90.
170. **Mavromara-Nazos, P., S. Silver, J. Hubenthal-Voss, J. L. McKnight, and B. Roizman.** 1986. Regulation of herpes simplex virus 1 genes: alpha gene sequence requirements for transient induction of indicator genes regulated by beta or late (gamma 2) promoters. Virology **149:**152-64.
171. **Maxon, M. E., J. A. Goodrich, and R. Tjian.** 1994. Transcription factor IIE binds preferentially to RNA polymerase IIa and recruits TFIIH: a model for promoter clearance. Genes Dev **8:**515-24.
172. **McCarthy, A. M., L. McMahan, and P. A. Schaffer.** 1989. Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. J Virol **63:**18-27.
173. **McGeoch, D. J.** 1987. The genome of herpes simplex virus: structure, replication and evolution. J Cell Sci Suppl **7:**67-94.
174. **McGeoch, D. J., A. Dolan, S. Donald, and D. H. Brauer.** 1986. Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. Nucleic Acids Res **14:**1727-45.
175. **McGeoch, D. J., F. J. Rixon, and A. J. Davison.** 2006. Topics in herpesvirus genomics and evolution. Virus Res **117:**90-104.
176. **McGregor, F., A. Phelan, J. Dunlop, and J. B. Clements.** 1996. Regulation of herpes simplex virus poly (A) site usage and the action of immediate-early protein IE63 in the early-late switch. J Virol **70:**1931-40.

177. **McKnight, J. L., T. M. Kristie, and B. Roizman.** 1987. Binding of the virion protein mediating alpha gene induction in herpes simplex virus 1-infected cells to its cis site requires cellular proteins. Proc Natl Acad Sci U S A **84:**7061-5.
178. **McKnight, S., and R. Tjian.** 1986. Transcriptional selectivity of viral genes in mammalian cells. Cell **46:**795-805.
179. **McLauchlan, J., A. Phelan, C. Loney, R. M. Sandri-Goldin, and J. B. Clements.** 1992. Herpes simplex virus IE63 acts at the posttranscriptional level to stimulate viral mRNA 3' processing. J Virol **66:**6939-45.
180. **McMahan, L., and P. A. Schaffer.** 1990. The repressing and enhancing functions of the herpes simplex virus regulatory protein ICP27 map to C-terminal regions and are required to modulate viral gene expression very early in infection. J Virol **64:**3471-85.
181. **Mellerick, D. M., and N. W. Fraser.** 1987. Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. Virology **158:**265-75.
182. **Mettenleiter, T. C.** 2004. Budding events in herpesvirus morphogenesis. Virus Res **106:**167-80.
183. **Mettenleiter, T. C.** 2002. Herpesvirus assembly and egress. J Virol **76:**1537-47.
184. **Metzler, D. W., and K. W. Wilcox.** 1985. Isolation of herpes simplex virus regulatory protein ICP4 as a homodimeric complex. J Virol **55:**329-37.
185. **Michael, N., and B. Roizman.** 1993. Repression of the herpes simplex virus 1 alpha 4 gene by its gene product occurs within the context of the viral genome and is associated with all three identified cognate sites. Proc Natl Acad Sci U S A **90:**2286-90.
186. **Michael, N., D. Spector, P. Mavromara-Nazos, T. M. Kristie, and B. Roizman.** 1988. The DNA-binding properties of the major regulatory protein alpha 4 of herpes simplex viruses. Science **239:**1531-4.
187. **Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear.** 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell **87:**427-36.
188. **Morse, L. S., L. Pereira, B. Roizman, and P. A. Schaffer.** 1978. Anatomy of herpes simplex virus (HSV) DNA. X. Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 X HSV-2 recombinants. J Virol **26:**389-410.
189. **Mou, F., T. Forest, and J. D. Baines.** 2007. US3 of herpes simplex virus type 1 encodes a promiscuous protein kinase that phosphorylates and alters localization of lamin A/C in infected cells. J Virol **81:**6459-70.
190. **Muller, C. W.** 2001. Transcription factors: global and detailed views. Curr Opin Struct Biol **11:**26-32.
191. **Muller, M. T.** 1987. Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. J Virol **61:**858-65.
192. **Nagy, Z., and L. Tora.** 2007. Distinct GCN5/PCAF-containing complexes function as co-activators and are involved in transcription factor and global histone acetylation. Oncogene **26:**5341-57.
193. **Nakajima, N., M. Horikoshi, and R. G. Roeder.** 1988. Factors involved in specific transcription by mammalian RNA polymerase II: purification, genetic specificity, and TATA box-promoter interactions of TFIID. Mol Cell Biol **8:**4028-40.

194. **Nikolov, D. B., H. Chen, E. D. Halay, A. Hoffman, R. G. Roeder, and S. K. Burley.** 1996. Crystal structure of a human TATA box-binding protein/TATA element complex. Proc Natl Acad Sci U S A **93:**4862-7.
195. **Nikolov, D. B., H. Chen, E. D. Halay, A. A. Usheva, K. Hisatake, D. K. Lee, R. G. Roeder, and S. K. Burley.** 1995. Crystal structure of a TFIIB-TBP-TATA-element ternary complex. Nature **377:**119-28.
196. **Nimonkar, A. V., and P. E. Boehmer.** 2003. Reconstitution of recombination-dependent DNA synthesis in herpes simplex virus 1. Proc Natl Acad Sci U S A **100:**10201-6.
197. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J Virol **53:**751-60.
198. **O'Hare, P., and G. S. Hayward.** 1985. Three *trans*-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J Virol **56:**751-760.
199. **Ohkuma, Y., S. Hashimoto, C. K. Wang, M. Horikoshi, and R. G. Roeder.** 1995. Analysis of the role of TFIIE in basal transcription and TFIIH-mediated carboxy-terminal domain phosphorylation through structure-function studies of TFIIE-alpha. Mol Cell Biol **15:**4856-66.
200. **Ohkuma, Y., and R. G. Roeder.** 1994. Regulation of TFIIH ATPase and kinase activities by TFIIE during active initiation complex formation. Nature **368:**160-3.
201. **Okamoto, T., S. Yamamoto, Y. Watanabe, T. Ohta, F. Hanaoka, R. G. Roeder, and Y. Ohkuma.** 1998. Analysis of the role of TFIIE in transcriptional regulation through structure-function studies of the TFIIEbeta subunit. J Biol Chem **273:**19866-76.
202. **Orphanides, G., T. Lagrange, and D. Reinberg.** 1996. The general transcription factors of RNA polymerase II. Genes Dev **10:**2657-83.
203. **Ozer, J., K. Mitsouras, D. Zerby, M. Carey, and P. M. Lieberman.** 1998. Transcription factor IIA derepresses TATA-binding protein (TBP)-associated factor inhibition of TBP-DNA binding. J Biol Chem **273:**14293-300.
204. **Panagiotidis, C. A., E. K. Lium, and S. J. Silverstein.** 1997. Physical and functional interactions between herpes simplex virus immediate-early proteins ICP4 and ICP27. J Virol **71:**1547-57.
205. **Papavassiliou, A. G., K. W. Wilcox, and S. J. Silverstein.** 1991. The interaction of ICP4 with cell/infected-cell factors and its state of phosphorylation modulate differential recognition of leader sequences in herpes simplex virus DNA. Embo J **10:**397-406.
206. **Parkinson, J., S. P. Lees-Miller, and R. D. Everett.** 1999. Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase. J Virol **73:**650-7.
207. **Paterson, T., and R. D. Everett.** 1988. Mutational dissection of the HSV-1 immediate-early protein Vmw175 involved in transcriptional transactivation and repression. Virology **166:**186-96.
208. **Paterson, T., and R. D. Everett.** 1988. The regions of the herpes simplex virus type 1 immediate early protein Vmw175 required for site specific DNA binding closely correspond to those involved in transcriptional regulation. Nucleic Acids Res **16:**11005-25.

209. **Paterson, T., V. G. Preston, and R. D. Everett.** 1990. A mutant of herpes simplex virus type 1 immediate early polypeptide Vmw175 binds to the cap site of its own promoter in vitro but fails to autoregulate in vivo. J Gen Virol **71 (Pt 4):**851-61.
210. **Pellet, P. E., and B. Roizman.** 2007. The Family Herpesviridae: A Brief Introduction, p. 2479-2499. *In* D. M. Knipe and P. M. Howley (ed.), Field's Virology. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.
211. **Pereira, L., M. H. Wolff, M. Fenwick, and B. Roizman.** 1977. Regulation of herpesvirus macromolecular synthesis. V. Properties of alpha polypeptides made in HSV-1 and HSV-2 infected cells. Virology **77:**733-49.
212. **Perry, L. J., F. J. Rixon, R. D. Everett, M. C. Frame, and D. J. McGeoch.** 1986. Characterization of the IE110 gene of herpes simplex virus type 1. J Gen Virol **67 (Pt 11):**2365-80.
213. **Phelan, A., J. Dunlop, and J. B. Clements.** 1996. Herpes simplex virus type 1 protein IE63 affects the nuclear export of virus intron-containing transcripts. J Virol **70:**5255-65.
214. **Poffenberger, K. L., and B. Roizman.** 1985. A noninverting genome of a viable herpes simplex virus 1: presence of head-to-tail linkages in packaged genomes and requirements for circularization after infection. J Virol **53:**587-95.
215. **Polvino-Bodnar, M., P. K. Orberg, and P. A. Schaffer.** 1987. Herpes simplex virus type 1 oriL is not required for virus replication or for the establishment and reactivation of latent infection in mice. J Virol **61:**3528-35.
216. **Poon, A. P., H. Gu, and B. Roizman.** 2006. ICP0 and the US3 protein kinase of herpes simplex virus 1 independently block histone deacetylation to enable gene expression. Proc Natl Acad Sci U S A **103:**9993-8.
217. **Preston, C. M.** 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant tsK. J Virol **29:**275-84.
218. **Preston, C. M., and M. J. Nicholl.** 1997. Repression of gene expression upon infection of cells with herpes simplex virus type 1 mutants impaired for immediate-early protein synthesis. J Virol **71:**7807-13.
219. **Preston, V. G.** 1981. Fine-structure mapping of herpes simplex virus type 1 temperature-sensitive mutations within the short repeat region of the genome. J Virol **39:**150-61.
220. **Puig, O., F. Caspary, G. Rigaut, B. Rutz, E. Bouveret, E. Bragado-Nilsson, M. Wilm, and B. Seraphin.** 2001. The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods **24:**218-29.
221. **Purnell, B. A., P. A. Emanuel, and D. S. Gilmour.** 1994. TFIID sequence recognition of the initiator and sequences farther downstream in Drosophila class II genes. Genes Dev **8:**830-42.
222. **Purves, F. C., D. Spector, and B. Roizman.** 1991. The herpes simplex virus 1 protein kinase encoded by the US3 gene mediates posttranslational modification of the phosphoprotein encoded by the UL34 gene. J Virol **65:**5757-64.
223. **Quadt, I., A. K. Gunther, D. Voss, M. Schelhaas, and D. Knebel-Morsdorf.** 2006. TATA-binding protein and TBP-associated factors during herpes simplex virus type 1 infection: localization at viral DNA replication sites. Virus Res **115:**207-13.
224. **Quinlan, M. P., and D. M. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol Cell Biol **5:**957-63.

225. **Reeves, R., C. M. Gorman, and B. Howard.** 1985. Minichromosome assembly of non-integrated plasmid DNA transfected into mammalian cells. Nucleic Acids Res **13:**3599-615.
226. **Rice, S. A., and D. M. Knipe.** 1988. Gene-specific transactivation by herpes simplex virus type 1 alpha protein ICP27. J Virol **62:**3814-23.
227. **Rice, S. A., and D. M. Knipe.** 1990. Genetic evidence for two distinct transactivation functions of the herpes simplex virus alpha protein ICP27. J Virol **64:**1704-15.
228. **Rice, S. A., M. C. Long, V. Lam, P. A. Schaffer, and C. A. Spencer.** 1995. Herpes simplex virus immediate-early protein ICP22 is required for viral modification of host RNA polymerase II and establishment of the normal viral transcription program. J Virol **69:**5550-9.
229. **Rice, S. A., M. C. Long, V. Lam, and C. A. Spencer.** 1994. RNA polymerase II is aberrantly phosphorylated and localized to viral replication compartments following herpes simplex virus infection. J Virol **68:**988-1001.
230. **Rigaut, G., A. Shevchenko, B. Rutz, M. Wilm, M. Mann, and B. Seraphin.** 1999. A generic protein purification method for protein complex characterization and proteome exploration. Nat Biotechnol **17:**1030-2.
231. **Rivera-Gonzalez, R., A. N. Imbalzano, B. Gu, and N. A. Deluca.** 1994. The role of ICP4 repressor activity in temporal expression of the IE-3 and latency-associated transcript promoters during HSV-1 infection. Virology **202:**550-64.
232. **Roberts, M. S., A. Boundy, P. O'Hare, M. C. Pizzorno, D. M. Ciufo, and G. S. Hayward.** 1988. Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (alpha 4) promoter and a specific binding site for the IE175 (ICP4) protein. J Virol **62:**4307-20.
233. **Roberts, S. G., and M. R. Green.** 1994. Activator-induced conformational change in general transcription factor TFIIB. Nature **371:**717-20.
234. **Roizman, B.** 1979. The structure and isomerization of herpes simplex virus genomes. Cell **16:**481-94.
235. **Roizman, B., L. E. Carmichael, F. Deinhardt, G. de-The, A. J. Nahmias, W. Plowright, F. Rapp, P. Sheldrick, M. Takahashi, and K. Wolf.** 1981. Herpesviridae. Definition, provisional nomenclature, and taxonomy. The Herpesvirus Study Group, the International Committee on Taxonomy of Viruses. Intervirology **16:**201-17.
236. **Roizman, B., D. M. Knipe, and R. J. Whitley.** 2007. Herpes Simplex Viruses, p. 2502-2600. *In* D. M. Knipe and P. M. Howley (ed.), Field's Virology, 5th ed. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphoa.
237. **Roy, A. L., S. Malik, M. Meisterernst, and R. G. Roeder.** 1993. An alternative pathway for transcription initiation involving TFII-I. Nature **365:**355-9.
238. **Roy, A. L., M. Meisterernst, P. Pognonec, and R. G. Roeder.** 1991. Cooperative interaction of an initiator-binding transcription initiation factor and the helix-loop-helix activator USF. Nature **354:**245-8.
239. **Ryu, S., S. Zhou, A. G. Ladurner, and R. Tjian.** 1999. The transcriptional cofactor complex CRSP is required for activity of the enhancer-binding protein Sp1. Nature **397:**446-50.
240. **Sacks, D. B., H. W. Davis, J. P. Williams, E. L. Sheehan, J. G. Garcia, and J. M. McDonald.** 1992. Phosphorylation by casein kinase II alters the biological activity of calmodulin. Biochem J **283 (Pt 1):**21-4.

241. **Sacks, W. R., C. C. Greene, D. P. Aschman, and P. A. Schaffer.** 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J Virol **55:**796-805.
242. **Sacks, W. R., and P. A. Schaffer.** 1987. Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. J Virol **61:**829-39.
243. **Sadowski, I., J. Ma, S. Triezenberg, and M. Ptashne.** 1988. GAL4-VP16 is an unusually potent transcriptional activator. Nature **335:**563-4.
244. **Samaniego, L. A., L. Neiderhiser, and N. A. DeLuca.** 1998. Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. J Virol **72:**3307-20.
245. **Samaniego, L. A., A. L. Webb, and N. A. DeLuca.** 1995. Functional interactions between herpes simplex virus immediate-early proteins during infection: gene expression as a consequence of ICP27 and different domains of ICP4. J Virol **69:**5705-15.
246. **Samaniego, L. A., N. Wu, and N. A. DeLuca.** 1997. The herpes simplex virus immediate-early protein ICP0 affects transcription from the viral genome and infected-cell survival in the absence of ICP4 and ICP27. J Virol **71:**4614-25.
247. **Sampath, P., and N. A. Deluca.** 2008. Binding of ICP4, TATA-binding protein, and RNA polymerase II to herpes simplex virus type 1 immediate-early, early, and late promoters in virus-infected cells. J Virol **82:**2339-49.
248. **Sandri-Goldin, R. M.** 1998. ICP27 mediates HSV RNA export by shuttling through a leucine-rich nuclear export signal and binding viral intronless RNAs through an RGG motif. Genes Dev **12:**868-79.
249. **Sears, A. E., I. W. Halliburton, B. Meignier, S. Silver, and B. Roizman.** 1985. Herpes simplex virus 1 mutant deleted in the alpha 22 gene: growth and gene expression in permissive and restrictive cells and establishment of latency in mice. J Virol **55:**338-46.
250. **Sedlackova, L., and S. A. Rice.** 2008. Herpes simplex virus type 1 immediate-early protein ICP27 is required for efficient incorporation of ICP0 and ICP4 into virions. J Virol **82:**268-77.
251. **Serizawa, H., J. W. Conaway, and R. C. Conaway.** 1994. An oligomeric form of the large subunit of transcription factor (TF) IIE activates phosphorylation of the RNA polymerase II carboxyl-terminal domain by TFIIH. J Biol Chem **269:**20750-6.
252. **Sermwittayawong, D., and S. Tan.** 2006. SAGA binds TBP via its Spt8 subunit in competition with DNA: implications for TBP recruitment. Embo J **25:**3791-800.
253. **Shao, H., M. Revach, S. Moshonov, Y. Tzuman, K. Gazit, S. Albeck, T. Unger, and R. Dikstein.** 2005. Core promoter binding by histone-like TAF complexes. Mol Cell Biol **25:**206-19.
254. **Shepard, A. A., and N. A. DeLuca.** 1991. A second-site revertant of a defective herpes simplex virus ICP4 protein with restored regulatory activities and impaired DNA-binding properties. J Virol **65:**787-95.
255. **Shepard, A. A., and N. A. DeLuca.** 1991. Activities of heterodimers composed of DNA-binding- and transactivation-deficient subunits of the herpes simplex virus regulatory protein ICP4. J Virol **65:**299-307.
256. **Shepard, A. A., A. N. Imbalzano, and N. A. DeLuca.** 1989. Separation of primary structural components conferring autoregulation, transactivation, and DNA-binding properties to the herpes simplex virus transcriptional regulatory protein ICP4. J Virol **63:**3714-28.

257. **Shepard, A. A., P. Tolentino, and N. A. DeLuca.** 1990. trans-dominant inhibition of herpes simplex virus transcriptional regulatory protein ICP4 by heterodimer formation. J Virol **64:**3916-26.
258. **Shevchenko, A., H. Tomas, J. Havlis, J. V. Olsen, and M. Mann.** 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc **1:**2856-60.
259. **Shieh, M. T., D. WuDunn, R. I. Montgomery, J. D. Esko, and P. G. Spear.** 1992. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. J Cell Biol **116:**1273-81.
260. **Shukla, D., J. Liu, P. Blaiklock, N. W. Shworak, X. Bai, J. D. Esko, G. H. Cohen, R. J. Eisenberg, R. D. Rosenberg, and P. G. Spear.** 1999. A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. Cell **99:**13-22.
261. **Skepper, J. N., A. Whiteley, H. Browne, and A. Minson.** 2001. Herpes simplex virus nucleocapsids mature to progeny virions by an envelopment --> deenvelopment --> reenvelopment pathway. J Virol **75:**5697-702.
262. **Smale, S. T.** 1997. Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. Biochim Biophys Acta **1351:**73-88.
263. **Smale, S. T., and D. Baltimore.** 1989. The "initiator" as a transcription control element. Cell **57:**103-13.
264. **Smale, S. T., and J. T. Kadonaga.** 2003. The RNA polymerase II core promoter. Annu Rev Biochem **72:**449-79.
265. **Smiley, J. R., D. C. Johnson, L. I. Pizer, and R. D. Everett.** 1992. The ICP4 binding sites in the herpes simplex virus type 1 glycoprotein D (gD) promoter are not essential for efficient gD transcription during virus infection. J Virol **66:**623-31.
266. **Smith, C. A., P. Bates, R. Rivera-Gonzalez, B. Gu, and N. A. DeLuca.** 1993. ICP4, the major transcriptional regulatory protein of herpes simplex virus type 1, forms a tripartite complex with TATA-binding protein and TFIIB. J Virol **67:**4676-87.
267. **Sodeik, B., M. W. Ebersold, and A. Helenius.** 1997. Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. J Cell Biol **136:**1007-21.
268. **Sossey-Alaoui, K., and A. K. Srivastava.** 1999. DCAMKL1, a brain-specific transmembrane protein on 13q12.3 that is similar to doublecortin (DCX). Genomics **56:**121-6.
269. **Sourvinos, G., and R. D. Everett.** 2002. Visualization of parental HSV-1 genomes and replication compartments in association with ND10 in live infected cells. Embo J **21:**4989-97.
270. **Spear, P. G.** 2004. Herpes simplex virus: receptors and ligands for cell entry. Cell Microbiol **6:**401-10.
271. **Spear, P. G., and R. Longnecker.** 2003. Herpesvirus entry: an update. J Virol **77:**10179-85.
272. **Spear, P. G., M. T. Shieh, B. C. Herold, D. WuDunn, and T. I. Koshy.** 1992. Heparan sulfate glycosaminoglycans as primary cell surface receptors for herpes simplex virus. Adv Exp Med Biol **313:**341-53.
273. **Steffy, K. R., and J. P. Weir.** 1991. Mutational analysis of two herpes simplex virus type 1 late promoters. J Virol **65:**6454-60.
274. **Steiner, I.** 1996. Human herpes viruses latent infection in the nervous system. Immunol Rev **152:**157-73.

275. **Stringer, K. F., C. J. Ingles, and J. Greenblatt.** 1990. Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TFIID. Nature **345:**783-6.
276. **Taatjes, D. J., A. M. Naar, F. Andel, 3rd, E. Nogales, and R. Tjian.** 2002. Structure, function, and activator-induced conformations of the CRSP coactivator. Science **295:**1058-62.
277. **Tanese, N., D. Saluja, M. F. Vassallo, J. L. Chen, and A. Admon.** 1996. Molecular cloning and analysis of two subunits of the human TFIID complex: hTAFII130 and hTAFII100. Proc Natl Acad Sci U S A **93:**13611-6.
278. **Thomas, M. C., and C. M. Chiang.** 2006. The general transcription machinery and general cofactors. Crit Rev Biochem Mol Biol **41:**105-78.
279. **Thompson, C. M., A. J. Koleske, D. M. Chao, and R. A. Young.** 1993. A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. Cell **73:**1361-75.
280. **Torok, K., K. Stauffer, and W. H. Evans.** 1997. Connexin 32 of gap junctions contains two cytoplasmic calmodulin-binding domains. Biochem J **326 (Pt 2):**479-83.
281. **Tsai, F. T., and P. B. Sigler.** 2000. Structural basis of preinitiation complex assembly on human pol II promoters. Embo J **19:**25-36.
282. **Ueno, H., K. Gonda, T. Takeda, and O. Numata.** 2003. Identification of elongation factor-1alpha as a Ca2+/calmodulin-binding protein in Tetrahymena cilia. Cell Motil Cytoskeleton **55:**51-60.
283. **Uprichard, S. L., and D. M. Knipe.** 1997. Assembly of herpes simplex virus replication proteins at two distinct intranuclear sites. Virology **229:**113-25.
284. **Uprichard, S. L., and D. M. Knipe.** 1996. Herpes simplex ICP27 mutant viruses exhibit reduced expression of specific DNA replication genes. J Virol **70:**1969-80.
285. **Valencia, C. A., W. Ju, and R. Liu.** 2007. Matrin 3 is a Ca2+/calmodulin-binding protein cleaved by caspases. Biochem Biophys Res Commun **361:**281-6.
286. **Verrijzer, C. P., J. L. Chen, K. Yokomori, and R. Tjian.** 1995. Binding of TAFs to core elements directs promoter selectivity by RNA polymerase II. Cell **81:**1115-25.
287. **Wadsworth, S., G. S. Hayward, and B. Roizman.** 1976. Anatomy of herpes simplex virus DNA. V. Terminally repetitive sequences. J Virol **17:**503-12.
288. **Wagner, E. K., and D. C. Bloom.** 1997. Experimental investigation of herpes simplex virus latency. Clin Microbiol Rev **10:**419-43.
289. **Wagner, E. K., J. F. Guzowski, and J. Singh.** 1995. Transcription of the herpes simplex virus genome during productive and latent infection. Prog Nucleic Acid Res Mol Biol **51:**123-65.
290. **Wagner, E. K., M. D. Petroski, N. T. Pande, P. T. Lieu, and M. Rice.** 1998. Analysis of factors influencing kinetics of herpes simplex virus transcription utilizing recombinant virus. Methods **16:**105-16.
291. **Warfield, L., J. A. Ranish, and S. Hahn.** 2004. Positive and negative functions of the SAGA complex mediated through interaction of Spt8 with TBP and the N-terminal domain of TFIIA. Genes Dev **18:**1022-34.
292. **Wassarman, D. A., and F. Sauer.** 2001. TAF(II)250: a transcription toolbox. J Cell Sci **114:**2895-902.

293. **Watanabe, T., K. Hayashi, A. Tanaka, T. Furumoto, F. Hanaoka, and Y. Ohkuma.** 2003. The carboxy terminus of the small subunit of TFIIE regulates the transition from transcription initiation to elongation by RNA polymerase II. Mol Cell Biol **23:**2914-26.
294. **Watson, R. J., and J. B. Clements.** 1978. Characterization of transcription-deficient temperature-sensitive mutants of herpes simplex virus type 1. Virology **91:**364-79.
295. **Weir, J. P.** 2001. Regulation of herpes simplex virus gene expression. Gene **271:**117-30.
296. **Wilcox, K. W., A. Kohn, E. Sklyanskaya, and B. Roizman.** 1980. Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. J Virol **33:**167-82.
297. **Wilkinson, D. E., and S. K. Weller.** 2003. The role of DNA recombination in herpes simplex virus DNA replication. IUBMB Life **55:**451-8.
298. **Woerner, A. M., and J. P. Weir.** 1998. Characterization of the initiator and downstream promoter elements of herpes simplex virus 1 late genes. Virology **249:**219-30.
299. **Woychik, N. A., and M. Hampsey.** 2002. The RNA polymerase II machinery: structure illuminates function. Cell **108:**453-63.
300. **Wright, K. J., M. T. Marr, 2nd, and R. Tjian.** 2006. TAF4 nucleates a core subcomplex of TFIID and mediates activated transcription from a TATA-less promoter. Proc Natl Acad Sci U S A **103:**12347-52.
301. **WuDunn, D., and P. G. Spear.** 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J Virol **63:**52-8.
302. **Xia, K., N. A. DeLuca, and D. M. Knipe.** 1996. Analysis of phosphorylation sites of herpes simplex virus type 1 ICP4. J Virol **70:**1061-71.
303. **Xia, K., D. M. Knipe, and N. A. DeLuca.** 1996. Role of protein kinase A and the serine-rich region of herpes simplex virus type 1 ICP4 in viral replication. J Virol **70:**1050-60.
304. **Xiao, H., A. Pearson, B. Coulombe, R. Truant, S. Zhang, J. L. Regier, S. J. Triezenberg, D. Reinberg, O. Flores, C. J. Ingles, and et al.** 1994. Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. Mol Cell Biol **14:**7013-24.
305. **Xiao, W., L. I. Pizer, and K. W. Wilcox.** 1997. Identification of a promoter-specific transactivation domain in the herpes simplex virus regulatory protein ICP4. J Virol **71:**1757-65.
306. **Yang, F., R. DeBeaumont, S. Zhou, and A. M. Naar.** 2004. The activator-recruited cofactor/Mediator coactivator subunit ARC92 is a functionally important target of the VP16 transcriptional activator. Proc Natl Acad Sci U S A **101:**2339-44.
307. **Yang, M., J. Hay, and W. T. Ruyechan.** 2008. Varicella-zoster virus IE62 protein utilizes the human mediator complex in promoter activation. J Virol **82:**12154-63.
308. **Yao, F., and R. J. Courtney.** 1989. A major transcriptional regulatory protein (ICP4) of herpes simplex virus type 1 is associated with purified virions. J Virol **63:**3338-44.
309. **Yao, F., and P. A. Schaffer.** 1994. Physical interaction between the herpes simplex virus type 1 immediate-early regulatory proteins ICP0 and ICP4. J Virol **68:**8158-68.
310. **Yokomori, K., C. P. Verrijzer, and R. Tjian.** 1998. An interplay between TATA box-binding protein and transcription factors IIE and IIA modulates DNA binding and transcription. Proc Natl Acad Sci U S A **95:**6722-7.
311. **Yudkovsky, N., J. A. Ranish, and S. Hahn.** 2000. A transcription reinitiation intermediate that is stabilized by activator. Nature **408:**225-9.

312. **Zabierowski, S., and N. A. DeLuca.** 2004. Differential cellular requirements for activation of herpes simplex virus type 1 early (tk) and late (gC) promoters by ICP4. J Virol **78:**6162-70.
313. **Zhang, Y. F., and E. K. Wagner.** 1987. The kinetics of expression of individual herpes simplex virus type 1 transcripts. Virus Genes **1:**49-60.
314. **Zhu, Z., and P. A. Schaffer.** 1995. Intracellular localization of the herpes simplex virus type 1 major transcriptional regulatory protein, ICP4, is affected by ICP27. J Virol **69:**49-59.