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The DNA binding and trans-regulatory properties of the herpesvirus immediate-early protein ICP4

DiDonato, Joseph Anthony, Ph.D.

The Ohio State University, 1990

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THE DNA BINDING AND TRANS-REGULATORY PROPERTIES
OF THE HERPESVIRUS IMMEDIATE-EARLY PROTEIN ICP4

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Joseph A. DiDonato, B.A., M.S.

****

The Ohio State University
1990

Dissertation Committee:
Mark T. Muller, Ph.D.
Philip Perlman, Ph.D.
Lee Johnson, Ph.D.
Berl Oakley, Ph.D.

Approved by

Program in Molecular Cellular
and Developmental Biology
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VITA

August 26, 1958....................... Born - Akron, Ohio

1980................................. B.A.  
Hiram College, Hiram, Ohio

1983................................. M.S.  
The University of Akron  
Akron, Ohio

1987-1990............................. Research Associate,  
Molecular, Cellular and  
Developmental Biology  
Program, The Ohio State  
University, Columbus, Ohio

1985-1986............................. Teaching Associate,  
Molecular, Cellular and  
Developmental Biology  
Program, The Ohio State  
University, Columbus, Ohio

1983-1985............................. Graduate Research Associate  
Molecular, Cellular and  
Developmental Biology  
Program, The Ohio State  
University, Columbus, Ohio
PUBLICATIONS


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<td>α</td>
<td>Immediate-early</td>
</tr>
<tr>
<td>β</td>
<td>Early</td>
</tr>
<tr>
<td>A4</td>
<td>an ICP4 containing complex</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyl-transferase</td>
</tr>
<tr>
<td>CIAP</td>
<td>Calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>Cu-OP</td>
<td>Copper- Phenantroline complex</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>gD</td>
<td>Glycoprotein D</td>
</tr>
<tr>
<td>hGH</td>
<td>Human growth hormone</td>
</tr>
<tr>
<td>HSV</td>
<td>Herpes simplex virus</td>
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<tr>
<td>ICP</td>
<td>Infected cell protein</td>
</tr>
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<td>IE</td>
<td>Immediate-early</td>
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<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>Equalibrium dissociation constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;r&lt;/sub&gt;</td>
<td>Relative binding affinity</td>
</tr>
<tr>
<td>M&lt;sub&gt;w&lt;/sub&gt;</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NP40</td>
<td>Nonidet P40</td>
</tr>
<tr>
<td>NPT</td>
<td>Non-permissive temperature</td>
</tr>
<tr>
<td>PI</td>
<td>Post infection</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethyly-sulphonyl flouride</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl alcohol</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>tk</td>
<td>Tymidine kinase</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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LIST OF SOLUTIONS

10x Lo NGB
67 mM Tris·Cl pH 7.9
33 mM Na Acetate

10x Binding buffer
50 mM Tris pH 7.9
5 mM EDTA pH 8.0
0.5% NP40
125 mM NaPO₄ pH 6.8

10x TBS
100 mM Tris
1.50 M NaCl
pH to 7.85 with HCl

10x Electrophoresis buffer
200 mM Tris pH 8.0
2 mM EDTA pH 8.0
50 mM NaCl

10x PBS (for 1L)
2.0 g KCl
11.5 g Na₂PO₄
2.0 g KH₂PO₄

2 M NET
2 M NaCl
20 mM Tris pH 8.0
10 mM EDTA pH 8.0

40% Acrylamide
40 g Acrylamide
0.5 g Bis-Acrylamide
volume to 100 ml
(for mobility shift gels)
STATEMENT OF THE PROBLEM

ICP4, the gene product of IE gene 3 of HSV-1 is a crucial regulatory protein and plays a pivotal role in the expression of early and late classes of HSV genes as well as in negatively regulating IE gene expression. Previously, ICP4 was shown to be a DNA binding protein and studies in this lab and in others has revealed that it is present in a sequence specific DNA-protein complex detected in vitro. The sequence on which the complex forms is located at the 5' transcriptional start site of immediate-early gene 3 (IE3). IE3 is the gene that encodes the protein ICP4. Genetic evidence points to a negative autoregulatory role for its own expression and that of a negative regulatory role for the expression of the other IE genes after ca. 2 hr post infection (PI). In the early and late class genes of HSV, ICP4 plays a stimulatory role in regard to gene expression. A consensus sequence to which the protein binds has been derived (Faber and Wilcox, 1986). Being a DNA binding protein it is easy to imagine how ICP4 might regulate its own expression but the picture becomes quite murky when the effect on the other IE genes and the early and late class genes is taken into account. Models describing how ICP4 regulates its own expression negatively have been likened to
the classical operator-repressor model, such as the lac repressor. This model however, does not explain its effect on the other IE genes to mention nothing about its ability to stimulate expression of the other classes of HSV genes.

Recently, a closely related IE protein of Pseudorabies virus was shown to stabilize the formation of preinitiation transcription complexes in vitro (Sawadogo and Roeder, 1985). It does this by stabilizing TFIID binding to TATA (Abmayr et al., 1988; Workman al., 1988). These findings may give a clue as to how ICP4 can have two different effects by acting through one pathway. Studies performed here were directed at biochemically characterizing the ICP4 containing DNA-protein complex and purification of the factor(s) involved in complex formation.

The physiological significance of DNA segments that form DNA-protein complexes containing ICP4 in vitro was examined in vivo using transient expression transfection assays. Subsequently, mutational alterations in these sequences and examination of them both in vitro and in vivo was performed. The results of these endeavors were used to formulate plausible models by which a single protein such as ICP4 could activate as well as repress expression of HSV genes.
CHAPTER I

REVIEW OF THE LITERATURE

Herpes viruses are large enveloped icosahedral double stranded DNA viruses which can be classified into three broad groups based upon DNA structure, host range, cytopathology, replicative cycle and characteristics of the latent infection (Roizman et al., 1973). The three groups are designated as alpha (α), beta(β) or gamma (γ) herpes viruses. All three groups contain herpes viruses which are able to infect man. There are six such viruses and they are designated as human herpesviruses 1 through 6. The commonly used names of these viruses are herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2), [human herpes viruses types 1 and 2 respectively], varicella-zoster virus (VZV) [human herpes virus type 3], Epstein Barr virus (EBV) [human herpes virus type 4] and cytomegalovirus (CMV) [human herpes virus type 5] and the most recently discovered human herpesvirus, human B-lymphotropic virus [HBLV] also known as HHV-6. HHV-6 has been isolated almost exclusively from patients suffering from acquired immunodeficiency syndrome (Salahuddin et al., 1986; Takahashi et al., 1989). HSV-1 and HSV-2 have been the most
experimentally studied of the human herpes viruses, due to a number of reasons; 1) isolation and characterization of HSV isolates over 50 years ago (Gruter, 1924), 2) ease of propagation of the virus in tissue culture, on readily available and easily grown tissue culture cells, 3) availability of genetic mapping of HSV viral genes using HSV-1 by HSV-2 intertypic recombinants (Halliburton et al., 1977).

VIRION STRUCTURE

The herpes virus virions are composed of four distinct morphological components. These components are known as the core which contains the viral DNA wound about a proteinaceous spindle, in the form of a toroid (Furlong et al., 1972; Heine, 1974). Surrounding the core is the icosahedral shaped capsid which has a diameter of ca. 100 nm and is composed of 162 capsomeres (Wildy et al., 1960). The capsid is covered by the tegument, which is amorphous in shape and is composed of protein. The tegument is structurally defined as the material located between the virus capsid and the viral envelope (Roizman and Furlong, 1974). The viral envelope, which surrounds the tegument, is usually ca. 280 nm in diameter (McCombs et al., 1971) and is composed of patches of the cellular membrane (Armstrong et
al., 1961). Normally, between 500 to 1000 infectious virus particles are produced per cell in tissue culture cultivation of the virus (DeLuca and Schaffer, 1988), while there is a thirty-fold higher number of virus particles produced (Everett, 1987).

**HSV-1 AND HSV-2 DNA STRUCTURE**

The entire genome of HSV-1 has now been sequenced and contains 152,260 nucleotide base pairs (McGeoch et al., 1985; McGeogh et al., 1986; McGeogh et al., 1988; Perry and McGeoch, 1988). The HSV-1 genome is a linear double stranded DNA that is 68% G + C rich. Earlier studies revealed the sequence arrangement of the DNA as two separate but contiguous regions of DNA known as the long and the short regions, which contain unique sequences of DNA bounded by inverted repeats, and comprise 82% and 18% of the HSV genome respectively. The majority of the HSV DNA lies in the long unique region and in the short unique region (Sheldrick and Berthelot, 1975). The reiterated sequences, that bound the unique long component (UL), comprise a total of ca. 12.6% of the DNA. The reiterated region that bounds the short unique region (Us) comprises only 8.6% of the DNA (Wadsworth et al., 1975). Due to the presence of inverted terminal repeats, at the ends of UL and Us, the production
of four isomeric forms of the virus can and are produced during a productive infection as a result of recombination in the repeated regions. Recombination, in such a manner, gives rise to four populations of viral DNA categorized as prototype (P), inverted short (IS), inverted long (IL) and inversion of the short and long (ILS) (Roizman et al., 1974; Hayward et al., 1975) each of which is infective (Roizman et al., 1979).

The HSV genome unlike the cellular chromatin is not arranged in a nucleosomal structure during a productive infection. This observation was made using microccoccal nuclease to digest isolated infected nuclei and probing of a DNA-protein gel Southern blot with HSV DNA (Muggeridge and Fraser, 1986; Deshmore and Fraser, 1989). However, HSV DNA present in latently infected neuronal ganglia when assayed as above, exhibited a nucleosomal-like organization (Deshmore and Fraser, 1989).

**HSV LIFE CYCLE**

The HSV virion attaches itself to the host cell via a cellular protein (fibroblast growth factor receptor)-viral glycoprotein interaction (Kaner et al., 1990), which then allows internalization of the virion (Sarmiento et al., 1979). Once internalized, the virion begins to uncoat its
nucleocapsid as it makes its way to the nucleus, at which point the viral DNA is transported into the nucleus by an unknown mechanism. After importation into the nucleus, the viral DNA is transcribed by the host cell's RNA polymerase II (Pol II), as indicated by the inhibition of viral as well as the bulk of cellular transcription by treatment of infected cells by α-amanitin (an inhibitor of the cellular RNA polymerase II) (Costanzo et al., 1977).

Viral protein synthesis occurs in three major waves temporally regulated in a cascade fashion (Honess and Roizman, 1974; reviewed by Roizman and Batterson, 1985). Elucidation of the three classes of viral polypeptides, the (immediate early [IE or α], early [β] and gamma [late] [γ] class polypeptides), was accomplished by the use of translational inhibitors in pulse-chase experiments, administered to infected tissue culture cells at different times after infection (Honess and Roizman, 1974; Honess and Roizman, 1975).

Polypeptides of the IE group are expressed maximally (at 3-4 hr post-infection) (PI) and at decreasing rates at later times. De novo viral protein synthesis is not required in order for IE proteins to be expressed (Honess and Roizman, 1974; Roizman et al., 1974). In addition, if amino acid analogues such as L-canavanine and azetidine-2-carboxylic acid were added to infected cells at 1 hr PI,
mainly IE polypeptides synthesis was detected to 18.5 hr PI, without expression of the late polypeptides and very little early class polypeptides, indicating a requirement for IE proteins in the expression of early and late class proteins. If these amino acid analogues were added at increasingly later times PI and the infected cells then labeled with $^{14}$C amino acids, it became apparent that $\beta$ proteins were required to: 1) observe a decline in IE protein synthesis and increased levels of host protein shut-off, 2) allow the late polypeptides to be expressed. As late polypeptides increased in their rate of synthesis, the $\beta$ proteins synthesis rate also declined. Maximal rates of $\beta$ polypeptide synthesis were observed at 5 - 7 hr PI while late proteins were maximally expressed at 9 - 12 hr PI (Honess and Roizman, 1974; Hones and Roizman, 1975).

During the synthesis of late polypeptides, newly made HSV DNA is incorporated into the viral nucleocapsid, inside the nucleus. Viral DNA is thought to be processed to unit length molecules by an unknown endonuclease, which cleaves circular or large head to tail concatameric HSV DNA (Jacob et al., 1979). This concatameric DNA most likely results from a rolling circle mode of HSV DNA replication, which has been detected at late times in the infection cycle (Ben-Porat and Tokazewski, 1977; Jacob and Roizman, 1977). The origins of HSV DNA replication have been mapped using
defective viral genomes and transfection studies with HSV DNA fragments and intact helper virus DNA. Two sites of HSV DNA replication origins were identified: one region is in the unique long region and the other is in a 90 base pair (bp) stretch of DNA in the reiterated short terminal repeats, thus it is present in 2 copies giving rise to a total of three origins of replication in the HSV genome (Vlazny and Frenkel, 1981; Mocarski and Roizman, 1982; Stow and McMonagle, 1983). Identification of which origin(s) are functional during replication still await elucidation.

Once the viral DNA is packaged into the nucleocapsid, it attaches to modified patches of the nuclear lamella and becomes enveloped in the process. The modified patches that are present on the nuclear lamellae likely consist of viral specified membrane proteins, on the outside surface and anchorage and tegument proteins on the inner surface. Enveloped virions contain very little if any host cell proteins, so it seems plausible that the proteins which comprise these modified patches are virally encoded (Aaronson and Blobel, 1975). After the nucleocapsid buds from the inner lamella of the nuclear membrane, the enveloped nucleocapsid then makes its way from the cell. The process of egress from the cell is not clear, some investigators believe the enveloped particle exits the cell by way of the cisternae of the endoplasmic reticulum, which
extends out to the cellular plasma membrane (Shwartz and Roizman, 1969). Another school of thought believes the enveloped particle exits the cell via the Golgi apparatus, much like a secreted protein might do (Johnson and Spear, 1982).

**EFFECTS OF VIRAL INFECTION ON THE HOST CELL'S MACROMOLECULAR METABOLISM**

A characteristic feature of HSV infection is the rapid cessation of host macromolecular metabolism. This shutdown is most clearly evident in the failure of the host cell to replicate cellular DNA (Roizman and Roane, 1964) and to direct host cell protein synthesis (Roizman et al., 1965; Sydiskis and Roizman, 1966). Host cell protein shutoff is due in part to at least two different mechanisms, one which requires no de novo viral protein synthesis, acts as a general protein shutoff mechanism and has been defined by the generation of viral mutant in this function (the viral host shutoff [vhs] mutants) (Fenwick et al., 1979; Read and Frenkel, 1983). This generalized protein shutoff function is associated with the virion and the viral protein responsible for shutoff is brought inside the cell with the capsid.
A second stage of host protein shutoff requires that de novo synthesis of viral polypeptides occur. These viral proteins have been defined to either the β or the late stage of viral protein synthesis. This class of virally encoded shutoff polypeptides discriminates between host and viral protein synthesis (Honess and Roizman, 1974, 1975; Nishioka and Silverstein, 1977; Silverstein and Engelhardt, 1979).

Host cell RNA metabolism is also affected by HSV infection. Ribosomal RNA synthesis is nearly shutoff, 45S ribosomal precursor is made and then methylated. Cellular mRNA continues to be made although by some unknown mechanism it fails to direct host protein synthesis (Wagner and Roizman, 1969) and is subject to degradation (Nishioka and Silverstein, 1973; Pizer and Beard, 1976; Sternberg and Pizer, 1982). This shutoff of host cell mRNA synthesis is associated with at least one viral function (Fenwick et al., 1979). Since there are fast and slow shutoff strains of HSV, it seems likely that there may be at least two virally encoded functions operational during the shutoff (Fenwick et al., 1979).
GENE EXPRESSION DURING HSV-1 INFECTION

Viral protein synthesis, as mentioned previously, is temporally regulated in a cascade fashion into three classes based upon their kinetics of synthesis (Honess and Roizman, 1974). The temporally regulated cascade, of protein expression, is also reflected at the mRNA level (Spear and Roizman, 1980; Wagner et al., 1982). Like host cell mRNA, HSV-1 mRNA is synthesized in the nucleus, is capped at the 5' end and polyadenylated at the 3' end. Additionally, the mRNA is internally methylated (Backenheimer and Roizman, 1972; Moss et al., 1977; Wagner and Roizman, 1969). Viral mRNA differs from cellular mRNA in the degree of splicing. Only a limited number of the HSV mRNA's are spliced, whereas the majority of the cellular mRNA's are spliced (Frink et al., 1981; Watson et al., 1981).

IMMEDIATE EARLY GENE EXPRESSION

There are five IE gene products synthesized immediately upon infection (Honess and Roizman, 1974). Failure to produce the IE class of proteins resulted in the loss in ability to transcribe the β and gamma classes of genes (Honess and Roizman, 1975; Swanstrom et al., 1975; Clements et al., 1977; Anderson et al., 1980). These
findings suggest that the gene products of IE genes may modify the host RNA polymerase II or the cellular transcriptional machinery, in some manner, in order to express the remainder of the viral genes. For this reason, the study of IE gene expression has become the focal point of regulatory studies in HSV-1.

The positions of the IE genes of HSV-1 have been mapped and their orientations discerned, relative to their location in the viral genome (Clements et al., 1979; Watson et al., 1979). The location of each IE gene is depicted (Figure 1). The various nomenclatures used for each IE gene and the properties exhibited by the gene product are listed (Table 1).

All IE genes share some common features, in regard to cis sequences, present in their promoter elements. These features include a minimal promoter element, which contains a TATA box sequence (Breathnach and Chambon, 1981), and a short region of sequence 5' to the TATA box [the length of this region is usually ca. 100 bp]. The minimal promoter is the least amount of promoter sequence needed to drive expression of a linked gene as measured by microinjection into Xenopus oocytes (Cordingly et al., 1983), stable transformation assay (Lang et al., 1984) or by transient transfection assay (Mackem and Roizman 1982; Gelman and Silverstein, 1986). The minimal promoter elements are also
Figure 1. Positional arrangement of the HSV-1 immediate early genes on the HSV-1 physical map. Numbers in the figure correspond to the number of that immediate early gene. Arrows indicate the direction of transcription of the corresponding gene. Abbreviations in the key explain the indicated regions of the HSV-1 physical map. Each immediate early gene encodes for a protein product which is listed in the box. The Vmw designations refer to the Glasgow nomenclature system and the ICP designations refer to the Chicago nomenclature system each of which is used interchangeably.
GENE: PRODUCT:

<table>
<thead>
<tr>
<th>GENE</th>
<th>PRODUCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>IE-1</td>
<td>Vmwl10 (ICP0)</td>
</tr>
<tr>
<td>IE-2</td>
<td>Vmwl63 (ICP27)</td>
</tr>
<tr>
<td>IE-3</td>
<td>Vmwl75 (ICP4)</td>
</tr>
<tr>
<td>IE-4</td>
<td>Vmwl68 (ICP22)</td>
</tr>
<tr>
<td>IE-5</td>
<td>Vmwl12 (ICP47)</td>
</tr>
</tbody>
</table>

$\text{TR}_L$ = The terminal repeat long region

$\text{TR}_S$ = The terminal repeat short region

$\text{IR}_L$ = The inverted repeat long region

$\text{IR}_S$ = The inverted repeat short region

$\text{UL}$ = The unique long region

$\text{US}$ = The unique long region

Figure 1
Table 1. Immediate-early protein products and their characteristics. The immediate-early proteins are listed as to their gene designation and thby both the Chicago nomenclature and the Glasgow designations. Additionally, the size and the function of the protein if known is given.
<table>
<thead>
<tr>
<th>Glasgow system Gene</th>
<th>Product</th>
<th>Chicago system Gene</th>
<th>Product</th>
<th>Size</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IE-1</td>
<td>Vmw110</td>
<td>α0</td>
<td>ICP0</td>
<td>110kd</td>
<td>gene expression</td>
</tr>
<tr>
<td>IE-2</td>
<td>Vmw63</td>
<td>α27</td>
<td>ICP27</td>
<td>63kd</td>
<td>DNA replication and late gene expression + some IE regulation</td>
</tr>
<tr>
<td>IE-3</td>
<td>Vmw175</td>
<td>α4</td>
<td>ICP4</td>
<td>175kd</td>
<td>Early and late gene activator, IE negative regulator</td>
</tr>
<tr>
<td>IE-4</td>
<td>Vmw68</td>
<td>α22</td>
<td>ICP22</td>
<td>68kd</td>
<td>enhances some Late gene expression</td>
</tr>
<tr>
<td>IE-5</td>
<td>Vmw12</td>
<td>α47</td>
<td>ICP47</td>
<td>12kd</td>
<td>no role known</td>
</tr>
</tbody>
</table>
not responsive to viral activation, and are sometimes referred to as the proximal promoter element. Inclusion of additional sequences 5' to the minimal promoter ([usually 50 - 100 bp] to the proximal promoter) gives rise to the distal promoter region. This region contains one or more G + C rich sequence motifs, the majority of which are binding sites for the transcription factor Sp1 (Dynan and Tjian, 1983), which can stimulate expression driven from such promoters (Jones and Tjian, 1985). Like the proximal promoter, the distal promoter elements are not responsive to viral activation. Addition of sequences [50 - 100 bp] 5' to the distal promoter forms the full IE promoter which contains in this last segment of DNA the cis sites that are responsive to viral activation and allow a linked gene to this promoter to be regulated as an IE gene (Mackem and Roizman, 1982a,b; Cordingly et al., 1983; Gaffney et al., 1985).

The sequence element that responds to viral activation has been identified and is known as the consensus sequence TAATGARAT (Mackem and Roizman, 1982a; Land et al., 1984; Bzik and Preston, 1986; Kristie and Roizman, 1987), where R is a purine. This sequence binds the cellular transcription factor NFIII (Pruijin et al., 1986), also known as OTF-1 (Oct-1) (Fletcher et al., 1987; Oneill et al., 1988; Gerster and Roeder, 1988) and in conjunction with
the virion stimulatory protein Vmw65 (65K\text{tiff}) (Cambell et al., 1984), forms a DNA–protein complex \textit{in vitro} (O'Hare and Goding, 1988; Preston et al., 1988; Gerster and Roeder, 1988). The ability to form this DNA–protein complex \textit{in vitro} is related to the ability of the virion structural component (65K\text{tiff}) to stimulate expression of IE genes, when the TAATGARAT sequence is present in the IE promoter (Mackem and Roizman, 1982a,b; Kristie and Roizman, 1984; Preston et al., 1984; Bzik and Preston, 1986), during the initial stages of HSV infection (Post et al., 1981; Batterson and Roizman 1983). After the initial burst of expression of IE genes, IE transcription subsides (at ca. 2 hr PI) to decreased levels. IE gene 3 transcription is the most severely down regulated of the IE genes (Honess and Roizman, 1974; Weinheimer and McKnight, 1987). Negative regulation of IE genes requires a functional IE3 gene product, Vmw175 (ICP4) (Preston, 1979; Dixon and Schaffer, 1980; Watson and Clements, 1980). Nuclear run off experiments of infected cell nuclei at different times post infection and \textit{in vitro} transcription experiments indicate that negative regulation by ICP4 occurs both at the transcriptional and post transcriptional level (Pizer et al., 1986; Weinheimer and McKnight, 1987). The results of this type of nuclear run off experiment are very difficult to interpret due to non-
template strand synthesis at times greater than 4 hours PI, which give rise to non-specific transcription products and render any transcription rates observed at these times uninterpretable. Another problem with this type of analysis is that a mutation in cis or a mutant transacting viral factor must be introduced back into the viral genome by marker rescue and the appropriate mutant isolated before analysis of function can be ascribed to a particular viral protein or DNA sequence. This is a very arduous and laborious task. A method that circumvents and simplifies this problem has been to utilize transient transfection expression assays. These assays employ isolated HSV promoters fused to indicator genes cotransfected with HSV regulatory genes. Following a reductionist approach, the function of each regulatory gene in HSV may be determined.

TRANSIENT EXPRESSION STUDIES INVOLVING GENE REGULATION OF HSV GENES.

Transient transfection assays allow the introduction of foreign gene(s) into tissue culture cells such that the level of expression of these foreign genes can be measured at either the level of RNA, protein or as an enzymatic activity (Gorman et al., 1982). This approach, to studying
either regulatory sequences or regulatory proteins or both, is ideal for examining the regulatory processes in HSV gene regulation. The use of isolated viral genes eliminates contributions of other viral proteins present in the cell during a productive infection, allowing the effect of specific genes or combinations of genes to be monitored in a somewhat "controlled" fashion (Post et al., 1981; Mackem and Roizman, 1982; Everett).

Transient expression assays, aimed at examining the effects of cloned IE gene products on viral transcriptional control, have yielded new findings. First, the gene products of IE genes 1, 2, and 3 are capable of transactivating early and late genes (Everett, 1984; O'Hare and Hayward, 1985a,b; Everett, 1986; Gelman and Silverstein, 1986). Second, the IE1 gene product can activate all three classes of HSV genes and cellular genes (Everett, 1984; O'Hare and Hayward, 1985a,b; Gelman and Silverstein, 1985). Third, IE gene 1 and IE gene 3 products act synergistically to strongly activate expression from early and late HSV promoters (Everett, 1984; Everett, Gelman and Silverstein, 1985; Everett, 1986). Fourth, the IE gene 3 product, ICP4, can stimulate indicator genes linked to early and late promoters (Everett, 1984; O'Hare and Hayward, 1985a,b; Gelman and Silverstein, 1986). Indicator genes that were linked to IE promoters were
regulated negatively by ICP4 (DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985b; Gelman and Silverstein, 1986; Gelman and Silverstein, 1987; Patterson and Everett, 1988a; Roberts et al., 1988; DiDonato and Muller, 12th Annual Herpesvirus Workshop, 1987, p293; this study).

The repressive activity of ICP4 was demonstrated conclusively by the use of temperature sensitive mutants (ts) of ICP4, in similar experiments using transient expression assays, with either plasmid borne copies of the tsICP4 or infection of transfected tissue culture cells with a tsICP4 strain of HSV. The tsICP4 failed to regulate indicator genes, linked to IE promoters, negatively when cells were grown at the nonpermissive temperature (NPT) (DeLuca and Schaffer, 1985; Gelman and Silverstein, 1986). Thus, it appears that in addition to its ability to transactivate early and late HSV genes, functional ICP4 is also necessary to regulate IE genes negatively. At the present time, it is not clear whether or not ICP4 directly or indirectly represses expression of IE genes, although DNA binding studies using DNA affinity purified ICP4 indicate that ICP4 binds directly to the IE promoter DNA (Kattar-Cooley, 1989; DiDonato and Muller, 1989) and could therefore play a direct role in negative regulation of IE genes.
ICP4, the gene product of IE gene 3 (IE3), is a large phosphoprotein whose entire amino acid sequence is known (Peirera et al., 1977; Preston, 1979; Watson and Clements, 1980; Wilcox et al., 1980; McGeough et al., 1986). Since this IE protein plays a crucial role in the activation of early and late genes and in the negative regulation of IE genes, it was of extreme importance that the DNA binding properties of such a multifunctional protein be examined in order to gain insight as to how ICP4 elicits its effects.

Early attempts to purify ICP4 indicated it was a DNA binding protein and after partial purification, ICP4 appeared to lose its ability to bind DNA (Freeman and Powell, 1982). The DNA binding ability was regained by addition of mock infected cell proteins to the partially purified preparation of ICP4. This result implied that a cellular protein(s) may be involved in aiding ICP4 to bind to DNA. Recent studies concerning the purification of ICP4 have found the native form of the protein, in solution, to be a homodimer with a molecular mass of approximately 350Kd (Metzler and Wilcox, 1985). Chromatographic fractions of partially purified ICP4 that were enriched 100 fold in ICP4 concentration relative to infected cell nuclear extracts, were used in DNA binding assays. The DNA-protein complex
(containing ICP4) was immunoprecipitated using antibodies directed against ICP4. Specific DNA-protein complexes containing ICP4 were identified using pBR322 DNA sequences and HSV DNA. HSV sequences forming complexes containing ICP4 were found to be sequences present in the promoter region of the glycoprotein D gene (gD) of HSV (Beard et al., 1986; Faber and Wilcox, 1986). Comparison of the sequences present in the immunoprecipitated DNA-protein complexes allowed a consensus binding site to be derived. The consensus binding site for ICP4 was found to be ATCGTCNNNYCGRC (Y= pyrimidine, R= purine, N= any base) (Faber and Wilcox, 1986). Further binding studies, using infected cell nuclear extracts and low ionic strength polyacrylamide gel retardation assays (also known as mobility shift assays), has demonstrated specific binding to the promoter element of the IE-1 gene (Kristie and Roizman, 1986b; Kattar-Cooley, 1989; DiDonato and Muller, 1989). ICP4 was shown to be a component of these specific DNA-protein complexes by retardation of the complex's migration through the gel by monoclonal antibody to ICP4 (Kristie and Roizman, 1986; Muller, 1987; Deluca and Schaffer, 1988; Patterson and Everett, 1988a; DiDonato and Muller, 1989, Resnick et al., 1989). The limits of the ICP4 interaction were localized by Exonuclease III digestion (Kristie and Roizman, 1986b), DNase I and chemical footprinting.
to a region in the IE-1 promoter that contains the ICP4 binding consensus derived by Faber and Wilcox.

ICP4 binding to other IE genes has also been demonstrated in the IE gene 2 promoter and in the distal promoter of IE3 by use of mobility shift assay and antibody retardation of DNA-protein complexes (Kristie and Roizman, 1986a,b). A site in the IE3 promoter was identified by mobility shift assay (Muller, 1987). Dimethyl sulphate chemical footprinting localized this binding site to the 5' transcriptional start site which contains a consensus ICP4 binding site (Muller, 1987). Binding of ICP4 to this site may play a role in negative autoregulation of IE3, in a manner similar to that seen for the large T antigen of Simian Virus 40 (SV40) (Tooze, 1982; Rio and Tjian, 1983).

DNA-protein complexes containing ICP4 have also been found in fragments of HSV DNA that do not contain the ICP4 binding consensus (Michael et al., 1988). The nonconsensus site DNA-protein complexes form on DNA sequences located in the long untranslated leader regions of some early and late HSV genes. Binding affinity of ICP4 to these nonconsensus sites appears to be effected negatively by posttranslational modification of the protein (Michael et al., 1988).

The contributions of host cell factors and their interaction with ICP4, to aid in DNA binding, remain unknown
at the present time. With the recent advent of DNA affinity chromatography, used as a tool in the purification of transcription factors to near homogeneity, the proteins responsible for ICP4/DNA complex formation should be purified in this manner (Rosenfeld and Kelley, 1986; Kadonoga and Tjian, 1986). ICP4 purified in such a manner appears to bind the ICP4 consensus site DNA alone without the need for host cell factors (Kattar-Cooley and Wilcox, 1989; DiDonato and Muller, 1989). Multiple DNA-protein complexes formed with infected cell nuclear extract or with affinity purified ICP4 have recently been identified. These multiple DNA-protein complexes exhibit different equilibrium binding affinities indicating protein-protein interaction (DiDonato and Muller, 1989). Perhaps, self association and DNA binding are important features of regulatory control of gene expression by ICP4.

**STRUCTURAL DOMAINS OF ICP4 THAT PERTAIN TO FUNCTION OF THE PROTEIN**

ICP4 is a large (175Kd) phosphoprotein (Periera et al., 1977) containing 1298 amino acids and its entire nucleic acid sequence known (McGeough et al., 1986). The large size of the protein may reflect a complex structural organization that is indicative of the many functions
ascribed to this protein. Recent studies have addressed the issue of ICP4's structural organization and its importance on the various functions of the protein. These studies have used in vitro mutagenesis of cloned copies of ICP4. The mutations include deletions at both the amino terminal and carboxyl terminal ends and small in-frame insertions, some of which, result in nonsense mutations. These various constructions were then assayed for stimulatory or negative regulatory ability in transient expression assays and in DNA binding assays using mobility shift assays (DeLuca and Schaffer, 1987; DeLuca and Schaffer, 1988, Patterson and Everett, 1988a,b; Shepard et al., 1989). An assortment of the mutants, in the plasmid borne copies of ICP4, were recombined into the virus and functional assays carried out using the recombinant virus. The same observations that were observed in the transient assays, with plasmid versions of the different ICP4 mutants, were also seen when the identical viral mutations were used to infect tissue culture cells (DeLuca and Schaffer, 1988; Shepard et al., 1989a). These observations further support the view that use of transfection experiments faithfully mimic the action of the cloned copy of ICP4 in vivo.

The results of these studies indicate ICP4 is divided into different structural domains. These regions include a nuclear localization domain, a DNA binding domain, a
phosphorylation site domain, a transactivation and auto repression domain and finally a domain that is important in viral DNA synthesis, late gene expression and intranuclear localization (Deluca and Schaffer, 1988; Patterson and Everett, 1988a,b; Shepard et al., 1989). The different domains are not mutually exclusive and there is a great deal of functional overlap between domains especially those concerned with autorepression, DNA binding and transactivation. At the amino acid sequence level, ICP4 does not share homology with any reported non-viral protein whose sequence is in the protein sequence library. ICP4 shares 5 regions of homology with the immediate early protein of varicella- zoster, a gamma human herpesvirus (McGeough et al., 1986) and two long regions of homology with the IE protein of Pseudorabies virus (PRV) (Vlcek et al., 1989). The PRV IE protein has been shown to increase the binding stability of the TATA binding factor TFIID to TATA (Abmayr et al., 1988). Perhaps ICP4 is also capable of this function.

Recent studies have further defined the DNA binding domain to a 228 amino acid region of ICP4. This region can be expressed as a fusion protein in E. Coli and is capable of binding consensus and nonconsensus ICP4 binding sites in vitro. This finding indicates ICP4 alone is capable of binding DNA in a sequence specific manner.
MATERIALS AND METHODS

Restriction enzymes and tRNA were from Bethesda Research laboratories, Inc., Gaithersburg, Md. Sepharose CL2B, poly(dA-dT)·(dA·dT), 1,10-phenanthroline, 2,9-dimethyl-1,10-phenanthroline, 3-mercaptopropionic acid and Acetyl Co-A came from Sigma Chemicals (St. Louis, Missouri). T4 polynucleotide kinase and Sequenase were obtained from United States Biochemical Corporation (Cleveland, Ohio), radioactive nucleotides were from New England Nuclear (Boston, Massachusetts), 14C-Chloramphenicol was purchased from ICN (San Jose, California) and electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, California). Human growth hormone (hGH) assay kits were from Nichols Institute (San Francisco, California).

Cells and viruses.

BHK cells were grown in Dulbecco modified Eagle medium (Flow Laboratories, Inc., McLean, Virginia) supplemented with 5% fetal bovine serum (GIBCO Laboratories, Grand
Island, New York) and 50 μg/ml Gentamicin. Stocks of HSV-1 (KOS strain) were prepared from low-multiplicity passage as described previously (Muller, 1987).

Transfections.

1) Human growth hormone transfection. The cells were seeded 1 day prior to transfection in 100mm petri dishes (1.0 x 10^6 cells per plate) and were transfected using the calcium phosphate precipitate method (Graham and van der Eb, 1973). Transfections with hGH gene constructions used 2.5μg of indicator gene per plate (100mm). Effector genes were added to the indicator as molar gene equivalents (MGE's) and then precipitated with CaPO_4. The DNA-calcium phosphate precipitate was allowed to remain on the cell monolayers for 4 hr at which time the medium removed and fresh medium was added. The medium was replaced at 24 hr later and the growth medium was harvested at 40-48 hr post transfection for human growth hormone assays. Harvested culture media was stored at -20°C until assayed.

2) Chloramphenicol acetyl-transferase (CAT) indicator gene transfections. BHK cells (3 x 10^5 cells) were plated in 60mm dishes the night before transfection. CAT containing gene constructions contained 4μg of indicator gene unless stated otherwise in the Figure legend. Transfections
followed the same procedure as that listed for the hGH transfections. The DNA-calcium phosphate precipitate was removed from the cell monolayer after 4 hours and the media was replaced. Cells were harvested at 36 to 44 hours post transfection. Cells were harvested by removal of the culture media and washing the monolayer with 3 ml ice-cold 1x PBS two times. The washes were discarded and one ml of 1x PBS was added to the plate and the cells were scraped from the plate with a rubber policeman. Cells were transferred to a microfuge tube (1.9 ml) and centrifuged for 30 sec to pellet the cells, the wash liquid removed and the cell pellet frozen at -80°C until cell extracts were made (no later than 1 week after harvesting).

Chloramphenicol acetyl-transferase assay.

Frozen cell pellets were thawed on ice. To each tube 50 µl of 50 mM beta mercapto ethanol (BME), 50 µl of 0.5 M Tris·Cl (pH 7.5), 50 µM thymidine and 250 µl water were added and the pellet resuspended. The resuspended pellets were sonicated on ice for 30 sec. Sonicated samples were centrifuged in the microfuge (14,000 x g) at 4°C for 5 minutes. The supernatant was drawn off and transferred to a new microfuge tube. Protein assays are then done using the Bio-Rad colorimetric assay. Equal amounts of protein are
assayed in each reaction, the difference in volume being made up with 0.25 M Tris·Cl. The reactions consist of cell extract up to 55 µl, 70 µl 1 M Tris·Cl pH 7.8, 20 µl 4 mM Acetyl Co A (freshly prepared), 1µl 14C-chloramphenicol (NEN 46.9 mCi/mmol., 0.1 mCi/ml). The extract was added last and reactions were carried out at 37° C for 45-60 min. Reactions were stopped with 1 ml of ethyl acetate. The reactions were vortexed for 30 sec and then centrifuged at room temperature for 5 minutes. The organic phase was drawn off and transferred to a new tube. The ethyl acetate was evaporated to dryness in a speedvac. The dried residue was resuspended in 30 µl of ethyl acetate and placed on ice until all the samples were resuspended. Samples (15 µl were spotted onto a thin layer (Silica gel) chromatography plate. The spots were allowed to dry and the plate was lowered into a 9:1 chloroform/methanol solvent and developed by ascending chromatography. The plates were removed, dried and exposed to Xray film (XAR5). Acetylated and non-acetylated spots were identified and the amount of radioactivity in each spot determined either by cutting the spots out and counting by liquid scintillation or by using the AMBIS detector. The percent conversion was calculated and the change in CAT specific activity determined. Since all reactions had equal amounts of protein, added to the incubation, the values could be compared directly. Values between duplicate
or triplicate samples varied by less than 10% of the values measured in each grouping.

**Human growth hormone assays.**

A radioimmunoassay was used to measure levels of human growth hormone (hGH) released into the culture medium. Assay conditions were as described by the commercial supplier of the kit (Nichols Institute). The assay can detect as little as 0.2 ng/ml of hGH and is linear in the range up to 50 ng/ml of hGH. All assays contained background controls (either no growth hormone gene or blanks) and various known concentrations of growth hormone to establish the linear range of the assay.

**S-1 analysis.**

Total cellular RNA was purified from transfected cells as described (Chirgwin et al., 1979). For S1 nuclease analysis (Berk and Sharp, 1977), 30 µg of total RNA was hybridized to a singly end-labeled DNA probe as described (Casey and Davidson, 1977) for 12 hr at 53° C. After hybridization, 300 µl of S1 digestion buffer (0.28 M NaCl, 4.5 mM ZnSO4, 50.0 mM sodium acetate [pH 4.6]) containing 200 units of S1 nuclease was added to each sample, mixed
rapidly then placed on ice. S1 digestions were performed at 37°C for 30 min, stopped by addition of 6 μl of 0.5 M EDTA, extracted with 1 volume of phenol-chloroform (1:1) and the aqueous phase was ethanol precipitated. The precipitate was washed with 70% ethanol and dried. Sequencing dye (Maxam and Gilbert, 1980) was then added to resuspend the pellet and the sample was loaded on a 12% sequencing gel. Following electrophoresis, the gel was dried and exposed to X-ray film at -80°C for 24 hr.

**Preparation of crude extracts.**

BHK cells growing in 100 mm tissue culture dishes were either mock infected or infected with HSV-1 at an input multiplicity of 10 plaque forming units per cell and cultures were harvested at designated times post infection. Cells were scraped into the tissue culture medium and collected on ice. The cells were centrifuged for 5 min at 800 x g (4°C) and the pellet washed twice with 50 volumes of cold Tris-saline (10 mM Tris-Cl [pH 8], 150 mM NaCl). Cells were resuspended in 1 pellet volume of cold sterile water containing 0.2 mM EDTA. An equal volume of cold lysis buffer (20 mM Tris-Cl [pH 8.2], 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM L-1-tosylamide-2-phenylmethyl chloromethyl ketone (TPCK), 2 M NaCl) was
added, vortexed briefly and the lysate placed on ice for 1 hr with periodic mixing. The lysate was then clarified by centrifugation at 100,000 x g (4° C) for 60 min. The supernatant was collected and dialyzed overnight at 4°C against several changes of a 0.3 M buffer A (20 mM Tris-Cl pH 8.5, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol and 0.5 mM PMSF supplemented with KCl to 0.3 M). The extract was then centrifuged (12,000 x g for 10 min.) to deposit any insoluble material and stored at -80° C in aliquots. The DNA binding activity was stable for 3 years under these conditions.

**Affinity Purification of ICP4.**

Chromatography was performed at 4° C. The DNA affinity column was prepared as described (Kadonaga, 1986) with slight modifications. Instead of coupling synthetic oligonucleotides, fragments containing the ICP4 target sequence were prepared from a plasmid, pBA-1, which has three copies of the 50 bp AvaI/BamHI sequence (-18/+27 of IE gene 3). The 150 bp fragment was gel purified and 450 µg were coupled to CNBr activated Sepharose CL2B (39). Infected cell lysates were prepared from 2 x 10^8 cells as described above and dialyzed against 0.3 M buffer A. The dialysate was centrifuged (15,000 xg, 5 min), and the supernatant
adjusted to a final salt concentration of 50 mM KCl using buffer A and loaded onto a DEAE-Sephacel column (3 ml volume in a 10 cc syringe) at a rate of 30 ml/h. The column was extensively washed with 50 mM buffer A and proteins were desorbed by increasing salt steps (in buffer A). Activity was located by mobility shift assays and the active fractions were pooled (most of the activity localized in the 0.5 M KCl step fractions) and diluted to a final concentration of 50 mM KCl with binding buffer B (5 mM Tris-Cl [pH 7.9], 0.05% Nonidet P-40 (NP40) (vol/vol), 0.5 mM EDTA, 12.5 mM sodium phosphate [pH 6.8]). Salmon sperm DNA was added to 40 μg/ml and the final concentration of the mixture was 5 mM Tris-Cl pH 7.9, 0.5 mM EDTA, 0.05% NP40, 12.5 mM NaPO₄, 50 mM KCl, 0.05 mM PMSF, 0.01 mM TPCK and 1% glycerol. The reaction proceeded on ice for 10 min and then was loaded at a rate of 4 ml/h onto an A4 sequence affinity column (1 ml column in a 3 cc syringe) pre-equilibrated with buffer C (5 mM Tris·Cl pH 7.9, 0.5 mM EDTA, 0.05% NP40, 12.5 mM NaPO₄, 50 mM KCl, 0.5 mM PMSF, 0.1 mM TPCK and 10% glycerol). The flowthrough was loaded over the column again. The column was developed with stepwise increments of salt in buffer C. Fractions were assayed by mobility shifts and peak fractions were pooled. Western blotting was performed to confirm the presence of ICP4. Silver staining revealed the presence of a single 170 kDa polypeptide, which
was the major staining species in this fraction (total recovery of 30 μg total protein, ICP4 ca. 50-80% homogenous).

**Western blotting**

Proteins were fractionated on a 10% SDS-PAGE gel and then electrically transferred to nitrocellulose (Schleicher and Schuell) in 25 mM Tris, 192 mM glycine at 0.6 A for 3.5 hr. The blot was blocked in 50 ml of 2% non-fat dry milk in TBS (100 mM Tris, 156 mM NaCl, pH 7.5) at room temperature for 2-3 hr. The blot was then transferred to 12 ml of the anti-ICP4 monoclonal antibody 58S (diluted 1:300) in BTBS (BSA [1 mg/ml] in TBS) for 1.5 hr at room temperature with gentle rocking. The blot is then washed with 20 ml of T-TBS (0.05% Tween 20 vol/vol in TBS) 5 changes for 5 min each. The blot is then incubated in 12 ml of the biotinylated antibody (diluted 1:500 in BTBS) for 1 hr at room temp with gentle rocking. The blot was then transferred to 25 ml of T-TBS and washed 3 x for 7 min each wash. The blot was then incubated in 12 ml of the ABC-AP solution (Vectastain kit, Vector laboratory San Diego, Ca.) for 30 min at room temperature with gentle shaking. The blot was then washed with 20 ml of T-TBS 3x for 5 min each wash. The blot was then rinsed briefly with 15 ml of TBS and then incubated in
10 ml of the substrate kit solution (Vector Alkaline Phosphatase Substrate Kit II, Cat. No. SK-5200) until bands develop in usually ca. 30 sec) the blot is then rinsed with 300 -500 ml of distilled water and dried.

Recombinant plasmids.

All new gene constructions were verified by DNA sequencing. The IE gene 3 promoter was present in the plasmid pJD1 described previously (Muller, 1987). The plasmid designated as p111, contains the gene for ICP0 (Vmw110) (Everett, 1987), and pgDCAT (Everett, 1983) contains the chloramphenicol acetyl transferase gene (CAT) under the control of the glycoprotein D promoter(-392 to +11), pIGA95 contains the IE2 promoter (-278/+1) driving expression of the chloramphenicol acetyl transferase gene (Gelman and Silverstein, 1987a), pIGA98 contains the minimal IE2 promoter (-84/+1) driving expression of the CAT gene (Gelman and Silverstein, 1987b). The construction pWT-GH, containing the recombinant growth hormone gene (pOGH) (Selden et al., 1986) was made by subcloning the region of IE3 from BamH1 (+27) to Sma1 (-333) from pJD1 (Muller, 1987) between the BamH1 and HincII sites of pOGH, thus putting the human growth hormone (hGH) gene under the control of the full IE3 promoter region from -333 to +27 relative to the 5'
cap site of IE3 (McGeough et al., 1986). The plasmid pD2-GH has a two base pair deletion from the ICP4 consensus binding site and was produced by cleaving pWT-GH with PvuI, and blunting the 3'overhangs with T4 polymerase. The blunt ends created were then resealed with T4 ligase (see text for sequence). The BamHI to HindIII fragment of the deletion mutant was then cloned between the BamHI and HindIII sites of pOGH.

The following constructions were also made:

1) The thymidine kinase promoter from Bgl II (+52) to Pvu II (-198) relative to the mRNA cap site (McKnight, 1980; Wagner et al., 1981) was isolated from pTK1 (Sanders et al., 1982) and subcloned between the Bam HI and Eco RV sites of Bluescript+ to make pBTK-P.

2) The Bam HI (+27) to Ava I (-18) fragment of the IE3 promoter element was isolated, blunted with Klenow and the resulting fragment subcloned into the filled-in Eco RI site of pBTK-P to make pBTK-9 and pBTK-8 containing two and three tandem copies, respectively, of the 50 bp IE3 fragment (pBTK-8 and pBTK-9 also differ in the orientation of the Bam HI/Ava I). [See Figure 6, panel A for the orientation of the insert.]

3) The blunted Bam HI/Ava I (+27/-18 of IE3) fragment was subcloned into the filled-in HindIII site of pBTK-P to make pBTK-6 and pBTK-4, which contain two or three tandem copies,
respectively, of the Bam HI/Ava I insert (pBTK-4 differs from pBTK-6 in orientation [see Figure 6, panel A]).

4) The recombinants pBA-2 and pBA-1 contain one or three copies, respectively, of Bam HI/Ava I (+27/-18 of IE3) cloned into the Eco RV site of Bluescript+. Both constructs are oriented such that the blunted Ava I end (-18) is toward the Eco RI site and the blunted Bam HI site (+27) is to the Hind III site of the Bluescript+ polylinker.

5) An indicator gene containing the recombinant growth hormone gene (pOGH) (Selden et al., 1986) and the IE gene 3 promoter was constructed by subcloning the region of IE3 from Bam H1 (+27) to Sma I (-333) between the Bam H1 and Hinc II sites of pOGH, thus putting the human growth hormone (hGH) gene under the control of the IE3 promoter region from -333 to +27 relative to the 5' cap site of IE3. The plasmid pD2-GH has a two base pair deletion from the ICP4 consensus binding site and was produced by cleaving pWT-GH with Pvu I, blunting the 3' overhangs by T4 polymerase fill-in. The blunt ends created were then resealed with T4 ligase (see text for sequence). The Bam HI to Hind III fragment of the deletion mutant was then cloned between the BamHI and HindIII sites of pOGH. From pWT-GH and pD2-GH, a second class of promoter constructions was made which contained the minimal promoter element of IE gene 3 from Bam HI (+27) to Eco RI (-108). These were constructed by restricting pWT-GH
or pD2-GH with Eco RI which releases the entire hGH gene and minimal promoter elements (from +27 to -108 of IE gene 3). This Eco RI fragment was then subcloned into the Eco RI site of Bluescript+ (Stratagene, San Diego, Calif.). Recombinants oriented in the same direction as the parental plasmids (pD2-GH and pWT-GH) were identified by DNA sequencing. They are designated as pEWT-GH (the wildtype version) and pED-GH (the D2 mutant version).

6) The recombinants pJD10 and pJD11 were constructed by cutting pWT-GH and pD2-GH with Bam HI and Hind III to release the +27 to -342 fragment which was cloned into Bluescript+ between the same sites. The wildtype sequence clone was designated as pJD10 and the D2 version was designated as pJD11. Both mutants were sequenced for verification.

7) The recombinants pJD8 and pJD9 were derived from pEWT-GH and pED-GH by digestion with Bam HI and Eco RI. The fragments were isolated and cloned between the same sites in Bluescript+. The minimal wildtype promoter version was isolated and designated as pJD8 and the D2 minimal promoter version isolated and designated as pJD9. The CAT gene was cloned into these constructs as described above for pJD11CAT.

8) The recombinant pJD40 was constructed by restricting pWT-GH with AvaI and isolating the (-353/-18 of IE3)
fragment and filling in the ends with Klenow. This blunted fragment was then cloned into the SmaI site of Bluescript+. A clone containing an additional Sma I, Pst I, Eco RI, Eco RV and Hind III site that flanked the insert in the polylinker was then isolated. This clone is oriented with the 5' promoter sequences (-353) at the BamHI side of the polylinker and the 3' end of the promoter (-18) toward the Xho I end of the polylinker of Bluescript+.

9) The recombinant pJD45 was made by restriction of pJD40 with Bam HI and partial digestion with Eco RI. The 390 bp fragment was band isolated from a polyacrylamide gel by electroelution and ligated to pBA2 which had been restricted with Bam HI and Eco RI. The resulting plasmid, pJD45 has the TATA sequence of IE3 spaced 22 bp further apart from the A4 site than in the wild-type IE3 promoter.

10) The recombinants pJD42 and pJD43 were made from pJD40 by cloning of the filled-in Bam HI to Ava I fragment of IE3 (+27/-18) into the Nco I site (-211) of pJD40. Recombinants were screened by mini-lysate analyses. The recombinant pJD42 has a single insert that is oriented with the +27 end of the insert toward the Eco RI site (-108), pJD43 also has a single insert and it is orientation is in the opposite direction. The CAT gene constructions of these three recombinants were made by digestion of the recombinant with Hind III to release the promoter element. The promoter was
then cloned into the Hind III site of a CAT gene construct that has the CAT gene cloned between the Hind III and the Kpn I sites of Bluescript+®. Mini-lysates were made from the recombinants and one with the proper orientation were selected. The designations are the same as the promoter plasmid with the CAT added to it, for example pJD40CAT, pJD42CAT and pJD43CAT.

11) The recombinants pJD32 and pJD33 were constructed the same way except pJD32 is a derivative of pJD10 and pJD33 is a derivative of pJD11. The Eco RI to Eco RI (-108/-355) fragment of pJD40 was isolated and then cloned into the Eco RI site of pBA2. The recombinant having the -108 site closest to the pBA2 insert was isolated. This recombinant pJD60 (also called old C1BA2) contains the A4 site adjacent to the -108 sequence. The recombinant pJD60 was restricted with Hind III to release the Hind III fragment which was cloned into the minimal IE3 promoter construct (-108/+27) version of pJD10 designated as pJD8 at the Hind III site. The clone having the BA2 portion of the fragment nearest the Eco RV site of the vector was isolated. This construct contains the (+27 to -108) of IE3 the (+27 to -18) and the (-108 to -333) of IE3 in that order. This construct is pJD32. pJD33 was made by digesting pJD32 with Eco RV and Sal I. This fragment was then cloned between the same site in the D2 minimal promoter construct pJD9 to give pJD33.
The CAT gene was cloned into the Bam HI site to give pJD32CAT and pJD33CAT after the correct orientation clones were selected.

12) The recombinants pJD34 and pJD35 are derived from pJD10 and pJD11 respectively. The recombinant pJD10 was digested with Eco RI and Nco I, the ends filled in with Klenow and the ends resealed with ligase resulting in a 99 bp deletion between -112 and -211 of IE3 to make pJD34. The same procedure was used on pJD11 to make pJD35. The CAT gene was cloned into each of these constructs as a Bam HI piece. The proper orientation clones were isolated and grown for harvesting DNA. These recombinants are pJD34CAT and pJD35CAT.

13) The minimal promoter IE1 construct pJD100 was constructed by isolation of the SMA I to Ava I fragment (-126/+51 relative to the IE1 transcriptional start site) from p111 and cloned into the Eco RV site of Bluescript+. The clone with the +51 end nearest to Bam HI was isolated and designated pJD100. Single stranded DNA was prepared from an M13 version of this clone in order to mutate the A4 site at -68. The oligo 5'GGGGGACGTCCACTGCC3' was used in an in vitro mutagenesis reaction. Recombinant phage that resulted were screened by dideoxy sequencing. The mutant delta A2 was isolated and the RF DNA isolated and cleaved with Eco RI and Hind III. This fragment was cloned between
those sites in Bluescript\textsuperscript{+} resulting in pJD101. The mutation arising from the mutagenesis gave rise to an Aat II site in the ICP4 core consensus sequence. The mutant pJD101 was digested with Aat II, the 3' overhangs digested away with T4 polymerase and the ends resealed with ligase to form pJD102. The sequence of the A4 region is listed in Figure 55. The CAT versions of these were made by cloning the CAT gene as a Bam HI fragment into the Bam HI site of each construct to form pJD100CAT, pJD101CAT and pJD102CAT after the proper orientation clones were isolated.

14) The recombinants RAB6 and RAB8 were made by the isolating the SSTI to Nco I fragment of pl11, making it blunt ended by mungbean nuclease treatment and cloning the fragment into the Eco RV site of a CAT gene containing plasmid (pICCAT) (Gelman and Silverstein, 1987a) in both orientations. The IEx promoter drives CAT expression in pRAB6 and the IE1 promoter drives CAT expression in pRAB8.

15) The recombinant pJgD was constructed by restricting pgDCAT with Hind III and Xma III to release a 132 bp fragment (from +11 to -121 relative to the gD 5' capsite [Everett, 1984]). The fragment was filled-in with Klenow fragment and blunt end ligated into the Eco RV site of Bluescript\textsuperscript{+}.

15. The recombinants p-108bend and pBA2bend were constructed in the following manner. The Bam HI to Eco RI
fragment of IE3 (+27/-108) was filled in by the Klenow fragment and cloned into the filled XbaI site of pBend2 (Zweib et al., 1989). A clone was isolated having the +27 site of IE3 toward the Eco RI site of pBend2. This clone was designated as p-108bend. pBA2bend was constructed by restriction of pBA2 with Eco RI and Hind III releasing the -18/+27 portion of IE3. The ends were filled and the fragment cloned into the filled Xba I site of pBend2. A clone was isolated and the orientation found to be opposite that of p-108bend.

**In vitro mutagenesis**

The mutant oligo spanning the A4 site in IE1 (described above) was used to extend the complimentary strand synthesis on the single stranded M13 recombinant containing the wildtype minimal IE1 sequence between Eco RI and Hind III. The procedure used was that of Su and El-Gewely (Su and El-Gewley, 1988) and was followed implicitly without variation. Mutants were screened by dideoxy sequencing.

**Missing base contact analysis**

G+A and C+T reactions were performed on 5' end-labeled DNA fragments identically as described in Brunelle and
Schlief (1987). The only modifications were the G+A modification reaction was carried out at 37°C for 25 minutes, the free DNA and bound DNA that was isolated after ethanol precipitation was washed 2x with 70% ethanol and dried. The dried pellets were then washed once with 25 μl of distilled water and then dried before piperidine cleavage at 90°C for 25 minutes. In order for the C+T reactions to work, the probes must be resuspended in distilled water and not TE buffer for the hydrazine reaction to work. Typically, 1-2 x 10^7 cpm of end-labelled probe was modified per reaction with an expected recovery of 75%. Experimental details are described in the text and in the Figure legend for that particular experiment.

**Detection of DNA-protein complexes.**

Mobility shift assays were performed as described previously (Singh et al., 1986; Muller, 1987). Probes were 5' end labelled with gamma-[^32P]ATP and T4 polynucleotide kinase (Maxam and Gilbert, 1980). The reaction mixtures (total volume, 20 μl) were assembled in binding buffer B which contained 30 to 100 mM KCl (contributed from the extract or purified protein fractions), 4 μg of poly(dA-dT)*poly(dA-dT), and 30,000 cpm (2 to 3 ng) of end-labeled DNA. In binding reactions where affinity purified ICP4 was
used as the protein source, PVA was added to a final concentration of 3% (vol/vol) and bulk DNA was reduced to 0 or 50 ng. Reactions were initiated by addition of protein and incubations were performed either on ice for 45 min or at 22° C for 30 min (the same results were obtained similar with both incubation regimens). At the end of the incubation period, 0.1 vol of sample dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 50% glycerol, 25 mM EDTA) was added and the samples were loaded onto a native 4% low ionic strength polyacrylamide gel (acrylamide-bis weight ratio 80:1) containing 6.7 mM Tris-Cl [pH 7.9], 3.3 mM sodium acetate. The gel was pre-run for 1 hour at 20 mA and after loading the samples, electrophoresis was carried out at 20 mA (with constant buffer recirculation) until the bromophenol blue dye front had reached the bottom of the gel. The gel was dried and exposed to Kodak XAR film with an intensifying screen, usually for 4 to 6 hours at -80° C. When quantitation was essential, soft laser densitometric scans of multiple exposures of the autoradiogram were used. The peaks under the curves were cut and weighed to determine the values. Alternatively and much simpler is the use of an AMBIS radioimaging detector (AMBIS, San Diego Ca.). The AMBIS detects radioactivity in the gel and quantifies it. The information is digitized and stored so that numerous
Footprinting analyses.

DMS interference assays were performed as described previously (Muller, 1987). Copper footprinting on DNA-protein complexes in situ was accomplished using 1,10-phenanthroline coordinated copper ion as a cleavage reagent (Kuwabara and Sigman, 1987). Low ionic strength polyacrylamide gels, described above, were used to separate bound and free DNA fragments. Following electrophoresis, the entire gel was rinsed with 50 mM Tris-Cl [pH 7.9], and submerged in 200 ml of the same buffer, followed by the addition of 20 ml of solution A1 (0.45 mM cupric sulfate, 2.0 mM 1,10-phenanthroline) and 20 ml of solution B1 (58 mM 3-mercaptopropionic acid). The gel was incubated for 12 min at room temperature with gentle agitation followed by addition of 20 ml of solution C1 (28 mM 2,9-dimethyl-1,10-phenanthroline) for 2 min at room temperature. The gel was then rinsed several times with distilled water and exposed to X-ray film overnight at 4°C. Bands of interest were excised from the gel and the DNA electroeluted. To prepare solution A1, 40 mM 1,10-phenanthroline (in 95% ethanol) was mixed with an equal volume of 9 mM CuSO$_4$ (in water) and the
mixture diluted 10 fold with water to yield a final concentration of 2 mM 1,10-phenanthroline, 0.45 mM CuSO₄. To prepare solution B1, neat 3-mercaptopropionic acid was diluted 200 fold with water to give 58 mM 3-mercaptopropionic acid. Solution C1 was prepared in 95% ethanol.
CHAPTER II

CHARACTERIZATION AND PURIFICATION OF FACTORS INVOLVED IN
ICP4-DNA COMPLEXES FORMED ON THE IE GENE 3 PROMOTER

INTRODUCTION

ICP4 is a component of a DNA-protein complex that forms at the 5' transcriptional start site of IE3 (Muller, 1987; Roberts et al., 1988; Faber and Wilcox, 1988; DiDonato and Muller, 1989). To study the interaction of the ICP4 DNA protein complex and other complexes that form on the DNA probes, gel retardation assays or mobility shift assays (MSA) were used (Fried and Crothers, 1981, Singh et al., 1986). MSA is sufficiently sensitive to detect sequence specific interactions between protein and nucleic acid at very low protein concentrations. The rationale behind the assay is as follows. Low concentrations of radioactively labeled probe (1-2 ng) are mixed with bulk non-radioactive DNA (usually poly [dI·dC] or poly [dA·dT]) at a ratio of approximately 1000 to 2000 parts bulk DNA to probe DNA. This titrates out proteins which bind to the DNA in a non-specific manner by electrostatic charge effects, thereby, allowing sequence specific DNA binding proteins the
opportunity to come in contact with the probe DNA. Proteins which recognize the probe in a sequence specific manner will bind to this DNA with an affinity several orders of magnitude greater than that for non-sequence specific DNA binding (due largely to electrostatic effects between protein and DNA). The mixture is then incubated with the protein source and after reaching equilibrium the reaction is fractionated onto a low ionic strength polyacrylamide gel.

The low ionic conditions and the gel matrix itself act to stabilize the complex against dissociation (Fried and Crothers, 1981). DNA bound to protein will migrate as discrete bands differently than the free DNA. The migration of the DNA-protein complex through the gel will depend upon the size, shape and charge of the protein and to some extent the length of the probe. This type of gel analysis allows visualization of stoichiometric differences in the number of protein species bound to the probe as a difference in the migration of the complex in the gel. Another feature of this system is that the relative binding affinities of a protein for two different probes can also be measured. Additionally, the dissociation rate of the DNA-protein complex can also be measured as long as the time of dissociation is longer than the time it takes to load the sample and have it migrate into the gel.
To confirm that a DNA-protein complex is sequence-specific, homologous and heterologous DNA fragments may be employed as competitor DNA's. These competitor DNA's are added in addition to the bulk DNA and probe usually in a 50 to 100-fold molar excess to the probe DNA concentration. The addition of competitor oligonucleotide will reveal if specific binding of protein to probe occurs. Homologous competitor should effectively titrate out the sequence specific DNA binding protein in question causing the probe-protein complex concentration to diminish. Heterologous oligonucleotide is used to demonstrate if a probe-protein complex is sequence-specific. If the interaction of probe and protein is specific, the heterologous oligonucleotide will have little or no effect on probe-protein complex formation relative to the homologous oligonucleotide; but if the complex is not sequence specific, then the heterologous oligonucleotide will effectively compete the probe for complex formation.

To identify a specific protein, as a component of the probe-protein complex, antibody directed against a particular protein can be used to determine if the protein is present in the complex. Binding of the antibody with the DNA-protein complex results in a decrease in electrophoretic mobility of the complex (Kristie and Roizman, 1986a; Muller, 1987). DNA affinity chromatography was used to identify the proteins along with ICP4 that bind
to the IE3 promoter. In the following sections, the biochemical nature of the ICP4 protein-DNA complex has been characterized. By examining conditions which effect ICP4-DNA complex formation a better understanding of how and when the complex forms, its relationship to other proteins and its dependence on surrounding DNA sequence was determined.
RESULTS

ICP4 PROTEIN CONTENT AND DNA-ICP4 COMPLEXES INCREASE AS HSV-1 INFECTION PROCEEDS.

Based upon methylation interference assays (Muller, 1987), the target sequence in IE3 that ICP4 binds to is as follows (relative to +1, the transcriptional start site) (McGeough et al., 1986):

-12 -10 -8 -6 -4 -2
5' C G C C C C G A T C G T C

Henceforth, the DNA/ICP4 complex will be referred to as the A4 complex and the ICP4 consensus binding site (containing ATCGTC) as the A4 site. The A4 complex was identified by mobility shift assays using defined probes; however, additional criteria (oligonucleotide competition and antibody induced shifts) were applied to confirm the formation of authentic A4 complexes. The reaction conditions were designed to detect ICP4 binding to the ATCGTC sequence above (Muller, 1987; DiDonato and Muller, 1989; DiDonato and Muller, this study); thus, binding conditions are specific for ICP4 binding to ATCGTC sites in IE3, IE1 and gD.
To determine the relative abundance of ICP4 during the course of infection and its reactivity to the A4 binding site, the following was done. Tissue culture cells were either mock infected or infected with HSV-1 strain KOS at a multiplicity of infection (MOI) of 20. Nuclear extracts were prepared at various times post infection (from 1 to 12 hrs PI). The extracts were analyzed for total ICP4 content by Western blotting. The ability of the extracts to form A4 complexes on the IE3 capsid was also assayed by MSA. Increasing amounts of ICP4 can be detected in infected extracts prepared (at 1 hr to 12 hrs PI) by Western blot (Figure 2, panel B). In this Western blot an equal volume of extract was fractionated on a 10% SDS-PAGE gel, (the protein content per lane may vary by a factor of no more than 2.5 fold), electrically transferred to nitrocellulose and probed with monoclonal anti-ICP4 antibody (Showalter et al., 1981). Bands were visualized by treatment of the blot with peroxidase (Vectastain). At 1 hr PI (Figure 2, panel B, lane 7) very little ICP4 could be detected by Western blot. At 2 hrs PI and later, appreciable amounts of ICP4 were detected. IE3 expression (transcription and translation) is known to be regulated negatively at this point in the infection (Honess and Roizman, 1974; Honess and Roizman, 1975). ICP4 appears to increase in concentration to 12 hrs PI (Figure 2, panel B, lanes 3 and 4), but does not increase at times later than 12 hr PI (data not shown).
Figure 2. ICP4 content and A4 complex forming ability of HSV-1 infected extracts prepared at increasing times post infection (PI). A. Either mock infected cell extracts (prepared hr. PI) or HSV-1 infected cell extracts (prepared at the times indicated in Figure 2) were used in mobility shift assays as described in Materials and Methods. One, two or four µl of each extract was incubated with the IE3 promoter (-108/+27) containing the ICP4 binding site. ICP4/DNA complexes are bracketed and designated "A" complex and the free DNA is bracketed as "free probe". B. A Western blot of a 10% SDS-PAGE fractionation of 5 µl of each extract prepared at the time indicated above each lane. The 58S monoclonal antibody directed against ICP4 was used as the primary antibody. The Western blot was developed with Vectastain (Vector Laboratories, San Diego Ca.). C. Western blot of a 10% SDS-PAGE fractionation of equal amounts (20µg) of each extract. The time of preparation of each extract is listed above the lane and the location of ICP4 is indicated by an arrow to the side. The blot was developed as in B.
MOBILITY SHIFTS WITH EXTRACTS PREPARED AT VARIOUS TIMES POST INFECTION

Figure 2
Figure 3. ICP4/DNA complex formation and ICP4 protein content examined at various times post infection. Western blot analysis of equal amounts (5μg) of extracts prepared at the times listed above each lane and fractionated on a 10% SDS-PAGE gel. The blot was incubated with the ant-ICP4 monoclonal antibody 58S and developed by peroxidase color development using Vectastain (Vector labs, San Diego Ca.). Lane 1 contains an ICP4 reference (1μl of 1M infected extract prepared 12 hr PI) and lane 8 contains the prestained molecular weight markers (Pharmacia); sizes are indicated off to the side. B. Mobility shift assays with mock and infected extracts. Equal amounts (5μg) of extracts prepared at the various times indicated above each lane were used in mobility shift assays. Reactions were completed, incubated, on ice for 30 min. and then incubated at room temperature (21°C) for 35 minutes. 1X mobility shift loading dye was added and the samples were then electrophoresed at constant current (20 mA) with buffer recirculation until the bromophenol blue was at the bottom of the gel. The gel was then dried and subjected to autoradiography. Lane 2 is free probe (IE3 promoter [-108/+27]), lane 3 is an ICP4/DNA complex containing reference lane. Lane 1 is identical to lane 3 except 58S monoclonal antibody had been added to the binding reaction to shift ICP4 containing protein/DNA complexes. Lane 10 is identical to lane 9 except 30ng of non-radioactive IE3 probe (-108/+27) was added to the binding reaction to act as a specific competitor (100 fold molar excess).
**B**

Mobility shift assays with mock and infected extracts

- A4 Oligo (30 ng) +
- 58S monoclonal Ab +

Hours PI extract was prepared

| Infected extract | 1 | 2 | 4 | 10 | 12 | 12 |
| Mock extract     | 10|

**Figure 3**

**A**

Western Blot analysis of HSV and Mock infected extracts at different times post infection (PI)

<table>
<thead>
<tr>
<th>Lane</th>
<th>12hr</th>
<th>10hr</th>
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- 200K
- 96K
- 66K

Free Probe

Figure 3
DNA binding activities of extracts were evaluated by A4 complex formation at the IE3 transcriptional start site. Incubating increasing amounts of nuclear extract with a 5' endlabeled probe yields A4 complexes only with HSV-1 infected extracts, furthermore, the intensity of the A4 complex increased from early to late times PI (Figure 2, panel C). ICP4 is a component of the A4 complexes (Figure 3). The various nuclear extracts (Figure 2) were used as sources of protein in mobility shift assays. Equal amounts of protein (5 ug,) from each time point, were incubated with the IE3 (-108/+27) probe in mobility shift assays (Figure 3, panel B). As a positive control, to demonstrate the presence of ICP4 in the A4 complex, anti-ICP4 monoclonal antibody was added to the binding reaction (Figure 3B, lane 1). Addition of anti-ICP4 antibody resulted in the retardation in mobility of the A4 complex. Multiple complexes were observed and all three of the DNA-protein complexes were supershifted by addition of the anti-ICP4 antibody (lane 1). This observation demonstrates that ICP4 is present in all of the A4 complexes. To distinguish among these different A4 complexes, the following convention was adopted. The fastest migrating A4 complex was designated as the 1° A4 complex, the middle complex as the 2° A4 complex and the slowest migrating complex as the 3° A4 complex. Mock infected cell extract failed to form a specific DNA-protein complex (Figure 3, panel B, lane 4). Extracts from
increasing times post infection yielded a higher intensity of A4 complex formation (Figure 3, panel B, lanes 5 through 9). A4 complex formation peaked at 12 hrs PI (Figure 3, lane 9).

To demonstrate the sequence specificity of the A4 complex formation, an oligonucleotide containing the A4 site was added to the binding reactions in 100 molar excess (30 ng) relative to the labeled probe (1 ng). A4 complexes were diminished (compare lane 10 with lane 9 in Figure 3, panel B). The amount of ICP4 present in each extract prepared at increasing times PI was examined by fractionation of equivalent amounts of protein (5 μg) used in the mobility shift assay (Figure 3, panel B) on a 10% SDS-polyacrylamide gel. Protein in the gel was transferred to nitrocellulose and the Western blot probed with the anti-ICP4 monoclonal antibody. Western blot analysis revealed an increasing in the amount of ICP4 at later times PI (Figure 3, panel A). This result correlates with the increase in A4 complex formation seen in the mobility shift assays of the same extracts at later times after infection (Figure 3, panel B). This lends support to the idea that the levels of ICP4 are proportional to the amount of A4 complex formation observed.
SENSITIVITY OF ICP4 DETECTION USING MOBILITY SHIFT ASSAYS AND WESTERN BLOTTING.

Mobility shift assays detect specific DNA-protein interactions at low protein concentrations (< 100 ng/ml) (Singh et al., 1986), thus it is highly sensitive method of detecting specific DNA-protein interactions. Probing of a Western blot with a specific antibody and visualization of the antibody-antigen reaction is also a sensitive way to detect small amounts (< 1 ng) of a specific protein using 125I-protein A and autoradiography for detection (Talbot et al., 1984). These two different methods were compared in an attempt to correlate the limits of detection using both procedures as they apply to ICP4.

An infected nuclear extract (prepared at 12 hr PI) was serially diluted with mock infected cell nuclear extract so that all samples contained equal amounts of total protein. Protein (5 μg) from each dilution point was fractionated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose for Western blot analysis using anti-ICP4 monoclonal antibody as the probe (Figure 4, panel B). Identical amounts of each dilution (5 μg) were also incubated with endlabeled IE3 probe (-108/+27) and fractionated on a mobility shift gel. The mobility shift assay could detect A4 complexes to a dilution point of at least 1:100 (50 ng protein) (Figure 4, panel A, lane 10).
Western blot analysis of the same dilution set fails to detect ICP4 at dilutions greater than 1:10 (500 ng protein) using peroxidase to develop the blot (Figure 4, panel B, lane 5). The mobility shift assay was at least 10-fold more sensitive than Western blot analysis using peroxidase to develop the ICP4-antibody complex. A4 complex formation was dependent upon infected cell proteins and not mock infected cell proteins (note the decrease in A4 complex formation at increasing dilutions of infected cell protein [Figure 4, panel A, lanes 5 through 10]).

SEQUENCE ELEMENTS AT THE IE3 TRANSCRIPTIONAL START SITE THAT ARE RESPONSIBLE FOR A4 COMPLEX FORMATION.

Previously, a consensus sequence for ICP4 binding had been proposed (Faber and Wilcox, 1986). This sequence is present at the capsite of IE3. ICP4 has been shown to be present in this specific DNA-protein complex (Muller, 1987; Faber and Wilcox, 1988; Roberts et al., 1988; DiDonato and Muller, 1989) known as the A4 complex. To investigate the presence of the consensus site in IE3 relative to its ability to form an A4 complex, a 2 bp deletion mutant in the ICP4 consensus sequence was constructed. This mutant was constructed because there is an available restriction site located within the consensus sequence. The mutant was created in the following manner, Pvu I was used to cleave
Figure 4. Comparison of Mobility Shift Assay and Western blotting in the detection of ICP4. Mobility shift assays were prepared as described in Materials and Methods. Infected cell extract prepared 12 hr. PI (5μg) was used as a source of protein and serially diluted as listed above each lane. Total protein concentration was kept at 5μg by addition of mock infected cell extract. A. Each dilution point (5μg total protein) was assayed by mobility shift assay and examined for A4 complex formation as the IE3 promoter (-108/+27). B. Additionally, 5μg of each dilution point was also fractionated on a 10% SDS-PAGE gel and electrically transferred to nitrocellulose. ICP4 content was assayed by Western blotting using 58S monoclonal antibody as probe. The amount of infected cell protein is also given for each dilution point.
Figure 4
the IE3 promoter leaving 3' end overhangs which contained the AT bp of the core ICP4 consensus sequence ATCGTC. The AT was then deleted by exonuclease digestion using T4 DNA polymerase leaving blunt ends which were resealed using T4 DNA ligase. This AT deletion mutant was designated as the D2-GH mutant. Both the wild type (WT-GH) and the D2-GH mutant promoters were used as probes (the Bam HI to Eco RI fragments, (-108/+27 of IE3) and incubated with infected cell extract and fractionated on mobility shift gels (Figure 5). The WT probe formed multiple A4 complexes that were retarded in mobility by anti-ICP4 monoclonal antibody (Figure 5, lanes 3 through 9) and were competed away with increasing amounts of non-radioactive A4 oligonucleotide (Figure 5, lanes 4 through 6) but not by heterologous oligonucleotide, which contained a strong topoisomerase I cleavage site (Figure 5, lanes 7 through 9). The D2 mutant probe incubated with anti-ICP4 antibody and infected cell extract formed an A4 complex inefficiently (Figure 5, lane 10). This complex was competed away by the A4 oligonucleotide (lanes 11 through 13). The 53 bp A4 oligonucleotide was capable of forming an A4 complex (Figure 5, lane 14) and was competed by cold A4 oligo (lanes 15 through 17). Key features of this experiment were the inability of the D2 mutant probe to form appreciable levels of A4 complexes and the ability of ICP4 to enter into multiple A4 complexes that could be retarded in mobility by
Figure 5. Binding specificity and localization of the ICP4 containing A4 complexes on the IE3 promoter. Mobility shift assays were prepared as follows. Infected cell extract (7 μg) prepared 12 hr post infection was incubated in binding reaction assembled without DNA. Either the anti-Vmw65 monoclonal antibody LP1 (ascites fluid 1 μg), anti-ICP4 monoclonal antibody (1 μg) or no antibody was added to the binding reactions and incubated on ice for 30 minutes. Radioactive probe was added to each reaction along with or without increasing amounts (5, 10 or 50 ng) of A4 oligo. or topo I oligo. The binding reaction was then placed at room temperature for 30 min. before addition of 1X mobility shift loading dye and fractionated on a 4% mobility shift gel. Lanes 1 through 9 contain wildtype IE3 promoter (-108/+27) as probe. Lanes 10 through 13 contain the D2IE3 promoter (-108/+27) as probe. Lanes 14 through 17 contain labelled A4 oligos as probe. A4 oligo and topo I oligo was added to reactions seen in lanes 4 through 9 and lanes 11, 12, 13, 15, 16 and 17. The type of oligo and amount is listed above each lane. Anti-Vmw65 monoclonal antibody was incubated in the reaction fractionated in lane 1 and anti-ICP4 monoclonal antibody was added to reactions seen in lanes 3 through 17.
Figure 5
anti-ICP4 monoclonal antibody. Note also, the A4 oligo competed the secondary (2° A4) and tertiary (3° A4) complexes before competing the primary (1° A4) complex (Figure 5, compare lanes 3 through 5 to lane 6). When one observes variations in electrophoretic mobility (with the same probe) it is usually due to differences in the stoichiometries of protein and DNA (see Fried and Crothers, 1981). One possibility is that multiple protein molecules bind at contiguous sites located in the DNA target sequence and these sites become filled only when protein to DNA ratios are sufficiently high to counteract anticooperativity effects (Berg et al., 1981; Record et al., 1981; Revzin and von Hippel, 1977). Alternatively, protein-protein interactions might tether ICP4 to another protein (or to itself) causing altered mobility, as happens when antibodies bind DNA/protein complexes. These possibilities were investigated further (see below and Chapter III).

MODULAR NATURE OF THE IE3 PROMOTER FRAGMENT TO CONFER THE ABILITY TO FORM AN A4 COMPLEX.

A probe derived from the BamH1 (+27) to HindIII (-338) region of the wild type IE3 promoter drives formation of the A4 complex when incubated with infected cell proteins as shown previously (Muller, 1987; this study) (Fig. 6, panel A, lane 1). The A4 complex was competed away by a
homologous non-radioactive probe (Muller, 1987; Deluca and Schaffer, 1988; DiDonato and Muller, 1989) or a synthetic oligonucleotide spanning from -15 to +16 of IE3 (henceforth, the "A4 oligonucleotide") (Fig. 6, panel A, lanes 2 and 3). A probe derived from the deletion mutant pD2-GH, in which 2 bases from the A4 site were deleted (-6 A and -5 T, see D2-GH above), failed to efficiently drive formation of the A4 complex (lanes 4, 5 and 6).

Heterologous promoters were then tested for their ability to form an A4 complex in vitro. Thymidine kinase (tk) promoter sequences derived from pTK-1 (Sanders et al., 1981) or pBTK-P (TK promoter subcloned into Bluescript+, see Materials and Methods) were tested for their ability to drive the A4 complex. Fragments were prepared from pTK-1 and pBTK-P that correspond to the TK promoter from +52/-198. (The probe from pBTK-P contained an additional 40 bp of vector sequence.) These TK promoter fragments were incubated with infected cell extracts in the presence and absence of the A4 oligonucleotide as competitor. The TK probes did not form significant levels of the A4 complex (Fig. 6A, lanes 7 through 10); however, this is not unexpected since the binding conditions were optimized for the ICP4 binding to ATCGTC and these probes lack the ICP4 consensus sequence. (A rather weak mobility shift was detected with the pTK1 probe (lane 7) which was not investigated further.) I next tested whether the addition
the IE3 region from +27/-18 would confer the ability of these probes to form the A4 complex. This sequence was subcloned into the Eco RI site at -79 (pBTK-8, pBTK-9) or the HindIII site at -197 (pBTK-4, pBTK-6) of pBTK-P and probes were prepared from the recombinant plasmids in the same manner as the pBTK-P probe. The recombinants differed in the orientation and number of copies of the IE3 +27/-18 region (see constructions below Fig. 6, panel A, and "Materials and Methods"). All recombinants containing the ICP4 target sequence were competent in their ability to drive the A4 complex (Fig. 6, panel A, lanes 11, 13, 15, 17) as attested by the observation that the complexes were competitively dissociated by a 100 fold excess of homologous oligonucleotides (lanes 12, 14, 16, 18). Furthermore, the complexes were shifted by the anti-ICP4 antibody (data not shown). The A4 oligonucleotide containing IE3 sequences from -15/+16 was also competent in forming the A4 complex (lanes 19 and 20) showing that a subset of the +27/-18 sequence is sufficient to form complexes. Cloning the A4 oligonucleotide into any sequence confers the ability of that sequence to bind ICP4 (J. DiDonato and M. Muller, unpublished data); therefore, sequences outside the -15/+16 domain are not essential in A4 complex formation as assayed by mobility shifts in vitro.

Multiple bands again were observed with various promoter elements containing the ICP4 consensus binding site
Figure 6. Modular nature of the ICP4 consensus binding sequence. Panel A: Restriction maps of the wild type IE3, mutant IE3 and TK promoters show the various TK promoter elements and insertion sites of the IE3 sequence (+27/-18) and orientation. There are two tandem repeats of the insert in pBTK-6 and pBTK-9, while pBTK-4 and pBTK-8 contain three tandem repeats of the insert. Binding reactions contained 8 μg of nuclear extract prepared from infected BHK cells harvested 14 h post infection as described in Materials and Methods. The probes used were as follows: Lanes 1-3, BamHI/HindIII fragment from wild type IE3 promoter (+27/-338); lanes 4-6, BamHI/HindIII fragment (+27/-338) from pD2-GH (the 2 bp deletion mutant of the IE3 promoter); lanes 7 and 8, a BglII/PvuII probe from pTK-1 (+52/-198); lanes 9 and 10 a SacI/SalI probe from pBTK-P (+52/-198 of TK sequence plus 40 bp of vector sequence); lanes 11-18 all contained the same SacI/SalI fragment corresponding to pBTK-P, however, it was isolated from different constructions; lanes 11 and 12 from pBTK-4; lanes 13 and 14 from pBTK-6; lanes 15 and 16 from pBTK-8; lanes 17 and 18 from pBTK-9. In lanes 19 and 20, the A4 oligonucleotide from -15 to +16 of IE3 was used as probe. The amount of unlabeled A4 oligonucleotide competitor (in ng) included in each reaction is indicated above each lane. Panel B: Binding reactions contained 5 μg of total protein which was either contributed by the infected nuclear extracts or by addition of mock infected nuclear extract such that the total protein per reaction remained constant. The BamHI/EcoRI probe from IE3 (+27/-108) was used. In order to keep total protein concentrations constant, infected cell nuclear extracts were diluted 0, 2, 5, 10, 20, 50, and 100 fold with mock infected cell nuclear extract, just prior to mobility shift assays. The positions of the 1°, 2° and 3° A4 complexes are marked on the left.
Free probe

"A" complex

Figure 6
and these additional bands (above the 1° A4 complex) were competed away with unlabeled A4 oligonucleotide. Furthermore, the major (1°) and minor (2°, 3°) bands all reacted with the anti-ICP4 antibody (data not shown) in identical fashion as the multiple A4 complexes in Figure 3, panel B did. The 2° and 3° complexes were not always detected (compare lane 1, Fig. 6, panel A), because their formation was sensitive to protein/probe ratios reflecting intrinsically different equilibrium binding constants (Fig. 6, panel B and below).

To demonstrate the effect of variations in protein to DNA ratios on formation of the three complexes, an infected cell extract was serially diluted but the total protein concentration was kept constant by addition of mock infected cell extract. The diluted samples were then incubated with a probe derived from the IE3 promoter (+27/-108). As the concentration of infected extract was increased, there was a commensurate increase in 1°, 2°, and 3° complexes; however, the equilibrium binding constants are clearly different between the three (Fig. 6, panel B, lanes 1 through 7). The relative equilibrium binding constant (defined below as $K_R$) can be determined from these data and is described (Huei-Nin et al., 1986) as:

$$K_R = K_{1°}/K_{2°} = ([C_{1°}]/[D_{1°}])/([C_{2°}]/[D_{2°}])$$

where $K_{1°}$ and $K_{2°}$ are equilibrium binding constants for the 1° and 2° complex, respectively, [C] is the concentration of
the 1° or 2° A4 complex, and [D] the concentration of free DNA. Since [D] is constant in each reaction, equation (1) is reduced to a simple ratio of 1° to 2° A4 complexes or 1° to 3° complexes. From the equilibrium binding curves and integrating the area under each complex (Fig. 6, panel B), the $K_r$ was calculated to be 0.53 for 2° complexes and 0.18 for 3° complexes (relative to 1.0 for the 1° complex).

Because the 3° was so weak compared to the 1° and 2° complexes, only the 1° and 2° complexes were characterized further. The probe used in Fig. 6, panel B has only one copy of the ICP4 target sequence, thus, the possibility that 2° complexes are being formed at contiguous A4 sites present in the TK clones which have multiple copies of the ICP4 binding site (see Fig. 6, panel A) can be ruled out. Furthermore, the 2° complexes were sensitive to the ratio of infected cell proteins to DNA rather than total protein, thus, the effect is quite specific.

**INCUBATION CONDITIONS WHICH AFFECT DNA-ICP4 INTERACTIONS.**

The D2 mutant failed to efficiently drive A4 complex formation (Figure 6, panel A, lanes 4 through 6) however, when anti-ICP4 monoclonal antibody (58-S) was present in the reaction, the intensity of the A4 complex increased (see Figure 5, lane 10). To investigate which components of the anti-ICP4 antibody (58-S) preparation were responsible for
the increase in A4 complex formation, the following experiment was performed. Infected cell extract in increasing amounts was incubated with a probe (+27/-110 of IE3) under conditions where total protein in the reactions was kept constant by addition of bovine serum albumen (BSA). Addition of two different purified anti-ICP4 monoclonal antibody preparations and the sodium phosphate buffer (buffer used with the antibodies) was also tested under conditions of constant protein concentration. As infected cell extract proteins in the reaction were increased, there was a proportional increase in the A4 complex (Figure 7, lanes 2 through 4). Addition of BSA, to keep total protein levels constant, had no effect of A4 complex formation (compare lanes 6 through 8 with lanes 2 through 4). Addition of the sodium phosphate buffer, under conditions of equal protein content, results in an increase in A4 complex (Figure 7, lanes 10 through 12). The increase could result from enhanced stability of the A4 complex or increased levels of active protein interacting with the probe or a combination of both.

Addition of both sources of anti-ICP4 antibody resulted in an increase in A4 complex formation (Figure 7, lanes 14 through 16 and 18 through 20). Anti-ICP4 antibody did not stimulate A4 complex formation as much as addition of only the sodium phosphate buffer (compare lanes 14 through 16, 18 through 20 to lanes 10 through 12). To further study the
Figure 7. Enhancement of ICP4 complex formation by addition of NaPO₄. Binding reactions were assembled on ice devoid of probe DNA and contained 0, 1 μl (8 μg), 2 μl (16 μg) or 4 μl (32 μg) of HSV-1 infected cell extract prepared 12 hr post infection. Total protein content of each reaction was adjusted to 32 μg by addition of bovine serum albumin (BSA) as indicated. Either two separate preparations of the anti-ICP4 monoclonal antibody 58S (1 μg) were added to the binding reaction as indicated above each lane. Additionally, NaPO₄ was added to reactions 9 through 12 to a final concentration of 12.5 mM, the same [NaPO₄] present in reactions containing antibody. After incubation on ice for 30 min. IE3 promoter (-108/+27) probe was added. Reactions were then incubated at room temperature for 30 minutes. 1X mobility shift dye was then added and the reactions fractionated at room temperature on 4% mobility shift gels. Free probe and A4 complexes are indicated by arrows.
12.5 mM NaPO₄, pH 6.8
58S (A)
58S (B)
BSA (8 mg/ml)
Infected cell extract

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Figure 7
effect sodium phosphate had upon A4 complex formation, different concentrations of sodium phosphate were incubated at various pH's with infected cell extract and probe in binding reactions. A4 complexes formed over a wide range of pH (from a pH of 6.0 to pH 9.0). The optimum pH was between pH 7.6 and pH 9.0 (Figure 8, Lanes 9 through 20). Also note, that as the sodium phosphate concentration was increased at each pH tested, the quantity of A4 complex also increased. A strong A4 complex formed at pH 6.0, which had an altered electrophoretic mobility (Figure 8, lane 1); however, the mobility of the complex gradually became normal as the sodium phosphate concentration increased (lanes 2 through 4). There was also an increase in non-specific complexes formed under binding conditions at pH 6.0. This apparent increase in A4 complex formation was most likely a result of non-specific association of ICP4 to the probe based upon the following observation. The quantity of A4 complexes formed at pH 6.0 was substantial (lanes 1 through 4), usually, when there is an abundant quantity of 1° A4 complex formed, 2° A4 and 3° A4 complexes also appear, and they were absent at this pH. Based upon the results of this experiment, the incubation regimens employed in all following mobility shift assays were the same as previously described, except for the addition of sodium phosphate to a final concentration of 12.5 mM unless otherwise stated.
Figure 8. pH optimization of A4 complex formation. Binding reactions were assembled at pH's ranging from pH 6.0 to pH 9.0. The pH of each reaction is listed above each lane. NaPO₄ concentration was also varied between 0 and 12.5 mM as listed above each lane. IE3 promoter (-108/+27) was used as probe. Binding reaction contained 16 µg of HSV-1 infected cell extract and 4 µg of poly dAdT DNA. Reactions were incubated at room temperature for 30 min., 1X mobility shift loading dye was added and the reactions fractionated on a 4% mobility shift gel with buffer recirculation at room temperature.
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**Figure 8**
PURIFICATION OF FACTORS INVOLVED IN A4 COMPLEX FORMATION.

To investigate whether or not ICP4 was the only protein responsible for A4 complex formation, it was important to purify all factors bound to the IE3 DNA probe. A method that would allow this type of "blind" purification was recently developed and is known as DNA affinity chromatography (Kadonaga and Tjian, 1986; Rosenfeld and Kelley, 1986). Multiple copies of the target binding site (oligomers) are covalently attached to a support matrix. The bound target sequence is then used to select specific factor(s) from a complex mixture of proteins. The matrix is washed and bound protein is eluted with a salt gradient. A wide variety of low abundance transcription factors have been purified using this method (Kadonaga and Tjian, 1986; Rosenfeld and Kelly, 1986) such as Spl (Briggs et al., 1986), CTF (Jones et al., 1987), AP1 (Lee et al., 1987) and ICP4 (Kattar-Cooley and Wilcox, 1989; DiDonato and Muller, this study).

Using the procedures outlined (Figure 9), factors involved in A4 complex formation were chromatographically fractionated. High salt (1 M NaCl) nuclear extracts of infected cells were prepared at 12 hrs PI (as described previously) (DiDonato and Muller, 1989). The high salt extract contained 45.0 mg of protein. The high salt extract was then dialyzed against a 0.3 M KCl stabilization buffer
Figure 9. Purification of A4 Complex forming protein(s).

Following the steps outlined, 1M NaCl extract (14 mg/ml, 3 ml) was prepared from 2.8 x 10^8 cells. The 1M NaCl extract (3 ml) was dialyzed against 0.3 M KCl stabilization buffer [see buffers and solutions] O/N at 4°C. The dialysate was clarified by centrifugation in an eppendorf centrifuge at 14,000xg at 4°C for 15 minutes. A total of 3.30 ml (7.0 mg/ml) of dialysate was recovered. One milliliter (7 mg/ml) of the clarified 0.3 M KCl dialysate was diluted to 50 mM KCl with stabilization buffer and loaded onto a 3.0 ml DEAE Sephacel column at 0.25 ml/minute. The column was washed with 50 mM KCl stabilization buffer and proteins eluted stepwise with increasing salt. Fractions were tested for A4 complex forming ability using 4 μl from each fraction in standard binding reactions using the IE3 promoter (-108/+27) as probe DNA. Fractions having maximal A4 complex forming ability (lanes 8 and 9) were pooled (0.38 mg/ml, 1.1 ml). 0.8 ml of the DEAE Sephacel pooled fraction was assembled into 8 ml of 1X binding buffer including 210 μg of salmon sperm DNA and loaded onto a 1 ml A4 oligo affinity column (see Materials and Methods for construction of column). The column was loaded at 0.15 ml/min. and the input cycled through the column 1x. The column was washed with 5 ml of 50 mM KCl binding buffer including 10% glycerol. Proteins were eluted stepwise with 5 ml each of binding buffer containing increasing salt. One-half milliliter fractions were collected and assayed for A4 complex forming ability by mobility shift assay. Binding reactions were performed under standard conditions except for the addition of polyvinyl alcohol (PVA) to 3% and elimination of poly dA·dT as bulk DNA. Fractions 27 and 28 were pooled and designated as 0.5 M elution fraction.
1. Infect cells with HSV-1.
2. Harvest cells, wash cell pellets with PBS buffer.
3. Resuspend cell pellet in 0.2 mM EDTA pH 8.0.
4. Add an equal volume of 2.0 M lysis buffer and extract proteins for 1 hr on ice.
5. Centrifuge lysate at 100,000 x g for 1 hr at 4°C.
6. Dialyze supernatant o/n at 4°C in 0.3 M KCl stabilization buffer.

7. **DEAE Sephadex liquid column chromatography**

8. Elute proteins from DEAE column with increasing salt.
9. Monitor elution fractions by Mobility shift assay for A4 complex formation.
10. Identify and pool active fractions.
11. Assemble pooled fractions into a 1x binding reaction and load onto an A4 DNA Affinity column.
12. Elute proteins from the A4 DNA affinity column with increasing salt and monitor the fractions for A4 complex formation by Mobility shift assay.

**Figure 9**
at 4 °C overnight and centrifuged at 30,000 x g for 20 minutes to eliminate any insoluble protein. The dialyzed extract contained 21.0 mg of protein. The majority (>80%) of the A4 complex forming factor(s) remained in the 0.3 M KCl supernatant, thus giving a 2- 3 fold purification after this step. This estimation was based on ICP4 content (Western blot) of the 1M and 0.3 M infected extract and comparative A4 complex forming ability in mobility shift assays (Figure 3, panel A, compare lane 1 with 4 and in Figure 3, panel B, compare lane 3 with 7).

The 0.3 M KCl fraction was diluted to 50 mM KCl and fractionated over a DEAE- Sephacel column (typically 1 ml of resin for 4 mg of protein). The column was extensively washed and proteins were eluted with increasing salt. Fractions were assayed by mobility shift assay. The majority of the A4 complex forming activity eluted in the 0.5M step (Figure 9, lanes 7 through 10). The most active fractions were pooled (the pooled DEAE- Sephacel fractions contained 3.4 mg of protein). DEAE- Sephacel fractionation resulted in an additional 5- 6 fold purification as compared to the 0.3 M KCl fraction (calculated from Table 2).

The pooled DEAE fractions were diluted to 50 mM KCl in 1x mobility shift binding buffer and slowly (4.0 ml/hr) loaded onto an IE3 transcriptional start site containing DNA affinity column (1 ml). The flowthrough was recycled over the column again as described in Materials and Methods.
Protein was eluted from the column with increasing steps of salt. Fractions were assayed by mobility shift assay. The A4 complex forming fractions were located in the 0.5 M KCl step (Figure 9, lane 27 and 28). Fractions from the DNA affinity column (equal volumes [25 µl]) were fractionated on duplicate 10% SDS-polyacrylamide gels. One gel was processed for Western blotting and the other was first stained with Coomassie blue and then silver stained (Figure 10, panel A, and panel B). Examination of the Coomassie blue stained gel revealed a complex pattern of proteins in the pooled DEAE fractions (Figure 10, panel A, DEAE lane). The 0.5 M step fraction (lane 2) from the DNA affinity column revealed a single faint band that was barely visible with an apparent molecular weight of 175K. Due to the small amount of sample recovered, detection of protein by SDS-PAGE was difficult. The silver stain of this gel revealed a major protein band which contained approximately 50-80% of the total stained material visible in the lane (Figure 10, panel C, lane 22), which corresponded to the only visible band in the 0.5 M elution lane on the Coomassie blue stained gel. There were also minor protein bands of lower molecular weight present in this lane of the gel. Total protein content of the pooled active fraction was determined to be 30 µg based upon Bio-Rad protein assay.
Figure 10. Analysis of A4 complex forming protein(s). A. An equal volume of each fraction (25 µl) designated as listed above each lane, was fractionated on a 10% SDS-PAGE gel and stained with Coomassie blue. Lanes marked DEAE (DEAE pool), and IN (0.3 M KCl dialysate) contained 1.5 µg and 14.0 µg protein respectively. Molecular weight markers (Rainbow molecular weight markers from Pharmacia) are in lane 10. B. A duplicate gel was run and analyzed by Western blotting using anti-ICP4 antibody as probe and peroxidase (Vectastain) to develop the blot. C. Silver stain of the gel in A. The position of molecular weight markers and ICP4 is indicated.
Coomassie Blue stained SDS-polyacrylamide gel

Western Blot of (A)

Silver stain of (A)

Figure 10
Western blot analysis using an anti-ICP4 monoclonal antibody revealed a single dark staining band in the 0.5 M KCl elution lane of the DNA affinity column (Figure 10, panel B, lane 12) in the identical region of the dark staining major band seen in the silver stained gel (Figure 10, panel A, lane 22). The major protein component of the 0.5 M elution step from the DNA affinity column appears to be ICP4 based upon the result of the Western blot data. I estimate the purity of the affinity ICP4 to be approximately between 50-80% homogeneous based on the results of Figure 10, panels A and C.

The lower molecular weight proteins could be degradation products of ICP4 which lack the epitope recognized by the anti-ICP4. This is a plausible explanation as Metzler and Wilcox described extensive proteolysis of ICP4 during its purification unless specific proteolytic inhibitors were included in the buffers (Metzler and Wilcox, 1985; DiDonato and Muller, unpublished observations). Alternatively, the lower molecular weight proteins bands could either be contaminants or proteins that associate with ICP4 to form the A4 complex. The latter possibility seems less likely with the recent evidence of Kattar-Cooley and Wilcox who have purified ICP4 to near homogeneity (>90%) (Kattar-Cooley and Wilcox, 1989). Also, Michael et al. (1988), have used the Southwestern blotting technique to identify a protein from infected cell extracts
with an apparent molecular weight of 175K that binds labeled IE3 probe (-108/+27) and other ICP4 binding site containing probes but not heterologous probes. This region of the Southwestern blot also is recognized by monoclonal antibody to ICP4 (Michael et al., 1988). DNA affinity chromatography resulted in roughly between a 1000 and 1700 fold purification of ICP4 from the high salt extract (see Table 2).

DETECTION AND STABILIZATION OF THE A4 COMPLEX FORMED WITH AFFINITY PURIFIED ICP4.

Previous attempts to purify ICP4 indicated that after two chromatographic steps, the fractions containing ICP4 failed to bind to double stranded salmon sperm DNA-cellulose columns. The DNA binding property of the partially purified ICP4 was regained by the addition of mock infected cell proteins (Freeman and Powell, 1982). This suggested a role for cellular proteins in association with ICP4 in order for ICP4 to bind to DNA.

When partially purified ICP4 (DEAE pool) was first chromatographed over the DNA affinity column, the fractions assayed by the mobility shift assay failed to form an A4 complex (data not shown). When 2 μg of BSA was added to the binding reaction mix and the fractions reassayed, a faint A4 complex was visible in the 0.5 M KCl elution step (Figure
Table 2. ICP4 purification scheme. The concentration of pooled fraction and the total amount of protein at each step of the fractionation is given. As a comparison, the fractionation scheme of Kattar-Cooley and Wilcox is also given. Their fractions correspond to roughly the same fraction steps that are listed for the fractionation done in this study.
Table 2

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>CONCENTRATION (mg/ml)</th>
<th>TOTAL PROTEIN (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) Cell lysate</td>
<td>14.06</td>
<td>45.0</td>
</tr>
<tr>
<td>B) 0.3 M KCl dialysate</td>
<td>7.0</td>
<td>21.0</td>
</tr>
<tr>
<td>C) DEAE-Sepharose peak</td>
<td>0.38</td>
<td>3.40</td>
</tr>
<tr>
<td>D) A4 DNA affinity Sepharose</td>
<td>0.0025</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Kattar-Cooley and Wilcox ICP4 purification
(J. Vir. 63:696-704)

| III) Triton washed nuclei | 4.8                   | 304.0              |
| VI) S400 peak             | 2.70                  | 2.7                |
| IX) DNA-cellulose peak    | 1.0                   | 0.20               |
Figure 11. Using affinity purified ICP4 in gel shift assays optimizing ICP4/DNA complex formation. Pooled 0.5M KCl BB A4 complex forming fractions identified from A4 DNA affinity chromatography was examined as to conditions of salt (KCl, MgCl\(_2\) and CaCl\(_2\)), protein (BSA) or volume exclusion substances (PVA) which allowed the greatest conversion of IE3 probe (-108/+27) to A4 complexes. The salt concentrations (mM), BSA (2 \(\mu\)g), anti-ICP4 monoclonal antibody (58S) (140 ng) and volume of affinity purified ICP4 is listed above each lane. Mobility shift assays were performed at room temperature and contained 5 ng of poly dA·dT except for lanes 1 and 2 which contained 4 \(\mu\)g of poly dA·dT as bulk DNA.
<table>
<thead>
<tr>
<th>DTT mM</th>
<th>+  +</th>
</tr>
</thead>
<tbody>
<tr>
<td>58S Ab</td>
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</tr>
<tr>
<td>CaCl$_2$ mM</td>
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</tr>
<tr>
<td>MgCl$_2$ mM</td>
<td>135 145 165 175 200 225</td>
</tr>
<tr>
<td>KCl mM</td>
<td>175</td>
</tr>
<tr>
<td>PVA%</td>
<td>3  3  3  3</td>
</tr>
<tr>
<td>BSA (ug)</td>
<td>2  2  2  2  2  2  2  2  2</td>
</tr>
<tr>
<td>INF extract</td>
<td>2.5 2.5 2.5 3.5 3.5 4.5 4.5 4.5 3.5 3.5 3.5 3.5 3.5 3.5 3.5 3.5 3.5 3.5</td>
</tr>
<tr>
<td>12 hr PI (ug)</td>
<td>3.6 3.6</td>
</tr>
<tr>
<td>Affinity purified ICP4 (ul)</td>
<td>2  3  4  5  6  7  8  9  10  11  12  13  14  15  16  17  18  19</td>
</tr>
</tbody>
</table>

Figure 11
Addition of exogenous protein was later shown not to be required for A4 complex formation. This observation was made as a result of adding polyvinyl alcohol (PVA) to the incubations instead of BSA. Different amounts of PVA ranging from 3 to 5% vol/vol were used (Figure 12) in mobility shift assays to determine the optimal concentration of PVA to add to the reactions. The optimum amount of PVA added the binding reaction resulting in the greatest conversion of probe to A4 complex was found to be 3% PVA (Figure 12, lane 4). Enhanced A4 complex formation with affinity purified ICP4 using PVA versus BSA (2 μg) was observed (Figure 11, compare lane 16 with lane 3, lane 17 with lane 7, lane 18 with lane 10 and lane 19 with lane 14).

In all cases, PVA greatly enhanced A4 complex formation with affinity purified ICP4. To demonstrate the specificity of enhancement and to rule out the possibility of non-specific binding of protein to probe, the following experiment was performed. Probes containing the IE3 probe (-108/+27) were incubated either with infected cell extract or affinity purified ICP4 containing 3% PVA in binding reactions (Figure 13). Anti-ICP4 antibody induced a shift of A4 complexes in both crude extracts and in affinity purified ICP4 reactions (Figure 13, lanes 5, through 11, 13, 14, 18, 19 and 20). When increasing amounts of A4 oligo were added as specific competitor DNA, the A4 complexes were competed (lanes 6 through 8, and 15 through 17) but when
Figure 12. Optimum polyvinyl alcohol concentrations for formation of ICP4/DNA complexes using affinity purified ICP4. Infected cell extracts (lanes 1 and 2) and affinity purified ICP4 (lanes 3 through 20) were included along with various amounts of PVA, MgCl₂, CaCl₂ or BSA as indicated above each lane. Lanes 1 and 2 contained 4 µg of poly dA·T and lanes 3 through 20 contained 5 ng of polydA·dT. 1°, 2° and 3° A4 complexes are indicated to the left. Free probe (IE3[-108/+27]) is also indicated.
<table>
<thead>
<tr>
<th>CaCl₂ mM</th>
<th>1 2 1 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂ mM</td>
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</tr>
<tr>
<td>PVA%</td>
<td>3 3 3 4 5 3 4 5 3 4 5 3 4 5 3 4 5 3 3 4 5</td>
</tr>
<tr>
<td>BSA (ug)</td>
<td>10</td>
</tr>
<tr>
<td>INF extract</td>
<td>3 6 3.6</td>
</tr>
<tr>
<td>12 hr PI (ug)</td>
<td>3.6 3.6</td>
</tr>
<tr>
<td>Affinity purified</td>
<td>2.5 2.5 2.5 2.5 3.5 3.5 3.5 4.5 4.5 4.5 3.5 3.5 3.5 3.5 3.5 3.5 3.5 3.5</td>
</tr>
<tr>
<td>ICP4 (ul)</td>
<td>1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20</td>
</tr>
</tbody>
</table>

Figure 12
Figure 13. Specificity of A4 complex formed by A4 affinity purified ICP4 in the presence of polyvinyl alcohol. Mock infected cell extract (32 μg), infected cell extract (32 μg), or affinity purified ICP4 (4 μl) was incubated in a mobility shift binding reaction on ice for 30 minutes with either anti-Vmw65 monoclonal antibody (LP1) or anti-ICP4 monoclonal antibody (58S) or no antibody as indicated. IE3 (-108/+27) or IE3 (-18/+27) probe was then added to reactions 1 through 10 and 11 through 20 respectively. Unlabeled A4 oligo (10, 30 and 50 ng) was added to the binding reactions as a specific competitor DNA (as indicated over the lane number) at the same time as the probe. SV40 72bp enhancer DNA (10, 30 and 50 ng) was added to the indicated binding reactions as an unlabelled non-specific competitor DNA. Binding reactions using affinity purified ICP4 also contained PVA to 3% vol/vol. Reactions 1 through 11 contained 4 μg of poly dA*dT and reactions 12 through 20 contained 5 ng of poly dA*dT.
Figure 13
heterologous DNA (72 bp Sph I- Sph I enhancer of SV40) was added no competition was seen (lanes 9 through 11 and 18 through 20). If complex formation were the result of non-specific binding of ICP4 to the probe, the SV40 competitor should compete as effectively as the A4 oligo and this clearly was not the case.

A4 complexes formed in the presence of PVA are bonafide ICP4 containing complexes based upon the criteria mentioned previously that define the A4 complex; i) the complexes must be observed only with probes containing the ICP4 binding domain; ii) complexes should dissociate upon addition of a molar excess of an oligonucleotide competitor containing the ICP4 binding domain (ICP4 consensus binding site); iii) DNA-protein complexes containing ICP4 should be shifted to a different position upon binding a monoclonal antibody directed against ICP4.

MONOVALENT AND DIVALENT CATION REQUIREMENTS FOR A4 COMPLEX FORMATION.

The monovalent and divalent cation requirements for A4 complex formation were investigated. Initially, the concentration of monovalent cation (K⁺ in KCl) was varied between 22.5 mM and 0.5 M in binding reactions. Monoclonal antibody to ICP4 was included in the reaction to authenticate A4 complexes. A4 complex formation was maximum
Figure 14. Optimum monovalent and divalent cation concentrations for ICP4/DNA complex formation using partially purified ICP4. Partially purified ICP4 eluted from DEAE Sephacel by a linear KCl salt gradient (active fractions 225 mM KCl) was used as a source of protein in mobility shift DNA binding reactions (lanes 2 through 19). Infected cell extract was used as a source of protein in the binding reaction in lane 1. Concentration of KCl, MgCl$_2$ and CaCl$_2$ were increased as indicated above each lane. Anti-ICP4 monoclonal antibody (1.4 µg) was included in reactions 1 through 9. All reactions contained 3 µg of poly dA·dT. Binding reactions were assembled minus the labeled DNA probe (IE3 promoter [-108/+27]) on ice and incubated 30 minutes. Probe was then added and reactions were then incubated at room temperature for thirty minutes before addition of tracking dye and fractionation on a 4% mobility shift gel. Primary (1°) and antibody shifted 1° A4 complexes are indicated as is free probe DNA.
Figure 14
from 22.5 mM to 100 mM KCl (Figure 14, lanes 2 through 4). At a salt concentration of 150 mM KCl and greater, the ability of the anti-ICP4 antibody to shift the A4 complex is abrogated (see Discussion). Appreciable amounts of A4 complex could be observed to 300 mM KCl and even at 0.5 M KCl slight A4 complex formation could also be seen. At low concentrations of Mg\(^{++}\), (<1.5 mM) (Figure 14, lanes 10 and 11) there was abundant A4 complex formation but at higher concentrations of Mg\(^{++}\), A4 complex formation was diminished (Figure 14, lanes 12 through 14). When Ca\(^{++}\) was included in binding reactions, A4 complex formation was substantial at concentrations ranging from 0.5 mM to 1.5 mM (Figure 14, lanes 17 through 19) but were reduced at 2 and 3 mM Ca\(^{++}\) (lanes 15 and 16).

When affinity purified ICP4 was used in binding assays and similar concentrations of KCl, MgCl\(_2\) and CaCl\(_2\) included in the binding reaction as in Figure 14 (above), comparable results were observed (Figure 15). Therefore, affinity purified ICP4 appears to have the same salt requirements and characteristics of A4 complex formation as does the partially purified extracts (pooled DEAE fractions). This observation lends itself to the notion that no additional factors present in the partially purified fractions contribute to A4 complex formation or stability that are not present in the affinity purified fractions which were substantially enriched in ICP4.
Figure 15. Optimum monovalent and divalent cation concentrations for A4 complex formation with affinity purified ICP4. A4 affinity purified ICP4 (lanes 4 through 19) or infected cell extract (7 µg) (lanes 2 and 3) were used as a source of protein in DNA binding reactions. KCl, MgCl and CaCl₂ concentrations were varied as indicated above each lane. The anti-ICP4 monoclonal antibody (58S) was included in binding reactions (lanes 3 through 12 and 19). Free probe is in lane 1. Binding reactions were assembled on ice without labeled probe and incubated 30 minutes further on ice. Labeled probe (IE3[-108/+27]) was then added and reactions incubated at room temperature for 30 minutes. Tracking dye was added and the samples fractionated at room temperature on a 4% mobility shift gel. 1° and 2° A4 complexes and their antibody shifted positions are indicated to the side as is the position of free probe DNA. Lanes 1, 2 and 3 are reference lanes for free DNA, 1° and 2° A4 complexes, and shifted 1° and 2° A4 complexes respectively.
Table 1

<table>
<thead>
<tr>
<th>CaCl&lt;sub&gt;2&lt;/sub&gt; mM</th>
<th>MgCl&lt;sub&gt;2&lt;/sub&gt; mM</th>
<th>KCl mM</th>
<th>58S Ab</th>
<th>Affinity purified ICP4</th>
<th>INF extract</th>
<th>Lane</th>
</tr>
</thead>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>50 62.5 75 87.5 100 112.5 125 137.5 150 50 50 50 50</td>
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<td>+  +  +  +  +  +  +  +  +  +  +  +</td>
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<tr>
<td>Shifted 2° A4</td>
<td>2° A4</td>
<td>1° A4</td>
<td>Free Probe</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

Figure 15
DISCUSSION

ICP4 is a component of a specific protein/DNA complex that is referred to as the A4 complex (DiDonato and Muller, 1989). A4 complexes are equivalent to ICP4 containing protein/DNA complexes described previously, (Faber and Wilcox, 1986a; Kristie and Roizman, 1986b; Muller, 1987). These complexes were demonstrated by immunoprecipitation of ICP4/DNA complexes formed by incubation of HSV-1 restriction fragments with infected cell extract (Faber and Wilcox, 1986) or by use of monoclonal antibody directed against ICP4 to retard the electrophoretic migration of ICP4 containing protein/DNA complexes through a mobility shift gel (Kristie and Roizman, 1986a, 1986b; Muller, 1987; Deluca and Schaffer, 1988; Paterson and Everett, 1989; Resnick et al., 1989).

A direct correlation between ICP4 content and A4 complex formation was clear (Figure 3, panels A and B). When equivalent quantities of infected cell protein were analyzed by Western blot, the ICP4 content during the course of HSV-1 infection increased until 12 hr PI and reached a plateau (Figure 3, panel B and Figure 2, panel C lanes 27 through 32 and data not shown). ICP4 containing complexes
formed more readily at late times PI (Figure 3, panel A). A4 complex formation increased between 4 to 10 hours PI as detected by mobility shift assay, to a more noticeable degree than the corresponding increase in ICP4 levels (Figure 2). ICP4 could become more active in complex formation at later times in infection in a number of ways; i) as a result of post-translational modification (Pereira et al., 1977; Wilcox et al., 1980; Preston and Notaranni, 1983); ii) perhaps the increased availability of a virally encoded protein needed for complex formation is now in excess; iii) the presence of a cellular factor which can be activated by viral products to enter into complex formation more readily.

The post-translational modification scenario is the most likely explanation for increased A4 complex formation at late times PI. There are several reasons for this assumption. First, less posttranslationally modified forms of ICP4 bind to certain ICP4 binding sites better than the slower migrating more modified forms of ICP4 based upon Southwestern blot results (Michael et al., 1988). Secondly, DNA affinity purified infected extracts, which are substantially enriched for ICP4 (Figure 10) and >90% homogeneous (Kattar-Cooley and Wilcox, 1989), can bind A4 site DNA. Third, a region of ICP4 has been identified and expressed, as a fusion protein in E. Coli, which is capable of binding DNA and forming A4 complexes (Wu and Wilcox,
1990). These data taken together almost entirely rule out options two and three and suggest that ICP4 alone interacts with the DNA to form A4 complexes; therefore, posttranslational modification could influence binding affinity (see below and Chapter III Discussion). The ICP4 used in the remainder of the experiments described in this study used ICP4 prepared at 12 hr PI and therefore is assumed to be posttranslationally modified maximally.

Mobility shift assays detect minute amounts of a specific DNA binding protein (Singh et al., 1986). Western blot analysis is also a sensitive technique used to detect specific proteins when $^{125}$I-protein A is used to detect the antibody-antigen complex (Talbot et al., 1984). Peroxidase development of antigen-antibody complexes is known to be 10 to 20 fold less sensitive as compared to $^{125}$I-protein A detection (Protocols in Molecular biology, Ausubel, F. ed.). The advantage of using peroxidase to develop the Western blot is the ability to visualize ICP4 on the transfer membrane opposite prestained molecular weight markers. Comparison of the level of sensitivity to which mobility shift assay and Western blot using peroxidase to detect ICP4 was determined. Mobility shift assay was at least one order of magnitude more sensitive than Western blot (using peroxidase) in detecting ICP4.
Binding Specificity

Binding specificity of a DNA binding protein to its binding site is usually demonstrated by the loss of complex formation as increasing amounts of unlabelled homologous competitor DNA is added to the binding reaction, but not when non-homologous competitor DNA is added (Fried and Crothers, 1981). Homologous competitor DNA (A4 oligo) decreased the amount of A4 complex formed on the IE3 transcriptional start site, but a heterologous oligo did not (Figure 5). ICP4 was identified as a member of the 1°, 2° and 3° A4 complexes by virtue of anti-ICP4 monoclonal antibody's ability to retard the electrophoretic migration of the complex. I was the first to demonstrate that alteration of the -6A, -5T in the IE3 transcriptional start site resulted in severe impairment of ICP4 to bind to this sequence, therefore confirming the importance of this sequence in A4 complex formation (Figure 5) and in negative regulation by ICP4 through this sequence in transient expression assays (DiDonato and Muller, 12th International Herpes Workshop, p293, 1987). These in vitro and in vivo findings were also supported by the results of other investigators (Deluca and Schaffer, 1988; Roberts et al., 1988). The A4 oligo containing the IE3 sequences -15 to +16 formed an A4 complex but not as efficiently as the WT IE3 probe (Figure 5). This may be a result of bases 5' to -15
or 3' to +16 which are absent and are required for increased binding affinity. This argument seems unlikely. Missing contact and Cu-OP footprinting data for the IE3 promoter indicate specific bases outside the -15 to +16 region do not affect binding affinity (Figures 22, 23 and 28; also see discussion in Chapter III). However, nonspecific electrostatic interactions between ICP4 and the DNA in the areas outside -15 to +16 may be responsible for the stabilizing ICP4 binding; therefore, somewhat longer probes would help stabilize binding.

Addition of anti-ICP4 monoclonal antibody to binding reactions was routinely used to authenticate the presence of ICP4, in an A4 complex. It was observed that addition of exogenous protein (ascites fluid or purified anti-ICP4 monoclonal antibody) could result in stimulation of A4 complex formation (Figure 5, lanes 1 and 3 and data not shown). This observation was examined further. Addition of crude mock infected cell extract did not enhance A4 complex formation, but anti-ICP4 monoclonal antibody did (Figure 7, lanes 15, 16, 19 and 20). Upon further examination, the sodium phosphate buffer rather than the anti-ICP4 antibody was found to enhance A4 complex formation (Figure 7, compare lanes 12 with 16 and 20).

This result may be explained in the following manner. ICP4 is known to be posttranslationally modified by phosphorylation and ADP-ribosylation (Pereira et al., 1977;
Wilcox et al., 1980; Preston and Notaranni, 1983; Faber and Wilcox, 1986b). Perhaps, the crude cellular extract contains a low concentration of phosphatase which during the course of incubation may dephosphorylate ICP4 and effect its ability to form an A4 complex. This is plausible for two reasons. First, addition of sodium phosphate, a known competitive inhibitor of some phosphatases, would inhibit the action of this phosphatase during the incubation. Secondly, I have examined the effect that calf intestinal alkaline phosphatase (CIAP) had on A4 complex formation when either affinity purified ICP4 or crude infected extracts were used as a source protein in binding reactions. CIAP effects the formation of A4 complexes negatively when either source of protein was preincubated with the phosphatase and then used in mobility shift assays (data not shown). These experiments need to be repeated using CIAP that can be removed from the binding reaction, so that the possibility of CIAP's physical presence rather than its enzymatic activity being responsible for lack of A4 complex formation can be determined (these experiments are in progress). Alternatively, sodium phosphate may simply be contributing ionic effects, which decrease ICP4's association with non-specific binding sites. This would increase the concentration of ICP4 available to bind specific binding sites.
The optimal sodium phosphate concentration and the pH of the binding buffer was determined to be 12.5 mM NaPO₄ and pH 7.9 respectively (Figure 8). A4 complex formation occurred over a wide range of pH (between 6.8 to 9.0). The pH 7.9 was chosen for all binding reaction because it is a physiological pH and is amenable to many different footprinting techniques and in vitro transcription reactions. At binding buffer pH 6.0, abundant quantities of complexes formed that resembled A4 complexes. These complexes migrated more rapidly than A4 complexes formed at higher pH. This discrepancy in migration was eliminated by addition of NaPO (12.5 mM) to the binding reaction. The variant migration may be a result of a change in the protein structure at the lower pH (see monovalent and divalent cation conditions, below) or due to a more basic charge of the protein at this pH. The increased complex formation may also result from non-specific binding of ICP4 to probe DNA. The last choice appears to be the most likely explanation for abundant A4-like complex formation as evidenced by the increase in the many more rapidly migrating non-specific complexes which are also greatly increased in intensity at this pH.
Affinity purification

Data from recent DNA binding studies reveal that ICP4 is part of the A4 complex, but other components of the complex (if any) have yet to be identified (Faber and Wilcox, 1986a; Kristie and Roizman, 1986a,b; Muller, 1987; Deluca and Schaffer, 1988; Roberts et al., 1988). Purification of factor(s) involved in A4 complex formation has been achieved using DNA affinity chromatography (Kattar-Cooley and Wilcox, 1989; DiDonato and Muller, 1989; this study).

SDS-PAGE analyses of A4 complex forming proteins eluted from the IE3 (-18/+27) DNA affinity column, revealed a single band with an apparent molecular mass of 175KDa. This single protein band was barely visible by coomassie- blue staining (Figure 10, lane 2). A silver stained version of the same gel revealed the appearance of a very darkly staining band corresponding to the only visible band by coomassie- blue staining. There were faster migrating fainter silver stained bands also visible in the lane. The major staining band corresponded to approximately 50 to 80 percent of the stained material in the lane. Western blot analysis of a duplicate gel identified the major staining band as ICP4 (Figure 10, panel B, lane 12).

The lack of other stained bands in the coomassie- blue stained gel and the minor staining of lower molecular
weight bands in the silver stained gel suggests but does not prove that ICP4 alone is responsible for A4 complex formation. The conclusion that ICP4 alone binds to DNA to form the A4 complex has also been reached by other laboratories, using the same method that was used in this study (Kattar-Cooley and Wilcox, 1989) and the Southwestern blotting technique (Michael et al., 1988). Mutation and deletion analysis within ICP4 resulted in the identification of a DNA binding domain that when mutated failed to bind the IE3 A4 sequence (Deluca and Schaffer, 1988; Patterson and Everett, 1988; Shepard et al., 1989a,b). Recently, expression of defined segments of the ICP4 binding domain, as fusion proteins in E. Coli, have shown that a subset of these fusion proteins can bind both consensus and nonconsensus ICP4 binding sites (Wu and Wilcox, 1990).

Taken together, the above results indicate that ICP4 alone can bind to the IE3 transcriptional start site and form an A4 complex without the need or aid of other proteins.

Assuming this to be the case, I calculated the yield of ICP4 from 2.8 X 10^8 BHK cells harvested at 12 hr PI to be between 10 to 20 μg and 0.02 to 0.05% of the total protein in the high salt fraction. Affinity purification of ICP4 resulted in a 1000 to 1700 fold enrichment of ICP4 relative to the high salt crude cellular fraction. The total number of molecules of ICP4 per cell was approximately 2 to 4 x 10^5 molecules/cell (calculated from Table 2). These values
compare favorably with those observed by Kattar-Cooley and Wilcox (1989) who recovered 200 μg of ICP4 from 9 × 10^9 Vero cells harvested a 6 hr PI. ICP4 was approximately 0.06% of the total protein in the high salt fraction. Affinity purification resulted in a 1450 fold enrichment of ICP4 relative to the high salt fraction (see Table 2 for a side by side comparison of the purification scheme). Each Vero cell was calculated to contain 1.1 × 10^5 molecules of ICP4/cell. For comparison, a cellular transcription factor, the major late transcription factor (MLTF) (Chodosh et al., 1986) is present at approximately 10,000 molecules/cell (Carthew et al., 1985).

When fractions from the affinity column were initially assayed in binding reactions, complexes were not observed (data not shown). The assays were repeated with BSA (2 μg) added to each reaction. A4 complex formation was barely detectable (data not shown; an example of which can be seen in Figure 11, lane 3). To increase A4 complex formation, polyvinyl alcohol (PVA) to 3% vol/vol was added to each reaction. PVA is thought to act by increasing the effective concentration of reactants in the reaction in a manner analogous to the way dextran sulphate increases the effective concentration of probe and target in a hybridization reaction. PVA addition significantly stimulated formation of A4 complexes (Figure 11, lanes 15 through 18). The affinity column fractions were reassayed
in the presence of 3% PVA and the fractions capable of A4 complex formation were identified (Figure 9, lanes 27 and 28). The specificity of A4 complexes formed by affinity purified ICP4, in the presence of PVA, was found to be identical to the specificity of A4 complexes formed by crude infected cell extract (Figure 13). Like the A4 complexes formed with crude cell extracts, A4 complexes formed with affinity purified ICP4 incubated with PVA were retarded in mobility by the anti-ICP4 antibody in mobility shift gel assays and competed by addition of homologous but not heterologous oligonucleotides. This result supports the idea that PVA enhances complex formation and does not lead to artifactual complex formation.

**Salt optima for A4 complex formation**

Monovalent and divalent salt optima for A4 complex formation by partially purified ICP4 (DEAE-Sephacel) and affinity purified ICP4 were quite similar. Partially purified ICP4, when assayed at KCl concentrations less than 120 mM KCl, exhibited maximum complex formation and declined as the salt concentration was increased (Figure 14). Affinity purified ICP4 reacts essentially identically (Figure 15, lanes 1 through 12).

Anti-ICP4 monoclonal antibody (58S) recognizes an epitope of ICP4 located within the 214 carboxyl terminal
amino acid residues of ICP4 (Deluca and Schaffer, 1988). The carboxyl terminal third of ICP4 contains a major portion of the temperature sensitive mutations that have been mapped in this protein (Dixon and Schaffer, 1980; Preston, 1981; Deluca et al., 1984). This region also bears a strong conservation of amino acid identity to the corresponding region of the varicella zoster (VZV) 140 KDa protein and the PRV IE protein (Vlcek et al., 1989; Deluca and Schaffer, 1988; Patterson and Everett, 1980a). The VZV protein can compliment these mutations in ICP4 (Felser et al., 1987; Felser et al., 1988) suggesting that this region of ICP4 does play an essential role in the virus life-cycle. The role this region plays in transactivation and repression of HSV gene expression is quite complex and confusing.

Insertion and deletion mutant analysis revealed, that as some portions of the region were altered or deleted, a detrimental effect to transactivation and/or repression was observed. If the deletion point was moved either 5' or 3', the effect on trans regulation was alleviated, in some cases (Deluca and Schaffer, 1988; Patterson and Everett, 1988a).

This region contains amino acid sequences (minidomains) that actually take part in trans regulation and it is also likely, that there are amino acid sequences that have nothing to do with trans regulation, but instead, ensure the proper folding of the protein to expose or sequester regions that do.
Evidence for the structural integrity contributions, made by this region, could be observed in a few of the deletion mutants of ICP4. These mutants are able to bind DNA, form A4 complexes and migrate to a position higher in the gel than the wild type ICP4/DNA complex (Patterson and Everett, 1988a; Wu and Wilcox, 1990). This result suggests an altered protein structure due to improper folding. The binding affinity of these mutants was affected as a function of the incubation temperature (Patterson and Everett, 1988b). Wild type ICP4 was also sensitive to temperature fluctuations in regard to the rate at which it binds to and dissociates from the DNA (Patterson and Everett, 1988a; Kattar-Cooley and Wilcox, 1989; DiDonato and Muller, data not shown). The differences in rate of association and the order of dissociation of ICP4 binding to the IE1 A4 site at 4°C and 23°C were striking. The A4 complex dissociated by 50% within 5 minutes at 23°C, whereas, it took 35 hr to dissociate to 50% of the initially bound DNA at 4°C. Dissociation rates were first order at 4°C while the rate varied with the concentration of ICP4 and time at 23°C (Kattar-Cooley, 1989).

Structural alteration of ICP4 conformation in this region in response to increased salt concentrations could explain the decrease in the ability of the anti-ICP4 monoclonal antibody to retard the migration of the A4 complex in mobility shift assays (Figures 14, lanes 4
through 9 and Figure 15, lanes 8 through 12). Salt concentrations of 150 mM (PBS), routinely used in Western blot analyses, do not have this effect on anti-ICP4 interaction with ICP4 (Figures 1, 2 and 3). Native ICP4 transferred to nitrocellulose in 150 mM KCl buffer on immuno-slot blots was detected by a 1:200 dilution of the anti-ICP4 monoclonal antibody (data not shown). In Western blot analyses, ICP4 is in a denatured state and the epitope is available for antibody binding. The above observations indicate that DNA bound ICP4 can alter its conformation in response to ionic conditions as evidenced by the inability of the anti-ICP4 monoclonal antibody to recognize its specific epitope located on the carboxy terminal region of ICP4. This ability to alter the structure of ICP4 bound to DNA, in response to salt concentration (Figure 14 and 15), could allow different areas of the protein to be exposed. Perhaps posttranslational modifications play a role in determining potential structural alterations. Such flexibility in protein structure could allow for a fine-tuned control of the trans regulatory activities of ICP4 during the virus life-cycle. Posttranslationally modified forms of ICP4 bind DNA fragments containing a nonconsensus ICP4 binding site with less affinity than less modified forms (Michael et al., 1988).
CHAPTER III

IDENTIFICATION AND CHARACTERIZATION OF ICP4 BINDING SITES: EVIDENCE FOR A RELATIONSHIP BETWEEN ICP4-DNA AND TFIID

INTRODUCTION

ICP4 has been implicated in the control of early and late gene expression in addition to its role in IE gene regulation (Preston, 1979; Dixon and Schaffer, 1980; Deluca and Schaffer, 1984). It seems likely that ICP4 would bind to all classes of HSV genes. Once identified as ICP4 binding sites, the exact location of the site should be determined by footprinting studies. DNase I is routinely used for such purposes but occasionally, the DNA binding protein in question does not bind efficiently under DNase I digestion conditions. To circumvent this problem, other nucleases such as Exo III (Kristie and Roizman, 1986b) or chemical nucleases (copper-phenanthroline (Cu-OP) and Fe-EDTA) can be used (Tullius and Dombrowski, 1986; Sigman, 1987).

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Use of nucleases such as DNase I reveal the boundaries of the bound protein, but not the intimate contacts the protein makes with the DNA. In the following experiments, I used the mobility shift assay to find ICP4 binding sites in promoters from all classes of HSV genes, in addition, I footprinted a number of these sites with copper-phenanthroline (Cu-OP), a chemical nuclease. Cu-OP footprints the DNA via free radical attack of the backbone and is sensitive to fluctuations in minor groove structure (Sigman, 1987). This footprinting method is capable of revealing intimate contacts made by the protein to the DNA (Marshal et al., 1981; Sigman, 1987; Kuwabara and Sigman, 1987).
RESULTS

ICP4 binds to various HSV-1 promoter elements.

The upstream region of the glycoprotein D gene (gD) and immediate early gene 1 (IE1) each contain ICP4 binding sites (Faber and Wilcox, 1986; Kristie and Roizman, 1986b). To confirm this mobility shift experiments were performed using probes derived from gD, IE1, and IE3. The gD probe [HindIII(-428) to HindIII(+11)] and the IE1 probe [SmaI(-129) to Avai(+51)] were capable of forming an A4 complex when incubated with infected cell extracts (Figure 16, lanes 13 and 17). The A4 complex was not formed when these probes were reacted with mock infected extracts (data not shown). To verify that the complexes were indeed due to ICP4 binding, I applied two criteria. The complexes were shifted with the anti-ICP4 antibody (Figure 16, compare lanes 2 with 3, 13 with 14, and 17 with 18) and the complexes were competed by the oligonucleotide containing the A4 consensus sequence (lanes 6, 7, 15, 16, 19 and 20). The gD probe formed several ICP4 containing complexes corresponding to 1°, 2° and 3° A4 complexes, seen with IE3 promoter (lanes 15
Figure 16. Mobility shifts with various promoters.
Binding reactions containing 3-5 ng of Bam HI to Eco RI (+27/-108) probe of PWT-GH (lanes 1 through 7) and pD2-GH (lanes 8 through 12); 10 ng of the Hind III to Hind III (-428/+11) probe of pgDCAT, lanes 13 through 16; and 3-5 ng of Sma I to Ava I (-129/+51) probe of p111 (ICPO), lanes 17 through 20, were incubated in standard binding reactions containing 8 μg of infected cell nuclear extract. Additionally, 2 μg of the anti-ICP4 monoclonal antibody 58S (Showalter et al., 1981) or 2 μg of the anti-Vmw65 monoclonal antibody LP1 (McCLean et al., 1982) was added to the binding reactions where indicated. Oligonucleotide (45 bp) containing a topoisomerase I cleavage site (PTI) or A oligonucleotide (A) (see Fig. 1) were added as competitor DNA (50 ng or 200 ng) as shown. The type of oligonucleotide is indicated above the lanes of the gel.
Figure 16
and 16). It may appear that the fastest migrating (1°) complex (lanes 15, 16) was not competed by the A4 oligo; however, these two reactions received inadvertently high levels of total probe (compare the intensity of free DNA in lanes 15, 16 with 13, 14) and based upon the ratios of free and bound DNA, the 1° complex was in fact effectively competed by the A4 oligonucleotide. I conclude that gD and IE1 promoters are competent to drive formation of the A4 complex in agreement with other laboratories (Faber and Wilcox, 1986; Kristie and Roizman 1986b). I have also confirmed these findings using purified ICP4 and the gD and IE gene 1 promoters (data not shown).

The mutant, pD2-GH contains a 2 bp deletion in the core of the ICP4 binding sequence. Wild type IE3 probe [Bam HI(+27) to Eco RI(-108)] formed a typical array of A4 complexes when incubated with infected cell extract (Figure 16, lane 1). Anti-ICP4 monoclonal antibody clearly reacts with these complexes (lanes 3 through 7). A probe (BamHI to EcoRI) derived from pD2-GH failed to form an A4 complex when incubated with infected cell extract (lane 8). Addition of the anti-Vmw65 antibody (LP-1) (McLean et al., 1982) to the reaction mixture stimulated complex formation (lane 9). This particular monoclonal antibody increased binding of ICP4 (possibly due to factors present in the ascites fluid); nonetheless, the level of binding is down considerably
compared to the wild type promoter. Anti-ICP4 monoclonal antibody did not increase specific binding to probe by ICP4 and in fact, no complex formation was observed (lanes 10, 11 and 12). From these data it was concluded that the two base pair deletion (-6A, -5T) substantially reduced the affinity of ICP4 for the A4 site in IE gene 3. (Muller, 1987, Faber and Wilcox, 1988; Deluca and Schaffer, 1988; Roberts et al., 1988; DiDonato and Muller, 12th Intl. Herpes workshop p293, 1987;). These data constitute rigorous biochemical evidence that I have modified a specific DNA/protein interaction by deleting the -6A and -5T from the IE3 capsit.

A Late HSV-1 gene also contains an ICP4 binding site.

A late gene, encoding the viral stimulatory protein 65Ktif, contains an ICP4 consensus site in its 5' untranslated leader region (Michael et al., 1988) [+154 relative to the 5' capsite (Campbell et al., 1984)]. This site was examined by mobility shift assay using a 5' end labeled SalI to AvaI (+194/+8) fragment of pMC1 (a plasmid that encodes the 65Ktif stimulatory protein [Preston et al., 1983] (lanes 16 through 20). Promoter fragments of IE3 (+62/-108) (lanes 1 through 5), gD (+27/-262) (lanes 6 through 10) and IE1 (+150/-125) (lanes 11 through 15) were
also included as positive controls to compare relative binding affinities of A4 complexes. Mock infected cell extract failed to form an A4 complex in all of the promoters tested (Figure 17, lanes 2, 7, 12, and 17). Multiple A4 complexes formed in the presence of infected cell extract in all the promoters tested (lanes 3, 8, 13, and 18). Complexes were effectively competed by the addition of non-radioactive A4 oligo (60 ng) to the binding reactions (lanes 4, 9, 14, and 19). Addition of the non-radioactive 72 bp Sph I/Sph I enhancer element of SV40 (60 ng) failed to compete for A4 complex formation (lanes 5, 10, 15, and 20). This experiment indicated that A4 complex formation occurred on all three gene classes of HSV-1. Results from other laboratories have also corroborated this result and additionally identify other late genes as targets for A4 complex formation (Michael et al., 1988; Tedder and Pizer, 1988a).

ICP4 binding sites in other immediate-early genes.

The sequence organization of a divergent promoter region of IE3 and IE4/5 is depicted (Figure 18). To investigate the facility of these two promoter regions to enter into A4 complexes, overlapping restriction fragments throughout the promoter regions were 5' end labeled and used
Figure 17. ICP4 binds to the immediate early, early and late classes of HSV genes. Probes indicated at the bottom of the figure were incubated in binding assays that included mock infected cell extract (14 μg) or infected cell extract (14 μg) and indicated above each lane. Included in the reactions as indicated above each lane was either no unlabeled competitor DNA, A4 oligo (60 ng) or SV40 72 bp enhancer sequence (60 ng). The amount of probe DNA was approximately 0.5 to 1.5 ng per reaction (20,000 cpm). Reactions were incubated at room temperature for 35 minutes and 1x mobility shift loading dye was then added. Samples were fractionated through a 4% mobility shift gel at room temperature at 20 mA constant current with buffer recirculation. Free probe lanes are in lanes 1, 6, 11 and 16. A4 complexes and free probe positions are marked. Lanes 1 through 5 contain the IE3 promoter (-141/+62), lanes 6 through 10, the gD promoter (-262/+27), lanes 11 through 15 the IE1 promoter (-125/+150) and lanes 16 through 20 contain the virion stimulatory factor promoter (+194/+8) as probes.
Figure 17
Figure 18. Sequence organization of promoters for IE genes 3 and 4/5. Relevant transcription factor binding site sequences are denoted. Black boxes labeled A are the position of the ICP4 binding site in IE3 and the suspected location of the site in IE 4/5. Boxes labeled TAATGARAT are sequences capable of binding Oct-1/65K_{tif} protein complex that transactivates expression of all IE genes (Goding and O'Hare, 1988). TATA denotes the position of the TATA box which binds the transcription factor TFIID. Numbers represent the position relative to the start sites of transcription for IE3 and IE4/5.
Figure 18
as probes in mobility shift assays. The IE3 region reported previously (Muller, 1987) was included here as a positive control to detect any additional A4 complex that may form when more purified preparations (DEAE fraction [see Chapter II, Figure 9]) were used as a source of ICP4. All IE3 promoter fragments include the A4 site at the IE3 caps; additional sites of DNA bound ICP4 would appear as a multifarious series of ICP4 containing complexes (similar to those in the gD promoter fragment which contains two ICP4 binding sites that are separated [see Figure 16]). All IE3 promoter fragments tested form the expected pattern of A4 complexes when incubated with partially purified ICP4 (Figure 19, lanes 2, 5, 8 and 11) but not with mock infected cell extract (lane 1). Addition of non-radioactive A4 oligo competed the A4 complexes (lanes 3, 6, 9 and 12) whereas, a heterologous oligo did not (lanes 4, 7, 10 and 13). Mock infected cell extracts failed to form an A4 complex with the IE4/5 promoter fragment (lane 14). Partially purified ICP4 drove A4 complex on two IE4/5 promoter fragments (-400/+108 and -51/+108) (lane 15 and 18); these complexes were competed by A4 oligo (30 ng) (lanes 16 and 19). ICP4 must therefore recognize a region of IE4/5 that is located between -51 to +108.
Figure 19. Localization of ICP4 binding sites in IE gene 3 and IE gene 4/5 promoters. DNA binding reactions containing either mock infected (2 μg) or partially purified ICP4 (2 μg) [DEAE pooled fractions] were preincubated on ice with A4 oligo (30 ng), a topoI cleavage site oligo (30 ng) or no unlabeled probe for 20 min before addition of the indicated labeled DNA probe (4 ng, 20,000 cpm) which covers the region of the IE3 and IE 4/5 promoter in an overlapping fashion. The binding reactions were then incubated on ice for an additional 45 minutes and 1X mobility shift loading dye was added. The sample remained on ice until loaded onto a 4% mobility shift gel at room temperature and fractionated at 20 mA with constant current and buffer recirculation. A4 complexes and free probe DNA positions are indicated to the left.
Figure 19
ICP4-DNA complexes form on sequences in the IE2 promoter and a novel IE-like gene.

The sequence arrangements for the immediate early gene 2 (IE2) and a new immediate early-like gene designated as IEx are depicted (Figure 20). IE2 contains a sequence that is closely related to the ICP4 consensus sequence (Faber and Wilcox, 1986) at -115 (Mackem and Roizman, 1982). Overlapping promoter fragments of IE2 spanning the region (-278 to +20) were prepared by restriction enzyme cleavage and end labeling. These fragments were tested for A4 complex formation by mobility shift assays after incubation with partially purified ICP4. The (-278/-182) probe formed an unique complex (Figure 21, lanes 1, 2, and 3) which failed to be competed by A4 oligo (5 ng) or a heterologous oligo (5 ng) (lanes 2 and 3) and therefore does not appear to be an ICP4 containing DNA/protein complex. A fragment containing the degenerate ICP4 binding site (-278/-84) formed a complex (lanes 4 and 6) which was competed by the A4 oligo (5 ng) (lane 5) but not by heterologous oligo (lane 6) suggesting this fragment contained an ICP4 binding site between (-182/-84). A probe containing sequences between -84/+20 formed an unique DNA/protein complex that migrated slower in the mobility shift gel (lane 7, 8, and 9) than the A4 complex (lane 10) and failed to be competed by either A4
Figure 20. Sequence organization of a novel immediate early-like gene divergent from IE gene 1. A. Sequence organization of the novel immediate early-like and IE-1 genes. The distances of potential transcription factor binding sites to the TATA boxes are indicated. The positions listed for the IE-1 promoter are relative to the transcriptional start site. B. Sequence organization of the IE gene 2 promoter. Positions listed are relative to the IE 2 transcriptional start site at the EagI restriction site. In both A and B the type of transcription factor binding site is indicated.
Figure 20
oligo (lane 8) or by a heterologous oligo (lane 9) indicating that this complex was not an A4 complex. The +47/-108 complex of IE3 included as a positive control (lane 10), was competed away by A4 oligo as expected (lane 11).

Analysis of sequences 5' to IE1 indicated the possibility of a divergent promoter element containing sequence elements reminiscent of an immediate early gene (i.e., TATA box, Sp1 sites, and a TAATGARAT element). This promoter mentioned previously, has been dubbed IEx. An ICP4 consensus sequence is located 3' to the proposed TATA box, and spaced almost identically as the IE3 A4 site/TATA (see Figure 18). The fragment (+35/-110) from IEx formed a very strong A4 complex with partially purified ICP4 (Figure 21, lane 12) which was competed by A4 oligo (lane 13) but not by the heterologous oligo (lane 14). This finding localizes a major ICP4 binding site in this novel promoter and has not been reported previously.

Footprinting of Complexes.

As noted above, multiple A4 complexes can be formed (for example see Figure 6, panel B); however, the basis for their formation is not clear. One possibility is that multiple A4 complexes are a result of interactions between
Figure 21. Identification and comparison of IE2 ICP4 binding site(s) with IE3 and IEx. Partially purified ICP4 (2 μg) [DEAE - Sephacel pooled fraction] was incubated with either no oligo, A4 oligo (5 ng) or topoI cleavage site oligo (5 ng) as indicated above each lane, on ice for 25 minutes in a standard binding reaction. DNA probes were then added to the binding reaction (IE2 promoter [-278/-182] lanes 1 through 3; IE2 promoter [-302/-84] lanes 4 through 6; IE2 promoter [-84/+38] lanes 7 through 9; IE3 promoter [-108/+47] lanes 10 and 11 and IEX promoter [-135/+35] lanes 12 through 14. Reactions were incubated 45 minutes further on ice. 1X mobility shift loading dye was then added to the reactions which were placed back onto ice until loaded onto a 4% mobility shift gel at room temperature. The samples were fractionated through the gel at 20mA constant current with buffer recirculation.
A4 OLIGO (5 ng) + + + + + + + +
TOPO I OLIGO (5 ng) + + + + + + + +
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Complexes

Free DNA

IE2 Promoter
Bam HI/Sma I -278/-182

IE2 Promoter
Hind III/Apa I -302/-84

IE2 Promoter
-84/+38 Apa I/Hind III

IE2 Probes

Figure 21
ICP4 (or ICP4 in combination with another protein) and uncharacterized binding sites in the target fragment. To evaluate this possibility, footprinting experiments were carried out using copper-1, 10, phenanthroline complex (Cu-OP) (Sigman, 1987). Cu-OP footprinting, which is similar to the iron-EDTA method (Tullius and Dombroski, 1986), gives high resolution and is ideal for footprinting studies on small DNA fragments which may be crowded with >1 protein. A fragment was prepared which contained the IE3 promoter sequences +27 to -108, it was incubated with an infected cell extract and fractionated on a preparative mobility shift gel. The low ionic strength gel was immersed in the Cu-OP solution to allow cleavages to proceed on the native complexes and DNA's in the gel matrix. The DNA's were then recovered by electroelution.

Free and bound DNAs (from A4 complexes) were analyzed on a sequencing gel which revealed a pattern of protection of the DNA in the 2° A4 complex (Figure 22, lane 3) that was very similar to the pattern observed in the 1° A4 complex (lane 5). Both protection patterns were different than the cleavage pattern of the free probe (lane 4). Differences in protection patterns in 1° and 2° complexes were not striking. Because of the intrinsic differences in binding equilibria (see Figure 6, panel B), partial protection was observed in the 2° complex (bracketed sequence, Figure 22,
Figure 22. Copper-Phenanthraline footprinting the IE3 A4 site (the non-coding strand). The specified probes were incubated with 20 μg of infected cell nuclear extract under standard binding conditions, loaded onto a preparative low ionic strength gel and fractionated at room temperature. The gel was treated with Cu-OP as described in Materials and Methods. The recovered DNA was analyzed on a 12% sequencing gel. Lanes 1, 2, 6, 7, and 11 contain designated sequence ladders. In lanes 1 through 7, the probe was +62/-108 (AvaII/EcoRI, labeled at +62) of IE3 from pWT-GH; lanes 8 to 11, the probe was -18 to +62 (AvaI to AvaII, labeled at +62) of IE3 from pWT-GH. The 1° and 2° A4 complexes of the coding strand (+62 to -108) are in lanes 5 and 3, respectively, and are labeled "1°B" and 2°B". Lane 9 contains DNA from a 1° complex derived from the non-coding strand (-18/+27). Lanes labeled "F" contained free probe. The nucleotide sequences are shown beside each gel and the brackets correspond to protected regions (transcriptional start site is noted as +1).
lane 3), whereas the 1° (lane 5) complex was fully protected. The data suggest that altered mobility of the 1° and 2° complexes was caused by variations in protein/protein interactions rather than protein-DNA interactions. In other words, ICP4 is interacting with its target sequence, and other proteins are interacting with the ICP4 bound to its binding sequence. The other interactive proteins could be ICP4 itself, possibly post translationally modified (Periera et al., 1977; Wilcox et al., 1980; Preston and Notarianni, 1983), or other viral or cellular proteins.

A peculiarity of the Cu-OP footprinting technique using ICP4 occurs when analyzing complexes in which the A4 site is near the 5' end of a fragment. In these experiments, I observed that smaller cleavage fragments (near the 5' ends) appear to be under-represented relative to free DNA fragments of equal size. This is particularly noticeable when a protein binding site is within 30-40 bp of the 5' end. As a result, the footprint appears to extend further in the 5' direction compared to experiments using different probes which place the site in the middle of the fragment. For example, in Figure 22, lane 9 the A4 site is within about 20 bp of the end and Cu-OP cleavage protection appears to extend into the 5' sequences; however, when the A4 site is about 60 bp from the end (Figure 23, lane 5) the 5' protection is not as pronounced (an additional example can
Figure 23. Copper - Phenanthroline footprinting the IE3 proximal promoter (the coding strand). A probe from pWT-GH (EcoRI/AvaII, -108/+62, labeled at -108) was incubated with 20 μg of infected extract or with 150 ng of affinity purified ICP4 under standard binding conditions and analyzed on a preparative low ionic strength gel. After electrophoresis, the gel was treated with Cu-OP and the A4 complexed DNA and free DNA was recovered. The DNA samples were analyzed on a 12% sequencing gel. Two staggered loadings of the gel were performed as marked above the gels. All lanes are marked as follows: G+A and G are sequencing ladders; F corresponds to free DNA, 2°B and 1°B are 2° and 1° complexes, respectively (crude vs. affinity purified ICP4 is as marked). In the second loading, only the results of incubation of probe with crude extract is seen (lanes 7 and 8). Protected A4 and hypersensitive TATA sites are marked with brackets.
Figure 23
be seen in Figure 24). It seems likely that native ICP4 interacts in some unknown way with DNA ends; therefore to avoid this complication, the results were confirmed using different probes containing A4 placed more centrally but still allowing resolution of the TATA sequence (data not shown).

One striking feature of the Cu-OP footprints is the cleavage hypersensitivity at the TATA box (Figure 22). The TATA box was hypersensitive to Cu-OP cleavage in free probe; however, the hypersensitivity was much more pronounced in both 1° and 2° complexes (compare lane 4 to lanes 3 and 5). Hypersensitivity was not unique to one particular shifted complex (compare lane 3 to lane 5). To examine the hypersensitivity more thoroughly, I repeated the experiment shown in Figure 22, (lanes 8 through 10) using a different probe on the same strand that extends into the TATA box (-108 to +27 of IE3). The TATA element hypersensitivity can be clearly seen (Figure 23, lanes 4, 5, 6, 7, 8). Hypersensitivity was observed with both crude nuclear extracts of infected cells and with affinity purified ICP4. To evaluate the generality of the hypersensitivity result, I repeated the analysis on the promoter of another gene, IE1 (ICP0). This promoter contains a consensus ICP4 binding site centered in the -61 region (Kristie and Roizman, 1986b; Kattar-Cooley and Wilcox, 1989; DiDonato and Muller, 1989).
I have confirmed this result by mobility shifts with the anti-ICP4 antibody (Figure 16, lanes 18-20) and Cu-OP footprinting (Figure 24). DNA in the A4 complex was hypersensitive to cleavage at the TATA box (Figure 24, lanes 4 and 5) showing that the phenomenon is not a peculiarity of the IE3 promoter/transcriptional start site. Hypersensitivity was less pronounced in IE1 compared to IE3, possibly because of a greater separation between the TATA element and ATCGTC in IE1.

DMS interference patterns between crude extracts and A4 DNA affinity purified ICP4.

To define the guanosine (G) residues that were critical for formation of the 1° and 2° A4 complexes, base alkylation was performed using dimethylsulfate (Muller, 1987; Siebenlist and Gilbert, 1980). Additionally, DNA target sites could be compared with purified ICP4 and crude extracts to examine whether ancillary proteins (in crude extracts) influence the DNA binding domain of ICP4. The probe used in the DMS interference analysis was from +27 to -18 of IE3. ICP4-DNA complexes and free DNA were separated on a preparative low-ionic strength polyacrylamide gel, the DNA was recovered and cleaved with piperidine (Maxam and Gilbert, 1980) followed by electrophoresis on a 12%
Figure 24. Cu-OP footprinting of the IE1 promoter. A probe derived from SmaI/AvaI fragment of IE1 (-129 to +51, labeled at -129) was incubated and processed under the same conditions as specified in the legend to Figure 23. DNA samples recovered from the Cu-OP treated preparative gel were analyzed on a 12% sequencing gel. Lanes 1 and 2 show the sequence ladders, lane 3 contained free DNA and 1' A4 complexes (as duplicates) were analyzed in lanes 4 and 5.
Figure 24
Figure 25. DMS interference of 1° and 2° A4 complexes. The methylated probe, Aval/BamHI of IE3 (-18 to +27, labeled at -18) was incubated with 20 µg of infected extract or with 150 ng of affinity purified ICP4 under standard binding conditions, followed by a preparative separation on a low ionic strength gel. DNA was recovered, repurified and cleaved with piperidine (Maxam and Gilbert, 1980) followed by analysis on a 14% sequencing gel. The G and G+A sequence ladders are marked. Lanes labeled B contain DNA from A4 complexes and F corresponds to free DNA. Three sets of reaction products were analyzed: lanes 3 through 6, with affinity purified ICP4 (lane 5 was from a 1° complex); lanes 9 through 12, with crude infected cell extract (lane 10 was from a 2° complex); lanes 15 and 16, with crude infected extract (lane 16 was from a 1° complex). Critical G residues are marked with an asterisk above the DNA sequence at the top.
sequencing gel. The G residues important in A4 complex formation appeared as lighter bands in the bound DNA relative to free DNA (marked by an asterisk in the DNA sequence above Figure 25). The results agree with the copper footprinting data (Figure 22). The G residues mapped in the 1° (lane 16) and 2° complex (lane 10) were essentially indistinguishable. Additionally, the G cleavage pattern seen in all of the bound lanes (be it those from incubated with crude cell extracts, lanes 10 and 16, or with purified ICP4, lane 5), were qualitatively the same. These results suggest the following. First, the footprints for 1° and 2° complexes were essentially identical; therefore these two must differ in some aspect involving only protein. Second, because affinity purified ICP4 formed multiple A4 complex bands, it is conceivable that multimeric forms of ICP4 were responsible; however I have not proven this. Third, affinity purified ICP4 generated an identical G cleavage pattern compared to the 1° and 2° A4 complex band shifts with crude extracts which is also consistent with the idea that ICP4 was self-associating in different ways.
Figure 26. Comparison of different A4 site containing promoters to cleavage at TATA. The 5' end labeled fragments prepared from pJgD (-136 to +41, labeled at -136) and pJD45 (+64 to -200, labeled at +64) were incubated with infected cell extract and subject to mobility shift analysis and CU-OP footprinting as described in Fig. 2. and in "Methods". Lanes 1, 2, and 4 contain the free DNA from the gD promoter; lane 3 contains the A4 complexed DNA. The G+A sequence ladder reference is in lane 5. Sequence positions in relation to the 5' transcriptional start site of gD are indicated to the right of lane 5. The A4 protected region as well as TATA are indicated with brackets. The free DNA of the insertion mutant of the IE3 promoter (pJD45) is in lanes 6 and 8. The A4 complexed DNA is in lane 7. The G+A and G sequence ladders are as marked. The A4 protected region, TATA and positions relative to the transcriptional start site of IE3 are marked to the right of lane 10.
<table>
<thead>
<tr>
<th>Gly D</th>
<th>IE3 WT +22 bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  2  3  4  5</td>
<td>6  7  8  9  10</td>
</tr>
<tr>
<td>F  F  B  F  G/A</td>
<td>F  B  F  G/A  G</td>
</tr>
</tbody>
</table>

Figure 26
To examine the effect of A4/TATA separation distance on the hypersensitivity at the TATA box, Cu-OP footprints were compared on probes containing the A4 site at a more distal location. In pJD45, the A4/TATA separation distance in IE3 was increased from 26 to 48 bp. The Cu-OP footprint on the mobility shifted A4 complex is shown (Figure 26, lane 7); the TATA region did not display cleavage hypersensitivity seen previously with wild type IE3 (Figure 22, compare lanes 4 and 5 with Figure 26, lane 7). The bracketed region (A4) corresponds to the ICP4 binding site. This site is located within 40 bp of the 5' end (fragment was labeled at +64) and as noted above, the protection appears to extend somewhat further 5'. TATA hypersensitivity was also examined in a native gD promoter which is trans-activated by ICP4 (see Everett, 1984 for review) and contains an A4 site separated from the TATA region by ca. 80 bp. Again, TATA box hypersensitivity was not detected in the Cu-OP footprints of A4 complexes derived from the gD promoter (Figure 26, lanes 1 through 4).

Examination of the sequence arrangement in the promoter for IEEx and the IE3 revealed the spacing between the A4 site and the TATA element in each promoter differs by only 2 bp.
Figure 27. Copper phenanthroline footprinting (Cu-OP) the A4 site in the IEx promoter. 5' end labeled fragments (10^6 cpm) prepared from p111 (-110 to +35 of IEx and -135 to +35 of IEx labeled at +35) were incubated with partially purified ICP4 (5 μg, 10 μg of poly dA·dT in a 50 μl binding reaction on ice 45 min) and subject to mobility shift assay at room temperature. Cu-OP footprinting was performed as described in the legend to Figure 22 and Materials and Methods. Lane 1, A4 complexed DNA from the IEx -110 to +35 fragment, lane 2, -110 to +35 IEx fragment (free probe). Lanes 3 and 4, the G+A and G sequence ladder reference for the -110 to +35 IEx fragment. Lanes 5, 6, 12 and 13 contain the G+A and G sequence for the -135 to +35 IEx fragment. Lanes 7, 9 and 11 contain free probe DNA (-135/+35 of IEx). Lanes 10 and 11 had more radioactivity loaded than did lanes 7, 8 and 9. Positions of TATA and the protected A4 site are marked with brackets. The contents of each lane is shown. Lanes designted B are A4 complexed DNA lanes, lanes marked with F are free DNA lanes and lanes marked with G or G+A are the corresponding sequence reference. Positions of bases relative to the IEx transcriptional start site are indicated to the left.
Figure 27
To investigate the effect ICP4 binding had on the conformation of the helix in the IEx TATA box, a Cu-OP footprinting experiment was performed as detailed above. Two overlapping IEx promoter fragments labeled at +35 and extending to -110 or to -140 were incubated with partially purified ICP4, fractionated on a mobility shift gel and the DNA cleaved by the Cu-OP reagent in situ. Free and protein bound DNA was isolated by from the gel slices by electroelution and ethanol precipitated. Purified DNA fragments were then resuspended in sequencing dye and fractionated on a 12% sequencing gel opposite sequencing ladders. The -110/+35 probe was protected by ICP4 over the ICP4 consensus site (Figure 27, lane 1). The longer probe (-140/+35) also exhibited protection by ICP4 over the consensus ICP4 binding site (lanes 8 and 10) as compared to the free probe (lanes 7, 9 and 11). This result indicated that there are no additional ICP4 binding sites between -110/-140 of IEx in addition to the binding site at the ICP4 consensus site. Note, that in the ICP4 bound lanes, the TATA region is much more hyper-reactive to cleavage than the TATA region in the free probe lanes (compare lanes 1, 8 and 10 to lanes 2, 7, 9 and 11). The hyper-reactivity of the TATA to cleavage by the synthetic nuclease activity of Cu-OP is indicative of a perturbation in the minor groove geometry of the DNA helix in the TATA region. This result is
identical to that seen in the IE3 promoter (Figure 22, lanes 3 and 5; Figure 23, lanes 4 through 8) and IE1 promoter (Figure 24, lanes 4 and 5).

**DNA sequence contributions to DNA/ICP4 complex formation.**

Previously, DMS alkylation interference was used to determine guanine residues important for A4 complex formation in the IE3 promoter (Muller, 1987; DiDonato and Muller, 1989; this study, Figure 25). This technique, although insightful, limits the analysis of protein/DNA interactions to only a single type of base (guanine). A more thorough analysis using a more recent technique, which tests the contributions of each base to the binding affinity, was therefore carried out. This technique, known as missing contact probing (Brunelle and Schleif, 1987), was applied to the IE3, IE1 and IEx promoters to determine the bases involved in ICP4/DNA complex formation. Briefly, 5' end labeled probe containing a single ICP4 binding site was sparingly depurinated with piperidine-formate or depyrimidinylated by hydrazine (see Materials and Methods). The G + A and C + T modified probe DNA was then incubated with partially purified ICP4 (5 μg of the DEAE-Sephacel fraction) in a standard preparative binding reaction (see Figure legends for Experimental details) and fractionated on
a 4% polyacrylamide mobility shift gel. Complexed and free DNA was located by autoradiography and the corresponding gel slices containing the DNA's were sandwiched against DEAE coated nylon-backed nitrocellulose (NA45 paper [Schliecher and Schuell]) and transferred onto the NA45 paper (see Materials and Methods). The DNA's were eluted from the NA45 paper, ethanol precipitated, dried, resuspended in 1 M piperidine and cleaved at 90°C for 25 minutes (Maxam and Gilbert, 1980). The samples were then desiccated to dryness and resuspended in sequencing sample dye. Equal amounts of radioactivity from the complexed DNA and the free DNA were fractionated opposite each other along with G and G+A sequencing ladders on a 12.5% sequencing gel.

The missing contact analysis for both the coding (Figure 28, lanes 1 through 8) and non-coding strand (lanes 9 through 17) of IE3 (-18/+27) are shown. Bases whose removal decreased binding affinity appeared as bands that were diminished or absent in the bound lanes compared to the corresponding band in the free DNA lane. Bases that upon removal increase the binding affinity of ICP4 to the site appeared as hyperreactive (increased intensity) bands in the bound lanes compared to the same bands in the free DNA lane. Lanes 1 through 3 contain the G + A modified DNA and lanes 6 through 8 contain the C + T modified IE3 DNA of the coding strand. Lanes 10 through 12 contain G + A
Figure 28. IE3 missing contact analysis. Fragments prepared from pBA2 (DiDonato and Muller 1989) endlabeled at the XbaI site and secondarily cleaved at the Sal I site (coding strand) along with endlabeled XhoI site and secondarily cleaved SstI (non-coding strand) which contain the IE3 promoter (-18/+27) were used to make probes which lacked both guanine and adenosine or cytosine and thymidine. Probe modification protocol was identical without any variance from that of Brunelle and Schleif 1987. 1.0 X 10^7 cpm of each probe was used in each base elimination reaction. Approximately 80% of the original amount of radioactivity was recovered after base elimination for use in experiments. Binding reactions consisted of 0.7-1.2 X 10^6 cpm/reaction incubated with 5 ug of partially purified ICP4 (DEAE-Sephacel fraction). Poly dA·dT was present in each binding reaction (4-5 ug) in 50 /u l and incubated on ice for 45 min. 1 X mobility shift loading dye was added and the reactions were fractionated on a preparative 4% mobility shift gel at 40 mA constant current with buffer recirculation at room temperature. Complexed and free DNA was visualized by autoradiography of the wet gel at 4° C for 3 hours. Areas of interest were then cut from the gel and transferred to NA45 paper (Schlieler and Schuell, New Hampshire) in 0.25 X TBE at 95V for 1.5 hr. at 4° C in a Biorad Transblot unit. The strips of NA45 paper (wet) were subject to autoradiography and all bands localized and then cut from the paper. Radioactivity was eluted from the NA45 by incubation in (2MNET) at 68° C for 2 hr. Samples were Phenol/chloroform extracted, ethanol precipitated, washed with 70% ethanol and dried. Samples were washed once with 25 uL distilled H2O and evaporated to dryness. Samples were then resuspended in 100 uL of 1 M piperidine, heated to 90° C for 25 min, vacuum desicated to dryness, washed with 25 uL distilled H2O and then evaporated to dryness. Samples were resuspended in sequencing loading dye and equal amounts of radioactivity were fractionated on a 12.5% sequencing gel. The gel was soaked in 10% methanol/12% acetic acid for 2 hrs, dried and subjected to autoradiography. See below, G+A base elimination probes are in lanes 1 through 3 and lanes 10 through 12. C+T base elimination probes are in lanes 6 through 8 and lanes 13 through 15. Free DNA is in lanes marked F, A4 complexed DNA is in lanes marked B. Lanes marked with G and G+A are sequence ladder reference for the indicated probe. Positions of bases relative to the IE3 transcriptional start site are indicated to the sides of each autoradiograph, bracketed areas denote the regions protected from digestion by Cu-OP footprinting (DiDonato and Muller, 1989). The actual nucleotide sequence of the coding strand is listed below the Figure and is the top sequence (reading 5' to 3', left to right). The lower sequence is the nucleotide sequence of the non-coding strand reading 5' to 3' right to left). Bases that effect binding affinity
are indicated by the symbols above the nucleotide sequence. Strongly negative and negative denote bases whose removal decreases binding affinity of ICP4 to this sequence. Strongly positive and positive denote bases whose removal increase the binding affinity of ICP4 to this sequence.
Figure 28
modified DNA and lanes 13 through 15 contain C + T modified DNA of the non-coding strand of IE3. The results of this experiment were quantified by radiographic imaging on an AMBIS radiographic image detector (see Materials and Methods) and analyzed by visual inspection of the autoradiogram.

The results of the visual inspection analysis are summarized as a DNA sequence cartoon (at the bottom of Figure 28) which indicates bases which either decrease or increase the binding affinity of ICP4 for the A4 site at the IE3 caps. As expected, the ATCGTC core consensus sequence was extremely important in formation of the A4 complex (note the diminished band intensities of this region in the protein bound lanes 2, 7, 11 and 14). The radiographic tracings of bound and free DNA lanes can be seen (Figure 29 and Figure 30) and are labeled. These tracings are the raw radiographic images of the AMBIS scan (Figure 28). The values obtained from the AMBIS scan were normalized to a specified band intensity of a residue that was located away from the A4 site and was unaffected in its reactivity to ICP4. The relative band intensity of each band in the free DNA and bound DNA lanes was then determined. The difference in normalized values between bound and free DNA for each base residue was determined and the fold difference between these values was plotted as a
Figure 29. IE3 coding strand missing contact analysis: Radioimaging scan of the cleavage intensities. The dried gel of Figure 27 was placed onto an Ambis radioimaging detector. The raw radioactivity of each band can be quantitatively measured as a peak on this scan. The area under each peak is commensurate with the amount of radioactivity of each peak. The right side of each scan is the 5' end of the fragment whose sequence is listed in the center of the Figure. The left end of the scan corresponds to the top of Figure 28, lanes 2, 3, 7 and 8. The right end of the scan corresponds to the bottom of Figure 28, lanes 2, 3, 7 and 8. Arrows denote bases to peak position. Free and Bound refer to radiographic scans of protein band lanes and free refers to free DNA lanes. Scans of the G+A base eliminated probe is seen in the top half of the Figure while the scans of the C+T base eliminated probe can be seen in the lower half of the Figure.
Figure 29
Figure 30. IE3 missing contact analysis of the non coding strand: Radiographic scan of cleavage intensity. The dried gel of Figure 27 was placed onto an Ambis radioimaging detector. The raw radioactivity of each band can be visualized as a peak on this scan. The area under each peak is commensurate with the amount of radioactivity of each peak. The right side of each scan is the 5' end of the fragment whose sequence is listed in the center of the Figure. The left end of the scan corresponds to the top of Figure 28, lanes 11, 12 and 14. The right end of the scan corresponds to the bottom of Figure 28, lanes 11, 12, 14 and 8. Arrows denote bases to peak position. Free and Bound refer to radiographic scans of protein band lanes and free refers to free DNA lanes. Scans of the G+A base eliminated probe is seen in the top half of the Figure while the scans of the C+T base eliminated probe can be seen in the lower half of the Figure.
Figure 30
Figure 31. Normalized cleavage intensities for the missing contact base analysis of the IE3 promoter. Radioactivity under each peak observed in Figures 28 and 29 was calculated and background radioactivity subtracted using Ambis software. The radioactivity of each band was normalized to a common single band present in each lane analyzed. This base was located outside of the region affected by ICP4 binding. The normalized values of each band in the free DNA lane was compared to the normalized values of the same band in the protein bound lane. The fold difference was then calculated for each band. If the values did not vary by ±1.1 fold the base was considered not to effect binding of ICP4 to the sequence. Bases whose removal has a negative effect on binding affinity greater than 1.1 fold is depicted as a black box above or below the base. Bases whose removal has a positive effect on binding affinity greater than 1.1 fold is depicted as a paper shaded box. The height of each box is representative of the fold difference the base has from a normalized value of 1.0.
Figure 31
histogram (Figure 31). Only values differing by an absolute value greater than 1.1 fold were considered significant. A plot of the data in such a manner allows an accurate and quantifiable assessment of the contribution to binding affinity each base makes. Bases that decrease binding affinity when removed are represented by filled bars, bases that increase binding affinity are represented by empty bars.

It is clear that the sequences immediately 3' to ATCGTC contribute quite significantly to the strength of A4 complex formation. This is surprising as the Faber and Wilcox consensus sequence places four N bases at these positions (ATCGTCNNNNNYCGRC). From the appearance of the plotted data (Figure 31) it appears that bases critical for A4 complex formation have a pseudo-symmetrical appearance; whereas, the actual nucleotide sequences themselves do not contain a dyad axis of symmetry.

Missing contact analysis was also performed on the IE1 promoter (-129/+51) (Figure 32) and the IEx promoter (-534/+35) (Figure 33). The same procedures were followed for these two probes exactly as described for the IE3 probes. Analysis of the IE1 missing contact experiment unexpectedly allowed the investigation of a 2° A4 complex in addition to the 1° complex. Missing contact analysis of 1° A4 complexes on the IE1 promoter can be seen (Figure 32). Bases which
Figure 32. IE-1 missing contact analysis. Endabeled fragment prepared from pJD100 (−129/+51) was used to make G+A and C+T base removal modified probes using conditions identical to those outlined in Figure 27. These modified probes were used in binding reactions under identical conditions to those listed in Figure 27. 1° and 2° A4 complexed modified DNA was recovered from the mobility shift gel and analyzed. Isolation and processing of bound and free DNA was also the same as in Figure 27. Piperidine cleaved DNA was then fractionated on a 10% sequencing gel. Lanes 3 through 5 and 6 through 8 contain G+A and C+T modified DNA respectively, DNA isolated from 1° A4 complexed DNA and free DNA is indicated above each lane. Free DNA that was G +A or C+T modified is in lanes 9 and 12 respectively. 1° A4 complexed DNA that was G+A modified or C+T modified is in lanes 10 and 13. 2° A4 complexed DNA that was G+A or C+T modified is in lanes 11 and 14 respectively. Lanes 1, 2, 15 and 16 contain the G and G+A sequencing ladder reference. Positions relative to the transcriptional start site are listed to the left as is the position of TATA and the A4 site protected in Cu-OP footprinting experiments (Figure 23). The nucleotide sequence of the IE-1 coding strand is listed below the Figure. Bases whose removal effects binding affinity is marked using symbols. The symbol key is located to the left of the figure and is the same as that seen in Figure 27. Symbol determinations were made by visual inspection of a variety of different exposures of the autoradiograph.
enhance binding affinity (open circle) or decrease binding affinity (filled circle) are located above the IE1 DNA sequence and were identified by visual inspection of the gel and comparison of the bound DNA lanes versus free DNA lanes.

G + A modified DNA of the bound and free probe is in lanes 3 through 5 and 9 through 11. C + T modified complexed and free DNA is in lanes 6 through 8 and 12 through 14. Due to the high percentage of G and A residues 3' to position -50, identification of bases that increase bind affinity when removed can not be identified due to lack of resolution in this area. Comparison of the G + A and C + T modified DNA of the 1° complex (lanes 10 and 13) and those of the 2° complex (lanes 11 and 14) exhibit no differences in bands that are increased or decreased in band intensities. This result implies as did those of the DMS interference assay of a 2° complex (Figure 25, lane 11) that no additional bases are involved in 2° complex formation compared to those required for 1° A4 complex formation. Multiple A4 complexes most likely arise via protein/protein interactions.

Like both the IE3 and IE1 promoters, the IEx promoter exhibits a decrease in binding affinity when bases within the ICP4 consensus sequence were removed (Figure 33, lanes 4 and 5). The missing contact analysis for the non-coding strand of the IEx promoter is diagramed below the
Figure 33. IEx missing contact analysis. G+A and C+T modified DNA was prepared from p111 (-511/+35*), in an identical manner to that listed in Figure 27. Binding reactions, mobility shift gel analysis, DNA recovery, piperidine cleavage and fractionation on a 13.5% sequencing gel was also identical to that described in Figure 27. Lanes 3 and 4 contain free and A4 complexed G+A modified DNA respectively. Lanes 5 and 6 contain the bound and free C+T modified DNA as indicated. Lanes 1, 2, 7 and 8 contain the G and G+A sequencing ladder references. The nucleotide sequence of the non coding strand of IEx is listed below the Figure. Bases affecting binding affinity of ICP4 to the site are shown.
Figure 33

= Strongly Negative
* = Negative

5'GCACCAAGCCGCTCTCCGGAGAGACGATGGCAGGAGCGCAT

Figure 33
autoradiogram (Figure 33), the filled circles over the DNA sequence correspond to bases that when removed decrease ICP4 binding affinity (as seen in lanes 4 and 5). Bases, which increase binding affinity by their removal, are marked with empty circles over the corresponding base in the DNA sequence and correspond to bases visualized in the bound DNA lanes (lanes 4 and 5). Dissimilar to IE3 and IE1 is the apparent lack of sequences 3' to the consensus sequence, which when removed, increase binding affinity (note the lack of hyper-reactive bands in lanes 4 and 5). Also, unlike IE3 and IE1 is the increased number of bases 3' to the consensus sequence, which when removed, decrease ICP4 binding affinity to this DNA (designated with filled arrows and asterisks over the DNA sequence). These dissimilarities, to the IE3 and IE1 results, may be a reflection of the IEx A4 site being a more "ideal" A4 site. (Note the increased propensity of the IEx probe to enter into A4 complexes in mobility shift assays as compared to IE1 [Figure 42, compare lanes 11 and 19 with lane 1]).

ICP4 complex formation results in DNA bending.

Important biological reactions such as replication, recombination and DNA packaging involve the winding of DNA around one or more protein components. This sort of
wrapping of the DNA around the protein, for example in the nucleosome (see Morse and Simpson, 1988 for a review), results in a continuous curvature of the DNA axis. Another type of alteration in the curvature of the DNA can be abrupt and result from either a characteristic of the DNA sequence (poly A tracts) called a static bend (Wu and Crothers, 1984) or by the binding of a sequence specific DNA binding protein. Bending induced by protein binding is known as a dynamic bend (Wu and Crothers, 1984; Shuey and Parker, 1986; Zweib et al., 1989). A characteristic of bent DNA, as it moves through a native agarose or native polyacrylamide gel, is that the migration of the bent DNA fragment becomes less as the bend in the DNA approaches the center of the fragment (Wu and Crothers, 1984).

A method routinely used to analyze the ability of a DNA sequence to bend, localizes the fragment in question between two direct repeats of DNA which contain an array of unique restriction sites. Cleavage of the recombinant plasmid with various restriction enzymes generates a battery of circularly permutated fragments of equal length (Shuey and Parker, 1986; Zweib et al., 1989). The circular permutation places the DNA sequence in question at varying positions from the ends of the fragment (Wu and Crothers, 1984; Shuey and Parker, 1986; Zweib et al., 1989). To detect protein-induced bending, circularly permutated end labeled DNA
fragments are separately incubated with a sequence specific DNA binding protein and fractionated on a mobility shift gel. The migration distance of the complexed and free DNA is then recorded. If binding of the protein to DNA induces a bend, the complexes that arise from the different permutations will migrate differently through the gel. The free DNA should migrate uniformly unless the fragment contains sequence elements that have intrinsic tendencies to bend (Wu and Crothers, 1984). The bend center of the fragment can be determined by plotting the migration distance of the complexes formed on the various fragments vs the location of the binding site from the ends of the fragment (Wu and Crothers, 1984; Shuey and Parker, 1986; Zweib et al., 1989).

To test if ICP4 binding to the IE3 promoter [the (-18/+27) or (-108/+27) constructions] induced DNA bending, the fragments were made blunt ended by filling in the 5' overhangs with the Klenow fragment and cloned into a filled Xba I site in the plasmid pBend2 (a specially designed plasmid used to test for bending). This plasmid contains two large direct repeats with multiple cloning sites separated by the unique Xba I and Sal I restriction sites (Zweib et al., 1989). The (-18/+27) construct was designated pBA2bend and the orientation of the insert is opposite that of p-108bend (see below). The (-108/+27)
construction was designated as p-108bend and the insert is oriented such that the +27 end of the insert is closest to the unique Eco RI restriction site in the pBend2 polylinker.

Five different restriction enzymes were used to cleave pBend2, pBA2bend and p-108bend. These fragments were 5' end labeled and isolated as described previously for use in mobility shift assays. Incubation of partially purified ICP4 (DEAE-Sephacel fraction [4 μg]) with probe fragments derived from pBend2 did not result in the formation of A4 complexes and served as a negative control and indicated a lack of any intrinsic ICP4 binding sites in the vector sequences present in the probe fragments (data not shown). The five different probes prepared from pBA2bend and p-108bend were incubated separately with partially purified ICP4 and fractionated on a mobility shift gel. Both constructions gave rise to probe fragments whose A4 complexes migrated with slightly different mobilities (Figure 34, panel B, lanes 1 - 5 and lanes 6 - 10). Also note the uniformity of migration of the free DNA in the same lanes which rules out the possibility of intrinsic bending due to sequence composition.

The mobilities of the DNA/ICP4 complex arising from the -108 bend fragments exhibit a decrease in migration as the ICP4 binding site was located nearer the center of the fragment (Figure 34, panel B, compare lanes 1 -5 and the
Figure 34. DNA bending by ICP4 binding to DNA. The plasmids pBA2bend and p-108bend were restricted with the restriction enzymes listed above each lane. These fragments were then incubated with partially purified ICP4 and fractionated on a mobility shift gel. The distance the bound and free DNA's migrated were measured and an $R_f$ was calculated. Panel A is the schematic of the insert's location relative to the ends of the fragment. Panel B. is a photograph of the autoradiogram of the mobility shift gel. Lane 1 is Rsa I, lane 2 is Sma I, Lane 3 is Eco RV, lane 4 is Xho I, and lane 5 is Bgl II cut p-108bend DNA fragments. Lane 6 is Bam HI, lane 7 is Rsa I, lane 8 is Sma I, lane 9 is Xho I and lane 10 is Bgl II cut pBA2bend DNA fragments. The complexed DNA is located high in the gel and the free DNA is at the bottom of the gel.
Figure 35. Mobility decreases as the ICP4/DNA complex moves toward the bend center. The relative distance of migration for the p-108bend and the pBA2bend fragments were plotted versus the distance from the ends the +1 site of IE3. The +1 site of IE3 is located within the A4 binding site of IE3. Panel A is the plot for pBA2bend and Panel B is the plot for p-108bend.
Figure 35

A. pBA2bend

B. p-108bend

R_t of A4 complex

bp from end of fragment

bp from end of fragment
schematic [panel A]). The restriction sites used to generate each fragment are listed in the Figure legend and a graphic representing this is depicted alongside (panel A). Fragments generated by pBA2bend also exhibited variation in electrophoretic mobility (lanes 6 - 10). The distribution of the migration pattern was inverted bell shaped, the slowest migrating complex arising from the Sma I cut probe (lane 8) which positions the ICP4 binding site approximately in the center of the probe fragment (see Figure 34, panel A).

Plotting the relative migration distance of the 1° complex versus the position of the IE3 (+1) transcriptional start site, which is located within the ICP4 binding site, the bend angle for pBA2bend was found to be located at or very near the ICP4 binding site (Figure 35, panel A). This observation is consistent with the idea that as the site for DNA bending was moved nearer the middle of the fragment, the greater the retardation in migration of the fragment through the gel (Wu and Crothers, 1984). The relative migration for the -108bend probe A4 complexes were plotted vs the distance from the end of the probe to the +1 transcriptional start site of IE3 (Figure 35, panel B). The bend angle of p-108bend fragments were also located at or near the ICP4 binding site judging by the decreased migration of the
complexes as the ICP4 binding site was located closer to the center of the probe fragment.
DISCUSSION

Previously, this laboratory reported that ICP4 specifically binds to a DNA sequence straddling its own transcriptional start site (Muller, 1987). Although this complex was detected in vitro, it was proposed that it might have some role in negatively regulating expression of IE3 during an HSV infective cycle (Muller, 1987). In this chapter, I have performed biochemical analyses on the complex in vitro and determined the base residues within the binding site that contribute to the binding affinity of ICP4. The identification and localization of ICP4 binding sites in all HSV IE genes is described as was their stereospecific relationship to TATA.

ICP4 forms multiple complexes.

An electrophoretically heterogeneous set of ICP4/DNA complexes were detected displaying different mobilities and intrinsically different binding affinities. I refer to these as primary (1°), secondary (2°) and tertiary complexes (3°). The following observations confirm these
complexes contained ICP4: i) they were only observed with probes containing ICP4 consensus recognition sequences (Faber and Wilcox, 1986; Kristie and Roizman, 1986b; Muller, 1987) or with sequences related to the ICP4 consensus sequence (see below: ICP4 nonconsensus sequence site binding); ii) they were competed with unlabeled probes containing the ICP4 consensus sequence (Muller, 1987) but not unrelated sequences; iii) they reacted with anti-ICP4 monoclonal antibody; iv) in crude cellular extracts their formation was dependent upon addition of infected cell extract and independent of uninfected cell proteins; v) they were detected with affinity purified ICP4.

Differences in the stoichiometry of protein (protein:DNA ratio) are known to produce complexes with altered mobility (Fried and Crothers, 1981). Probes containing a single ICP4 site generated multiple band shifts as did probes containing several contiguous ICP4 sites (Figure 6). It is likely that additional DNA binding sites cannot explain the multiple band shifts (DiDonato and Muller, 1989). Furthermore, the different band shifts displayed indistinguishable DNA footprinting patterns independent of the promoter (IE1 or IE3) analyzed.

Presently, the precise relationship between the various mobility complexes is not known. Alternate explanations such as proteolysis or varying degrees of post-translational
modifications can not be ruled out (Wilcoxon et al., 1980; Sorger et al., 1987; Weiderrecht et al., 1987; Michael et al., 1988; Yamamoto et al., 1988). The following arguments, suggest the different mobilities are due to variations in the ratio of ICP4 subunits to DNA. First, ICP4 in its native form is a homodimer (Metzler and Wilcoxon, 1985) and can self-associate; thus, it is possible that protein/protein interactions might generate tetramers or octamers of the ICP4 monomer subunit. Second, affinity purified ICP4 also generates primary and secondary complexes; therefore, it is less likely that an unrelated contaminant protein is responsible for mobility shifts. Third, the relative mobility shift of the three complexes appears to be logarithmic which suggests the primary complex differs from the secondary and tertiary complexes in molecular mass (Fried and Crothers, 1981). Finally, tetrameric forms of affinity purified ICP4 have been recently reported (Kattar-Cooley and Wilcoxon, 1989).

The mutant pD2-GH, which is deleted for two bases of the ICP4 core sequence, was impaired in A4 complex formation. This mutant was also insensitive to the presence of ICP4 in co-transfection experiments (see Chapter 5); therefore, a mutation that significantly diminished binding in vitro rendered that regulatory site dysfunctional in vivo. An identical mutation in the IE3 promoter was
recently reported (Deluca and Schaffer, 1988; Roberts et al., 1988) and that mutation, when introduced back into the viral genome, exhibited elevated levels of ICP4 production early in infection (Deluca and Schaffer, 1988).

ICP4 is a large multifunctional phosphoprotein containing 1298 amino acid residues that migrates on SDS-polyacrylamide gel with an apparent molecular mass of 175 kDa (Courtney and Benyesh-Melnick, 1974; Morse et al., 1978; Wilcox et al., 1980; Preston and Notarianni, 1983; Faber and Wilcox, 1986b; Deluca and Schaffer, 1988). Recent mutational analyses of the gene encoding ICP4 (Deluca and Schaffer, 1987,1988; Patterson and Everett, 1988a) have revealed regions of functional importance. Interestingly, a region for DNA binding, trans-activation and negative regulation map to the same region the protein (Deluca and Schaffer, 1988; Patterson and Everett, 1988).

A plausible model consistent with the data is based upon the following ideas. The ICP4 consensus sites reported by Faber and Wilcox (1986a) in IE gene 3 (Muller, 1987; Faber and Wilcox, 1988; DiDonato and Muller, 1989), IE gene 1 (46, DiDonato and Muller, 1989; this work, Figures 24 and 32) and IEx (Figures 27 and 33) are high affinity binding sites for ICP4 and although nonconsensus binding sites may exist, these differ from the IE1, IEx and IE3 sites in that they are displaced distally from the TATA box. Similarly,
the A4 core sequence, ATCGTC is repeated at a number of
sites in the HSV-1 genome; however, only in IE1, IEx and IE3
is it located close to the TATA box. The central role of
the TATA box in eukaryotic promoter function (Breathnach and
Chambon, 1981) and its close proximity to ICP4 binding sites
in IE genes 1 and 3, then incriminates TFIID (Sawadogo and
Roeder, 1985; Horikoshi et al., 1988,) in regulation.

The TATA box is extremely important in immediate early,
early and late class HSV-1 promoters as well (Coen et al.,
1986; Johnson and Everett, 1986; Everett, 1987;). In
general, mutations in this element (which would predictably
decrease the binding affinity of TFIID) cause a greater
decrease in transcription than mutations in other promoter
elements; for example, in HSV-1 tk (Coen et al., 1986),
mouse β-globin (10), adenovirus E1B (100) and major late
promoters (Corden et al., 1980; Hu and Manley, 1981; Concino
et al., 1983). ICP4 may affect TFIID/TATA association
through protein/protein interaction, as occurs with the
yeast factor GAL4 (Gill and Ptashne, 1988; Horikoshi, 1988);
thus, there are precedents where a specific DNA binding
protein affects neighboring transcription factor binding.
Furthermore, the pseudorabies immediate early protein has
been postulated to act by facilitating formation of the
preinitiation transcription complex through TFIID (Abmayr et
al., 1988; Workman et al., 1988).
A realistic view is that ICP4 most likely binds to its consensus binding site by itself; viz., the complexes detected by mobility shift experiments contain only ICP4 subunits. The in vitro binding and footprinting data (Muller, 1987; DiDonato and Muller, 1989; this study) and results regarding the mutagenesis of the ICP4 binding site, lead us to propose that the binding of ICP4 at the transcriptional start site elicits negative autoregulation of IE3 (Gelman and Silverstein, 1987a; Muller, 1987; O'Hare and Hayward, 1987; Deluca and Schaffer, 1988; Faber and Wilcox, 1988; Roberts et al., 1988; DiDonato and Muller, 1989; DiDonato and Muller, 12th Intl. Herpes Workshop p293, 1987).

A number of different models can be invoked to explain these findings. For example, simple stearic hindrance based upon prokaryotic repressor/operator models might explain the data; however, ICP4 may actually down regulate other IE genes (Preston, 1979; Dixon and Schaffer, 1980; Gelman and Silverstein, 1987a) which lack the ICP4 binding domain at the transcriptional start site. Recent preliminary data using GAL4 and the GAL4-ICP4 and GAL4-ICP0 fusion protein encoding plasmids in transient expression assays indicate the binding of a large protein to the DNA at the location of the A4 site in IE3 is not the major determinant in negative regulation of a linked gene driven from this promoter.
Footprinting ICP4 and TATA.

I propose that ICP4 binding to its cognate DNA site in IE3, IE1 and IEx either directly or indirectly (see below) modulates TFIID/TATA interactions. Because ICP4 is a homodimer, and presumably symmetrical, it is reasonable to propose that ICP4 may influence TATA/TFIID interactions when 5' or 3' of the TATA box. Native ICP4 as a dimer has a stokes radius of 8.74 nm (Metzler and Wilcox, 1985). This stokes radius corresponds to 52 bp of DNA sequence that can be covered by the protein when bound to the helix. In IE3 and IEx the A4 site is approximately 26 bp and 24 bp away (2.5 helical turns) from their TATA while the IE1 site it is 35 bp (3.5 helical turns) away, thus, all three ICP4 binding sites are on the same face of the helix relative to TATA. A summary of the separation distances between TATA and the footprinted A4 sites in IE1, IEx and IE3 is presented (Figure 36). At this separation distance between A4 and TATA, detectable alterations in the minor groove structure of TATA occurred only when ICP4 was bound. Minor groove
structural alterations could influence TFIID/TATA site interactions.

The chemical footprinting data (Figure 22, compare lanes 3 and 5, with lane 4) revealed that ICP4 binding at the +1 site of IE gene 3 induced a conformational change in the TATA element that was manifest as a hypersensitive region. Similar results were seen when ICP4 was specifically bound to the -61 region of IE gene 1 (Figure 24) and the +1 site of IEx (Figure 27). These data are summarized in Figure 36. Since this phenomenon was observed with affinity purified ICP4, it seems likely that ICP4 alone was responsible for the perturbation in the TATA region conformation. Note also that the ICP4 footprint ends at the A of the ATCGTC and does not extend into the TATA box. Furthermore, I have delineated the sequence elements required to form the ICP4/DNA complex. A 31 bp sequence which does not include the TATA box (from -15 to +16 of IE3) is necessary and sufficient to confer recognition by ICP4 on any given sequence. This observation revealed the modular nature of the ICP4 binding domain and demonstrated that sequence elements outside this domain are not essential in defining high affinity ICP4 binding in vitro.
Figure 36. Spacing between the A4 site and TATA in IE1, IE3 and IEx. The stereospecific alignment and the distance from TATA of the IE1, IE3 and IEx promoters is outlined.
Figure 36
ICP4 binding to other IE genes.

ICP4 binding to nonconsensus binding sites has previously been reported for the IE2 promoter and a number of late genes (Kristie and Roizman, 1986a; Michael et al., 1988; Michael and Roizman, 1989). Results obtained in this study further localized the binding of ICP4 in the IE2 promoter (Figure 21). The binding site is located at least 90 bp 5' of TATA and no further 5' than 165 bp. Additionally, an ICP4 binding site in the IE4/5 promoter has been identified which has not previously been reported in the literature (Figures 19, 39 and 40). The IE4/5 A4 site is located within 45 bp of TATA based upon recent experiments (data not shown and Figure 42). It is not known whether or not ICP4, when bound, induces a conformational change in helix geometry at TATA in these promoters (these experiments are in progress). The DNA sequence relationship between consensus and nonconsensus ICP4 binding sites is under investigation at the present time. Missing contact analysis results are being used in aiding in the derivation of a more accurate ICP4 consensus binding sequence (based on an algorithm that compares ICP4 binding sites) which predicts both consensus and nonconsensus binding sites (see below).
Binding strengths of ICP4 binding sites

Missing contact analysis (Brunnelle and Schleif, 1987) of the IE1, IEx and IE3 ICP4 consensus binding sites revealed which bases were important for stable ICP4 binding. Consistent with the relative strength of binding ($K_r$) (see Chapter IV) the IEx promoter contains no apparent base residues (at least on the noncoding strand) that decreased ICP4 binding affinity (Figure 33). The IE1 promoter contains base residues that decreased ICP4 binding affinity (Figure 32), while the IE3 proximal promoter (+1 site) contains an increased number of base residues detrimental to ICP4 binding affinity (Figure 28). These observations reflect the $K_r$'s observed (Table 3), where relative binding affinities were ranked highest to lowest (IEx > IE1 > IE3).

The missing contact analysis of the IE1 promoter indicated 2° complexes and 1° contacted the same bases during complex formation; therefore, multiple complex formation was probably due to some other event such as protein-protein interaction or post-translational modification of ICP4 rather than an altered binding pattern of ICP4 to the DNA. These results support the DMS interference (Figure 25) and Cu-OP footprinting data (Figures 22, 23, 24, and 27) concerning 1° and 2° A4 complexes discussed above. Critical bases which alter ICP4
binding affinity have been determined for these three strongest ICP4 binding sites (Figure 37). The position and identification of these residues have been compared to a data base of other known ICP4 binding sites (Figure 38).

A statistical comparison software package (Joseph Spitzner, TEAM Associates, Westerville, Ohio), used previously to determine an eukaryotic topoisomerase II cleavage site consensus sequence (Spitzner and Muller, 1989), is being utilized in the development of an ICP4 consensus binding sequence. This project is nearly completed (DiDonato et al., manuscript in preparation). The Faber and Wilcox ICP4 consensus sequence is 5'ATCGTCNNNYCGRC3' (R= purines and Y= pyrimidines); our consensus sequence is 5'RTCCTCNNYC/GG3'. Binding strength is determined by base residues that increase binding affinity versus those which decrease binding affinity. The software package calculates a matrix mean score (Spitzner and Muller, 1989) for a particular sequence which is determined by a base identity and location comparison to the data base of ICP4 binding sequences. The higher the matrix mean score, the higher the predicted probability of binding ICP4 to the sequence. Matrix mean scores calculated for the sequences listed in Figure 38 agree with the their ranking by $K_T$ (Table 3), thus indicating that the consensus sequence
Figure 37. Missing contact analysis for the IE1, IE3 and IEx promoters. The missing contact data of Figures 28, 29 and 30 are summarized here as a comparison base for base as they are aligned at the A4 site.
Figure 37
Figure 38. ICP4 binding sites. The strongest ICP4 binding sites are listed in aligned fashion relative to the ATCGTC of the A4 site. These sequences supply the bulk of the footprinted ICP4 binding sites used in the calculation of a more accurate ICP4 consensus binding sequence (DiDonato, Spitzner and Muller, in preparation).
<table>
<thead>
<tr>
<th>Relative Bases</th>
<th>1234567890</th>
<th>1234567890</th>
<th>1234567890</th>
<th>1234567890</th>
<th>1234567890</th>
</tr>
</thead>
<tbody>
<tr>
<td>IE3</td>
<td>ATGAGCCCGA</td>
<td>GGACGCCCCG</td>
<td>ATCGTCCACA</td>
<td>CGGAGCGGCGCTGCGCGACAC</td>
<td></td>
</tr>
<tr>
<td>IE3x</td>
<td>ATATATGCGC</td>
<td>GGCTCTGCCC</td>
<td>ATCGTCTCTC</td>
<td>CGGAGCGGCTTGGTGGG</td>
<td></td>
</tr>
<tr>
<td>IE1</td>
<td>GACGCGCCGC</td>
<td>CATTTGGGGA</td>
<td>ATCGTGACTG</td>
<td>CGGCCCCTTGAGGAGGGA</td>
<td></td>
</tr>
<tr>
<td>65Ktif</td>
<td>GATCGCATCA</td>
<td>AAAGCCCGAT</td>
<td>ATCGTCTTTC</td>
<td>CCGTATCAACCCAC</td>
<td></td>
</tr>
<tr>
<td>gD(-112)</td>
<td>TAGCCTGGCC</td>
<td>GTGTCAGACT</td>
<td>ATCGTGATTA</td>
<td>CGGACACACCGAGAGGAG</td>
<td></td>
</tr>
<tr>
<td>pBR322 (a)</td>
<td>CTAACAATGC</td>
<td>GCTCATCGTC</td>
<td>ATCGTGATCC</td>
<td>TGCCACCGTCACTGGGAT</td>
<td></td>
</tr>
<tr>
<td>pBR322 (b)</td>
<td>TGCCGGGCTT</td>
<td>CTTCCGGGAT</td>
<td>ATCGTGATT</td>
<td>CGCAGCATCGCCAGTCA</td>
<td></td>
</tr>
</tbody>
</table>

Figure 38
and use of the matrix mean score to predict ICP4 binding potential are accurate (data not shown).

ICP4 most likely binds to DNA through a single binding domain as evidenced by the ability to derive a single ICP4 consensus sequence and relate binding strength to the consensus via the matrix mean score. Recently, a 238 amino acid region of ICP4 has been identified and expressed as a fusion protein in \textit{E. Coli}, it can bind both consensus and nonconsensus ICP4 binding sites (Wu and Wilcox, 1990). This result further supports the idea of a single DNA binding domain. What is the effect on DNA as a consequence of ICP4 binding? The data suggest that ICP4 alters the geometry of the TATA box but does not physically protect the site. It is known that perturbations of the DNA helical geometry can be induced by protein binding, such as in the case of DNA wrapping in the nucleosome (reviewed by Morse and Simpson, 1988) or by binding of transcription factors such as heat shock transcription factor (Shuey and Parker, 1986a, 1986b; Sorger and Pelham, 1988). These factors when bound to their recognition sequence cause DNA bending (Shuey and Parker, 1986a). It is reasonable to suggest that these large perturbations of DNA conformation will impose additional constraints on any site specific DNA binding protein. Unless the DNA binding protein itself possesses a large degree of conformational flexibility, its equilibrium
association will be altered. Other investigators have speculated that ICP4 and TFIID may be functionally related (Patterson and Everett, 1988a; Roberts et al., 1988); however, this is the first evidence for a relationship between ICP4/DNA interactions and TFIID.

It is possible that the affinity of TFIID for the TATA site is altered when ICP4 is bound and it remains to be established whether ICP4 strengthens or weakens TFIID/TATA affinity. I imagine that at low abundance (i.e., immediately post infection), ICP4 might even facilitate TFIID/TATA interactions as reported recently for the pseudorabies immediate early protein (Abmayr et al., 1988) through a mechanism that does not involve binding at the A4 site. Since the sequence motif ATCGTC exists in a number of other regions of the genome, these sites could titrate ICP4 thereby keeping the regulatory A4 site or sites (near TATA) unoccupied. ICP4, like many other DNA binding proteins, finds its specific site through non-specific DNA binding and searching (Revzin and Von Hippel, 1977; Berg et al., 1981; Record et al., 1981). Perhaps the non-specific binding mode is important in activation. As ICP4 levels increase, high affinity A4 sites become occupied and movement of RNA polymerase II into the gene is attenuated. Alternatively, structural alteration in ICP4 as a consequence of
posttranslational modifications may contribute to both activation and repression (see DNA bending—below).

In the IE1 promoter, the A4 site displays the same stereo-specific alignment as in IE3 and IEx relative to TATA and is located 5' of TATA. Furthermore, ICP4 binding at -61 of IE1 is extremely stable (Table 3) and also induces hypersensitivity at the TATA element as seen in IE3 and IEx. ICP4 does not activate the IE1 promoter but represses it (Gelman and Silverstein, 1987a; Resnick et al., 1989) (see Figures 52 and 53). Transient expression results (Chapter 5) indicate ICP4 binding at -61 of IE1 modulates and restrains transcription of the IE1 promoter. Mutational analyses of the A4 site in the IE1 promoter confirm this observation (Figure 56). A distance dependent effect was observed in IE3 upon moving the A4 site 22 bp away from TATA in the mutant pJD45: TATA hypersensitivity in the A4 complexes was abolished (Figure 26). In addition, negative regulation in transient assays was abolished in the insertion mutant (Figure 53). A close correlation was observed between ICP4 mediated autorepression and stereo specific and or distance dependent placement of A4 and TATA. Repositioning a DNA binding in the insertion mutant could cause a variety of defects in normal regulation of the gene. I therefore analyzed a wild type promoter (gD) in which the A4 site is normally situated 80 bp away from TATA. As with
the insertion mutant, gD did not display TATA hypersensitivity in the A4 complex (Figure 26).

**DNA bending by ICP4.**

ICP4 binding induces a slight bend in the DNA at or near the ICP4 binding site (Figure 34, panel B and Figure 35). The degree of bending observed for the pBA2bend fragments is not as great as exhibited by the p-108 bend fragments, as exemplified by the magnitude of A4 complex migratory retardation. This may be due to the size of pBA2bend fragment (174 bp) which is only 20 bp longer than the persistence length of DNA (ca. 150 bp); thus, bending of this fragment is likely to be more difficult to detect than bending of the 274 bp p-108bend fragment which would have increased flexibility due to increased length.

Other DNA binding proteins bend DNA upon binding their cognate recognition sites. Examples are the heat shock transcription factor (Shuey and Parker, 1986), the cAMP activator protein (CAP) (Wu and Crothers, 1984), the lactose operon repressor protein (lac) and the galactose operon repressor (gal) (Zweib et al., 1989). These proteins recognize their binding sequences, which have a high degree of dyad symmetry and usually bind as dimers. ICP4 also binds DNA as a dimer (Michael and Roizman, 1989) and is
capable of forming higher oligomers when purified (Kattar-Cooley and Wilcox, 1989). Although the ICP4 binding site (A4 site) does not contain a dyad symmetry element in its DNA binding site, it appears ICP4 binds to the site in a pseudo-symmetrical fashion. This observation was made from the pattern of ICP4-DNA base residue contacts seen in the missing contact experiments (Figure 31). Perhaps ICP4 can sense a structural symmetry of the DNA sequence which is not apparent from its linear sequence.

How bending facilitates the function of the bound protein for the most part, still remains unclear. To bend the DNA upon binding of the protein, energy is required. The energy used to bend the DNA is required to allow maximum contact between critical DNA base residues and the protein (Huei-Nin et al., 1986). The energy required to do this is stored in the protein/DNA complex and can be released by loss of protein-DNA contacts. The free energy that has been released can be used to do mechanical work such as opening the DNA helix to initiate replication, initiate transcription or perhaps to alter the DNA conformation of nearby cis binding sites.

Applying these possibilities to ICP4 and IE3 in particular, the following observations were made. First, ICP4 binding sites near HSV origins of replication have not been reported. Secondly, nonconsensus ICP4 binding sites
have been identified in a number of early and late genes in the untranslated leader sequences and in the coding region (Michael et al., 1988; Tedder et al., 1989). These sites are clustered and extend over a range of ca. 100 bases and are >80 bp away from TATA (Michael et al., 1988). Perhaps multiple binding of ICP4 to these regions results in DNA bending and stored free energy which could be released upon ICP4's exit from the DNA, the free energy in sum may aid in the opening of the DNA helix at the transcription initiation start site of these genes. Alternatively, these multiple ICP4 binding sites may serve to organize these genes into a higher order DNA structure via ICP4-ICP4 interaction.

A higher order DNA structure could make these genes inaccessible to the transcriptional complex. ICP4 present at late times in infection has a decreased affinity for nonconsensus ICP4 binding sites (Michael et al., 1988); furthermore, ICP4 is posttranslationally modified as evidenced by its slower migration in SDS-PAGE gels (Figures 2 and 3) (Wilcox et al., 1980; Faber and Wilcox, 1986b; Michael et al., 1988). Release of ICP4 from these sites would then open up these genes to access by the transcriptional machinery at the proper point in the HSV infection cycle. The gal repressor in E. Coli represses the gal operon through formation of a loop which contains two
separated gal operator sites (Zweib et al., 1989). This loop is thought to organize into a higher order structure that excludes RNA polymerase activity and is mediated as a result of DNA bending. Higher order structure formation as a result of repression loops mediated through ICP4 on IE genes is not the way in which these genes are repressed.

Repression can be observed from promoters with only a single ICP4 binding site, therefore, another mechanism appears to be in operation for the IE genes. Bending of the DNA at the IE3 consensus ICP4 binding site may influence cis binding sites such as TATA. DNA bound ICP4 alters TATA helix geometry in IE3, IE1 and IEx (Figures 23, 24 and 27) (DiDonato and Muller, 1989). ICP4 is presumed to alter its structure in response to salt concentration (Figures 14 and 15), temperature (Kattar-Cooley and Wilcox, 1989; Patterson and Everett, 1988b) and self association (Shepard et al., 1989b). These structural alterations may result from posttranslational modifications (Periera et al., 1977; Wilcox et al., 1980; Preston and Notaranni, 1983). For TATA helix conformational change to occur, free energy must be transferred to the area.

There are three possible ways to transfer the free energy to TATA: i) transfer of free energy directly through the DNA to TATA; ii) transfer the energy via ICP4 to TATA, where ICP4 and the DNA interact and minor groove structure
is altered; iii) another protein, not ICP4, interacts with TATA only when ICP4 is bound. The first method requires free energy to be released. Unless the protein contains stored energy in the form of structural stress, the free energy would have to be supplied by the DNA bend. If this were the case, the bend would no longer exist, yet mobility shift experiments detect bent DNA with or without the TATA region being present. This does not appear to be a viable hypothesis. The second method is more attractive. Binding of ICP4 to the helix results in DNA bending. The protein alters its structure to stabilize bending. The structural alteration brings a region of ICP4 into close proximity of TATA and they interact resulting in alteration in the TATA helix geometry. The third possibility remains to be tested. Use of a more purified preparation of ICP4 is necessary to determine if ICP4 alone is responsible for TATA helix perturbation.

ICP4 is known to have an extended asymmetric ellipsoid shape (Metzler and Wilcox, 1985). According to its stokes radius ICP4 could cover a maximum of 52 bp, of which, 14 to 15 bp are accounted for in the footprints. This would leave a 38 bp span which ICP4 could cover before the TATA would be out of reach. This observation could explain why IE1, IEx and IE3 exhibit TATA hyperactivity but the IE3 mutant where TATA is separated from the binding site by 48 bp (pJD45,
Figure 26) does not. Deformation of TATA could result in an altered TFIID binding affinity which would effect expression driven from this promoter, most likely in a negative manner. An addendum to this idea is that ICP4 may actually interact with TFIID: ICP4 is located on the opposite face of the helix from TATA, structural alteration of TATA may cause TFIID to bind the sequence from an orientation different from normal. This different orientation could be favorable for interaction with ICP4, in which case, TFIID would be on the opposite face of the helix on which to form the transcription complex. Similarly, when ICP4 is >80 to 100 bp away from TATA there is latitude for flexibility of the DNA and stereo-specific alignment may not be so critical, in this case TFIID and ICP4 interaction may result in increased expression.
CHAPTER IV

DETERMINATION OF THE EQUILIBRIUM DISSOCIATION BINDING
CONSTANTS FOR THE IE3 ICP4 BINDING SITES AND THE RELATIVE
BINDING AFFINITIES OF OTHER IE GENE ICP4 BINDING SITES

INTRODUCTION

DNA binding proteins bind to their cognate recognition
sequences with a characteristic binding affinity. Often,
there are many such sites that vary in DNA sequence. It is
often important to relate the binding strength of one site
to another. Previously, the only way to do this was to
calculate the equilibrium dissociation constant ($K_d$) for a
site using filter binding (Riggs et al., 1970). This
process is extremely arduous and is not ideal for analyzing
a large number of sites. Further, the method cannot
distinguish between single complexes, multiple complexes, or
non-specific complexes that are trapped. A method, now
routinely used, to calculate the relative binding affinities
($K_r$) of different binding sites with fewer drawbacks than
the filter binding method is the mobility shift assay
Different probes can be compared directly in the same binding reaction. The $K_T$ can be determined, with this method, independent of the active protein concentration and the specific activities of each probe (Fried and Crothers, 1981). Probe lengths should be variable, so that each complex can be distinguished by mobility shift gel analysis. Multiple protein/DNA complexes can be visualized and their relationship with the single complex determined.

A nonconsensus binding site for ICP4 located in the 5' upstream portion of the IE3 promoter (-211/-108) was reported (Kristie and Roizman, 1986a; Michael et al., 1988). This nonconsensus site is a reportedly a strong ICP4 binding site, that remained undetectable by other laboratories. The following experiments were initiated in an attempt to determine the relative binding strengths of a variety of wild type and mutant versions of IE ICP4 binding sites. In addition, the binding affinity of the IE3 nonconsensus site and the consensus ICP4 binding site (at the 5' transcriptional start site of IE3) were determined.
RESULTS

Relative binding affinities of consensus vs nonconsensus ICP4 binding sites in the IE3 promoter.

Mobility shift assays were performed to determine if ICP4 bound to the upstream portion (5' to the -108 position) of the IE-3 promoter. Mock infected crude nuclear extracts and partially purified ICP4 (DEAE fraction) were compared in the presence of antibodies (as indicated in Figure 39). Complex formation on WT IE-3 transcriptional start site probe (-108/+27), IE-3 (-108/-211) and the IE-3 (-108/-342) fragments was first determined (Figure 39 lanes 9-12, 14-17, 19-22). The WT IE-3 transcriptional start site probe formed a complex unique to partially purified infected cell extract (lane 10), which was not retarded in mobility by a monoclonal antibody (MOPC 21) and is not reactive with ICP4 (lane 11). Addition of anti-ICP4 (58-S), to the partially purified infected extract, resulted in retardation of the A4 complex migration indicating ICP4's presence in the complex (lane 12).
The (-108/-211) fragment gave rise to a DNA/protein complex formed only with protein derived from infected cells (lanes 15 and 16). Furthermore, the complex contained ICP4, as evidenced by the retardation in migration of the complex by anti-ICP4 antibody (lane 17). The larger probe (-108/-342) gave rise to the same DNA/protein complex pattern as the (-108/-211) probe, indicating no new additional ICP4 binding sites were present between positions -211 to -342. The increase in complex formation on the larger probe (lanes 19 and 20) may be a result of increased binding affinity of the protein for the larger fragment as compared to that of the shorter (-108/-211) fragment.

Upon identification of the ICP4 binding site in the distal IE3 promoter, a series of experiments was devised to determine and compare the relative binding affinities of the WT and D2 versions of the IE3 A4 site and the distal nonconsensus ICP4 binding site. The following experiments utilize the presence of Hind III restriction sites at the -342 and +51 boundaries of the IE3 promoter, between which lie both ICP4 sites. End-labeling the Hind III sites and cutting the fragment with Eco RI generated two fragments with identical specific activities. The -108/+51 fragment contains the ICP4 binding site at +1, the -342/-108 fragment contained the nonconsensus ICP4 binding site. Equivalent amounts of radioactivity of each probe (identical numbers of
probe fragments) were mixed in a binding reaction and fractionated on a mobility shift gel. The percent conversion of each probe to A4 complex was measured and the relative binding affinity ($K_r$) was determined.

This analysis was performed with the WT IE3 promoter and the IE3 AT deletion mutant D2 (a mutant IE3 promoter which has a 2 bp AT deletion in the core ICP4 consensus sequence at the -6A, -5T). Both pJD10CAT and pJD11CAT (which contain the IE3 promoter elements from -330/+27 of the wild type [WT] and the -6A -5T deletion mutant [D2], respectively) were restricted with Hind III (releasing the -342/+51 Hind III/Hind III fragment from each construction). The fragments were 5' end labeled thus the specific activity of labeling was identical for both ends of the fragment. A portion of the -342/+51 fragment was saved and the remainder cut with Eco RI resulting in two fragments (a -108/+51 and -342/-108 fragment). The -108/+51 probe contained the ICP4 binding site at the 5' transcriptional start site and the -342/-108 probe contains the nonconsensus ICP4 binding site. The probes were isolated and used in mobility shift assays. A4 complex formation on each probe was assayed in separate binding reactions with partially purified ICP4. Mixed probe binding reactions were also performed (equivalent amounts of radioactivity of each probe were added to the mixed binding reactions hence, an equal number of fragment molecules for
each probe was present). Binding reactions were analyzed by mobility shift. The dried gel was also placed on an AMBIS beta scan unit to quantify the radioactivity present in the gel.

The WT IE3 (-342/+51) probe forms an A4 complex (Figure 39, lane 1) as did the (-108/+51) probe (lane 2) and the (-342/-108) probe (lane 6). A4 complexes formed on the -108/+51 and the -342/-108 probes were competed by addition of A4 oligo (5 ng) to the binding reactions (lanes 3 and 7) thus authenticating the DNA/protein complexes as A4 complexes. The A4 complex formed on the -342/-108 probe migrated more slowly through the gel than the A4 complex on the -108/+51 fragment (compare the positions of the A4 complexes in lanes 2 and 6). The difference in A4 complex mobility allowed the A4 complexes formed by each probe to be discerned (lanes 4 and 5). The AMBIS beta scan was used to quantify relative binding affinities of the -108/+51 and the -342/-108 probes using the previously described formula

\[ K_r = \frac{K_1^o}{K_2^o} = \frac{([C1^o]/[D1^o])}{([C2^o]/[D2^o])} \]

where \( C1^o \) and \( C2^o \) is the radioactivity present in the A4 complexes on the -108/+51 and -342/-108 probes respectively. \( C2^o \) and \( D2^o \) are the radioactivity in the free probes (-108/+51 and -342/-108). The percent conversion of each probe in separate binding reactions was 61.6% for the 1° complex and 7.1% for the 2° complex of the -342/+51 probe.
Figure 39. Identification and relative binding affinity ($K_r$) of the proximal and distal ICP4 binding sites in IE3. End-labeled fragments of identical specific activity were prepared by Hind III restriction of pJD10CAT, releasing the -342/+51 fragment. This fragment was isolated and cut with Eco RI which results in two fragments, one which contains the IE3 consensus ICP4 binding site (-108/+51) and the nonconsensus binding site (-342/-108). Equal counts (15,000 cpm) hence equal numbers of probe fragments were incubated either singly or mixed (1:1) in a binding reaction containing partially purified ICP4 (2 µg) and 4 µg of poly dA·dT. Reactions were incubated on ice for 45 minutes, tracking dye added and the samples were fractionated at room temperature over a 4% mobility shift gel running at 20 mA constant current with buffer recirculation. The dried gel was subjected to autoradiography and analysis on the Ambis radioimaging detector unit. Lane 1 contains the -342/+51 IE3 promoter. Lane 2 and 3 contain the proximal IE3 -108/+51 promoter. Lanes 4 and 5 contain both the IE3 proximal (-108/+51) and distal (-342/-108) promoter elements. Lanes 6 and 7 contain the IE3 distal (-342/-108) promoter. Partially purified ICP4 was added to binding reactions as indicated above the lanes. Lanes 8 through 22 demonstrate the presence of ICP4 in complexes forming on the proximal IE3 promoter (lanes 8 through 12) and on the distal IE3 promoter (lanes 13 through 22). Lanes 8 through 12 contain the IE3 proximal promoter (-28/+51) incubated with no protein (lane 8), mock infected extract (2 µg) (lane 9), partially purified ICP4 (2 µg) (lanes 10 through 12) with MOPC 21 monoclonal antibody (70 ng) (lane 11) or 58S monoclonal antibody (70 ng) (lane 12). Lanes 13 through 17 contain the distal IE3 promoter (-211/-108) incubated with no protein (lane 13), mock infected extract (2 µg) (lane 14), partially purified ICP4 (2 µg) (lanes 15 through 17), MOPC 21 monoclonal antibody (70 ng) (lane 16) or 58S monoclonal antibody (70 ng) (lane 17). Lanes 17 through 22 contain the IE3 distal promoter (-342/-108) incubated with no protein (lane 18), mock infected extract (2 µg) (lane 19), partially purified ICP4 (2 µg) (lanes 20 through 22), MOPC 21 antibody (70 ng) (lane 21) or 58S antibody (70 ng) (lane 22). Binding reactions were performed and fractionated as described above. The dried gel was then subject to autoradiography at -80° C.
Figure 39
(lane 1), 22.6% for the -108/+51 probe (lane 2) and 3.2% for the A4 complex on the -342/-108 probe (lane 6). The percent conversion to A4 complex on the -108/+51 probe in the mixed binding reaction was 26.1% and 21.1%, the -342/-108 probe was 3.7% and 3.4% respectively (lanes 4 and 5). The K_r of the -108/+51 fragment relative to that of the -342/-108 probe (consensus:nonconsensus) was 23.6/3.5 or 6.6+/− 0.4 (see Appendix A).

The same experiment was repeated with the D2 promoter (-342/+51) [described above] to determine the K_r of the D2 (-108/+51) probe to the nonconsensus ICP4 binding site in D2 IE3 (-342/-108). Additionally, the K_r of the wild type IE3 consensus site to that of the AT deletion mutant D2 was determined. This was accomplished by direct comparison of their respective K_r's to the nonconsensus ICP4 binding site (-342/-108 fragment). The -108/+51 probe of D2 formed a weak A4 complex (Figure 40, lane 3) as did the D2 (-342/+51) probe (lanes 1 and 2) and the D2 (-342/-108) probe (lane 7). These A4 complexes were competed by addition of A4 oligo (lanes 4 and 8). The A4 complexes formed on the D2 probes (-108/+51 and -342/-108) migrate to different positions in the mobility shift gel (Figure 40, lanes 3, 5, 6 and 7) allowing resolution of each A4 complex in the mixed binding reactions (lanes 5 and 6).
The percent conversion to A4 complex for the D2 (−342/+51) probe was 5.4% and 4.3% (lanes 1 and 2) the conversion of the D2 (−108/+51) probe to A4 complex was 1.7% (lane 3) and that of the D2 (−342/−108) probe was 2.9% (lane 7). The percent conversion to A4 complex in the mixed probe binding reactions for the D2 (−108/+51) probe was 1.8% and 1.5% (lanes 5 and 6) and 3.3% and 3.1% for the D2 (−342/−108) probe (lanes 5 and 6). A $K_r$ of 0.50 +/- 0.1 for the D2 (−108/+51) was calculated using the D2 (−108/−342) probe as the standard (see Appendix B). Since the −108/−342 region of both the wild type and D2 construction are identical, the relative binding affinities of WT (−108/+51) to D2 (−108/+51) were determined using the following equation.

\[ (2) \quad K_r = \frac{K_r (WT)}{K_r (D2)} \]

$K_r (WT)$ is the binding affinity of the wild type (−108/+51) probe relative to the wild type (−342/−108) probe. The $K_r (D2)$ is the binding affinity of D2 (−108/+51) relative to the D2 (−342/−108) probe. The $K_r (WT)$ was 6.6 (determined from the data in Figure 39), the $K_r (D2)$ was 0.52 (determined from Figure 40). A direct comparison of the $K_r (WT)$ and $K_r (D2)$ is a valid measure of the difference in the relative binding affinity for ICP4 between these two sites. The calculated $K_r$ was then 6.6/0.52 or 12.7. Therefore, the wild type consensus ICP4 binding site had a binding affinity nearly 13 fold greater than that of the D2
Figure 40. Relative binding affinities ($K_r$) of the proximal D2 mutation and the distal ICP4 binding site in IE3. Probe preparation was identical to that described for lanes 1 through 7 in Figure 35 except that pJD11CAT which contains the $-6A, -5T$ deletion (D2 mutation) in the IE3 consensus ICP4 binding sequence. Lanes 1 and 2 contain the IE3 D2 promoter fragment (342/+51) (15,000 cpm) incubated with partially purified ICP4 (2 μg). Lanes 3 and 4 contain IE3 D2 (-108/+51) (15,000 cpm) incubated with partially purified ICP and no protein respectively. Lanes 5 and 6 contain IE3 D2 (-108/+51) and (-342/+51) fragments (15,000 cpm each) incubated with partially purified ICP4 (2 μg). Lane 8 is free IE3 D2 (-342/-108) probe. Except for the specified extracts added to each reaction listed above, all incubation conditions and electrophoretic fractionations were identical to those described for lanes 1 through 7 in Figure 39.
Figure 40
mutant (-108/+51) and 6.6 fold greater than that of the nonconsensus binding site (-108/-211) region of IE3.

**Mutations in the IE1 promoter affect A4 complex formation.**

The IE1 promoter contains an ICP4 consensus binding site centered at the -61 site relative to the 5' transcriptional start site (Kristie and Roizman, 1986b; DiDonato and Muller, 1989; Kattar-Cooley and Wilcox, 1989). To determine the importance of the core ICP4 consensus site in A4 complex formation on the IE1 promoter, mutant IE1 plasmids were constructed. The ICP4 core consensus sequence was progressively altered and the relative binding affinity for ICP4 to these sites was determined. The mutants were then used to generate probes for binding studies. The construction pJD100 contains the -126/+51 region of IE1 cloned into the Eco RV site of pBluescript\(^+\). *In vitro* mutagenesis of the A4 consensus site changed the wild type IE1 consensus sequence (in pJD100) from 5'GGGAATCGTC...3' to 5'GGGGGACGTC...3' (see Materials and Methods). The altered consensus site construction was designated as pJD101. pJD101 was restricted with Aat II, which cuts the sequence 5'GACGT C3' leaving a 4 bp 3' overhang; the latter was treated with T4 DNA polymerase and religated with T4 ligase
to form pJD102. pJD102 contains the 4 bp deletion in the mutant A4 site (5'GGGGG.....C...3').

The IE1 promoters from each of these constructions were released from the plasmids by digestion with Eco RI and HindIII. The fragments were 5' end labeled and isolated. The various IE1 probes were incubated with either a mock infected cell extract or with partially purified ICP4 and analyzed by mobility shift. The WT IE1 probe (-138/+59) failed to form an A4 complex with mock infected cell extract (Figure 41, lane 1) but did when incubated with partially purified ICP4 (lane 2). This complex was competed by addition of A4 oligo to the binding reaction (lane 3). The mutant IE1 promoter, in pJD101, (pJD101 also is known as the -T construct) contains two transitions (A -> G) and a transversion (T -> A) mutation in the IE1 ICP4 consensus binding site (see description above). Probes prepared from pJD101 were used in binding reactions. Incubation of these probes with mock infected cell extract failed to form an A4 complex (lane 4), but did when partially purified ICP4 was used (lane 5). The complex could also be competed by addition of A4 oligo to the binding reaction (lane 6). The abundance of the A4 complex (lane 5) is less than that of the WT IE1 A4 complex (compare lanes 2 and 5). Probe prepared from the 4 bp IE1 deletion mutant pJD102 was also used in binding assays (pJD102 is also known as the d1°
Figure 41. Formation of A4 complexes at WT and mutant IE1 ICP4 binding sites. Probe DNA's were prepared by Eco RI and Hind III digestion of WT IE1 promoter (-129/+51) construction pJD100, the IE1 mutant pJD101 (-129/+51; the -T construction) and an IE1 deletion mutant pJD102 (-125/+51; the dl° construction). These probes ca. 1 ng (20,000 cpm) were incubated either with mock extract (2 µg), partially purified ICP4 (2 µg) or partially purified ICP4 preincubated with 90 ng of unlabeled A4 oligo for 30 minutes on ice. All binding reactions contained 4 µg of Poly dA·dT and were incubated on ice for 45 min, tracking dye was added and the samples electrophoresed on a 4% mobility shift gel at room temperature, 20 mA constant current with buffer recirculation. The gel was dried and subject to autoradiography. Lane 1 through 3 contains wild type IE1 probe (-129/+51) incubated with mock infected extract (lane 1), partially purified ICP4 (lane 2), partially purified ICP4 preincubated with A4 oligo (lane 3). Lanes 4 through 6 contain the IE1 -T probe incubated with mock infected extract (lane 4), partially purified ICP4 (lane 5) and partially purified ICP4 plus A4 oligo (lane 6). Lanes 7 and 8 contain the IE1 deletion probe dl° incubated with mock infected extract (lane 7) and partially purified ICP4 (lane8). Positions of the A4 complexes and free DNA are indicated.
Formation of A4 complexes at WT and mutant IE1 ICP4 binding sites.

<table>
<thead>
<tr>
<th>WT</th>
<th>-T</th>
<th>-T Del 4b</th>
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<tbody>
<tr>
<td>IE1</td>
<td>IE1</td>
<td>IE1</td>
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</tbody>
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GAATCGTC GGGACGTC GGG...C

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<tr>
<th>M I C</th>
<th>M I C</th>
<th>M I</th>
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<td>1 2 3 4 5 6 7 8</td>
<td></td>
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</table>

ICP4 complex

Free DNA

Figure 41
construct). Incubation of the pJD102 probe (-125/+59) with mock infected cell extract resulted in no complex formation (lane 7). Incubation of probe with partially purified ICP4 did form an A4 complex, but was extremely weak (lane 8).

The probability of the various IE1, IE4/5 and IEx promoter constructions to form A4 complexes were compared by mobility shift assays. The promoter fragments of PJD100 (-129/+59), pJD101 (-129/+59) and pJD102 (-125/+59) were incubated with mock infected cell extract or with partially purified ICP4. Binding reactions were performed in the presence or absence of A4 oligo. An identical incubation scheme was followed using the promoter fragments of IEx (-105/+35) and IE4/5 (-51/+108). As an added control, to authenticate A4 complex formation, the anti-ICP4 monoclonal antibody was also included in indicated binding reactions with partially purified ICP4. A4 formation on the IE1 probes indicated as the consensus ICP4 binding site was altered (pJD101 and pJD102) the ability to form an A4 complex decreased (Figure 42, compare lane 2 with lanes 5 and 8). The mobility of the A4 complex was retarded by addition of anti-ICP4 monoclonal antibody (lanes 16, 17 and 18) and effectively competed by addition of A4 oligo to the binding reactions (lanes 3 and 6). The IE4/5 promoter was capable of A4 complex formation (lane 14). The complex's mobility could be retarded by anti-ICP4 antibody (lane 20)
and competed by addition of A4 oligo to the binding reactions (lane 15). ICP4 appeared to have the greatest relative binding affinity for the IEx promoter in comparison to the other promoters tested. This conclusion was based upon the abundance of A4 complex observed (lane 11). The complex's mobility was further retarded by the anti-ICP4 antibody (lane 19) and was competed by A4 oligo but not to as great an extent as the other promoters (compare lane 12 with lanes 3, 6, and 15).

**Determination of the relative binding affinity of various ICP4 binding sites.**

The relative binding affinity of ICP4 for the IE3 (-108/+47), IE4/5 (-400/+108) and the various IE1 WT and mutant promoters (-138/+59) was determined using a mixed probe binding reaction as described above (Figure 39 and Figure 40). Either the IE3 or the IE1 probes were mixed with the IE4/5 probe (equivalent mounts of radioactivity of each probe were included in the binding reaction) and incubated with partially purified ICP4 and fractionated on a mobility shift gel.

The quantity of A4 complex formed on each probe was determined by soft laser densitometric scanning of the autoradiograms. Multiple autoradiographic exposures of the
Figure 42. Formation of ICP4/DNA complexes in different IE genes: Comparison of relative binding affinities. Binding reactions containing probes from either IE1 and two IE1 mutants (-129/+51), IE4/5 (-51/+108) or IEx (-105/+35) were incubated with either mock infected extract (2 μg), partially purified ICP4 (2 μg) or partially purified ICP4 preincubated with anti-ICP4 monoclonal antibody (58S, 1 μg) or A4 oligo (90 ng) for 30 minutes. All binding reactions were performed on ice. Binding reactions were fractionated on a 4% mobility shift gel under the same conditions described in Figure 41. Lanes 1 through 8 are identical to those described in Figure 41. Lanes 10 through 12 and lane 19 contain the IEx probe (-105/+35) incubated with mock infected extract (lane 10), partially purified ICP4 (lane 11), partially purified ICP4 plus A4 oligo (lane 12). Partially purified ICP4 preincubated with anti-ICP4 monoclonal antibody (58S, lane 19). Lanes 13 through 15 and lane 20 contain the IE4/5 probe (-51/+108) preincubated with mock extract (lane 13), partially purified ICP4 (lane 14), partially purified ICP4 preincubated with A4 oligo (lane 15), partially purified ICP4 preincubated with anti-ICP4 monoclonal antibody (58S) (lane 20). WT IE1 (lane 16), IE1 (pJD101) (lane 17) and IE1 (pJD102) probe (lane 18) was incubated with partially purified ICP4 which had been preincubated with anti-ICP4 monoclonal antibody prior to addition of probe. Positions of the A4 complex and free DNA are indicated.
<table>
<thead>
<tr>
<th>WT</th>
<th>-T</th>
<th>dl°</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC</td>
<td>MIC</td>
<td>MIC</td>
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</table>

<table>
<thead>
<tr>
<th>IE1</th>
<th>IEx</th>
<th>IE4/5</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>MIC</td>
<td>MIC</td>
<td>I I I I I I I I I I</td>
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**Figure 42**
dried gel were used for scanning. The area under each peak of the densitometric scan (corresponding to bands in the gel) was excised and weighed. The comparison of the relative conversion of free probe to A4 complex was determined for each probe in this manner. From the percent conversion to A4 complex, the $K_r$ for each probe was determined using the IE4/5 promoter as the standard ($K_r$ of IE4/5=1.0). The $K_r$ of the IE3 promoter (-108/+47) was 13.1 (Figure 43, lane 4). The $K_r$ of the pJD102 probe (IE1 - 125/+59) was 0.02 (lane 9), that of the pJD101 probe (IE1 - 129/+59) was 1.0 (lane 8). The WT IE1 promoter $K_r$ could not be directly calculated due to a problem in resolving the A4 complexes resulting from the IE1 and the IE4/5 probes (lane 6). An estimation of the $K_r$ of WT IE1 was calculated based on the following observations. First, the percent conversion of WT IE1 probe was calculated to be 41% (lane 7). The percent conversion for the IE4/5 promoter in lanes 8 and 9 averaged 1.5%. These values were used to calculate the WT IE1 $K_r$ using equation 3.

$$3.) \quad K_r (WT \ IE1) = \frac{\% \ conversion \ WT \ IE1 \ A4 \ complex}{\% \ conversion \ IE4/5 \ A4complex}$$

Use of equation 3 resulted in a calculated $K_r$ (WT IE1) of 41.0/1.5 or 27.3 as compared to the IE4/5 promoter.

The calculated $K_r$'s for the different IE promoters indicates a hierarchy of relative binding affinities of ICP4
to the different ICP4 binding sites. The WT IE1 (-138/+59) probe is 2.1x as "good" a binding site for ICP4 as the IE3 (+47/-108) probe, 28.3x as "good" as pJD101 (-129/+59) probe, ca. 1100x better than the pJD102 (-125/+59) probe and 27.3x as "good" as the IE4/5 probe (-400/+108). The $K_r$ of the IEx promoter was not determined, but by visual inspection of the IEx A4 complexes (Figure 42, lanes 11 and 19), they appeared to form at least 3 to 5x more abundantly than the WT IE1 probe A4 complex. The relative binding affinities of the different IE promoters are listed in Table 3.

**Calculation of the equilibrium dissociation constant for ICP4.**

The formation of the ICP4/DNA complex on the IE3 transcriptional start site occurs as a bimolecular reaction: ICP4 + DNA $\rightarrow$ ICP4/DNA. At equilibrium, the ratio of ICP4 bound DNA to free DNA reflects the relative affinity of ICP4 for its specific DNA sequence (Riggs et al., 1970). To determine the absolute binding affinity of ICP4, bound and free DNA over a wide range of initial DNA concentrations and fixed protein concentrations must be determined. The ratio of bound to free DNA is then plotted against the concentration of bound DNA. This type of
Figure 43. Relative binding affinity of the IE3, IE1 and IE4/5 ICP4 binding sites for ICP4. Probes were prepared for IE3 from pJD1 (Muller, 1987) and are designated IE3 short (-108/+48) and IE3 long (-330/+27). Probe for the IE4/5 promoter (-400/+108) was prepared from pJD2 (see Materials and Methods). Probes for the WT and mutant IE1 promoters (-129/+51) were prepared from pJD100 (WT IE1), pJD101 (-T probe), and pJD102 (dl° probe). All binding reactions contained partially purified ICP4 (2 μg) incubated with 20,000 cpm of each probe either singly or in mixed binding reactions as designated above each lane. Incubation was performed on ice for 45 min, tracking dye was added, samples placed back on ice until loaded onto a 4% mobility shift gel at 4° C run at 20 mA constant current with buffer recirculation. The gel was dried and subject to autoradiography. Autoradiograms were scanned with a soft laser densitometer (LKB). Peaks corresponding to the complexed and free DNA's were cut out and weighed to obtain values of peak band intensity. These values were then used to determine $K_r$ (see Chapter IV, Determination of the relative binding affinities of various ICP4 binding sites). Lane 1 contains the IE3 long probe. Lane 2 contains WT IE1 and IE3 long probe. Lane 3 contains the short IE3 probe. Lane 4 is the short IE3 probe and the IE4/5 probe. Lane 6, 8 and 9 are the IE4/5 probe with the IE1 (pJD100), IE1 (pJD101) and IE1 (pJD102) probes respectively. Lane 7 is WT IE1 probe. Lane 10 is WT IE1, IE4/5 and IE3 long probes.
Table 3. Relative binding affinities of various IE promoters. The IE fragments listed were assayed in a mixed binding reaction and fractionated. The percent conversion of each fragment was compared against the other fragment in the reaction to arrive at the $K_r$. In the first column, the relative binding affinities were related to the nonconsensus site between -108 and -211 of IE3. The values in the second column were related to the IE4/5 binding site as the standard.
Table 3

<table>
<thead>
<tr>
<th>PROMOTER</th>
<th>$K_r^a$</th>
<th>$K_r^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IE1 (-126/+51)</td>
<td></td>
<td>27</td>
</tr>
<tr>
<td>IE3 (-108/+51)</td>
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<td>13</td>
</tr>
<tr>
<td>IE3 (-108/-342)</td>
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<td>2</td>
</tr>
<tr>
<td>IE3 [D2] (-108/+51)</td>
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</tr>
<tr>
<td>IE1 -T (-126/+51)</td>
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<td>1</td>
</tr>
<tr>
<td>IE1 dl° (-122/+51)</td>
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<td></td>
</tr>
<tr>
<td>IE4/5 (-400/+108)</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

a The relative binding affinities calculated using the IE3 -108/-342 fragment as the standard ($K_r=1.0$). Data is listed in Appendices A and B.

b The relative binding affinities calculated from Figure 43 using the IE4/5 (-400/+108) fragment as the standard $K_r=1.0$. The values of the IE3 -108/-342 and the D2 fragment (-108/+51) were extrapolated from comparing the values in column A to the measured value of IE3 -108/+51 in column B.
analysis is known as a Scatchard analysis (Scatchard, 1949). The slope of the graph is equivalent to $-1/K_d$ and the X intercept is equal to the concentration of active DNA binding protein in the reaction. Mobility shift assays were carried out to determine bound DNA versus free DNA. This system has been used previously to determine binding affinity constants and equilibrium dissociation constants (Freid and Crothers 1981; Chodosh et al., 1986).

The analysis was performed on the -108/+27 and -108/-342 fragments labelled at the -108 site; therefore, the specific activities of the DNA's were identical. DNA concentrations were determined by use of a mini fluorometer. The -108/+27 WT IE-3 probe concentration was varied between 2.78 pM and 5.56 nM and the -108/-342 probe was varied between 27.8 pM and 8.34 nM. The ICP4 concentration in each reaction was constant (but unknown) because the protein source was only partially purified. Binding reactions were carried out on ice in a 20 \(\mu\)l reaction containing: 30 mM KCl (contributed by the extract), 12.5 mM NaPO\(_4\) pH 6.8, 5.0 mM Tris-Cl pH 7.9, 0.5 mM EDTA pH 8.0, 0.05 % NP40 and 4.0 \(\mu\)g poly dA·dT. The reaction was carried out for 1 hr and then fractionated on a mobility shift gel at 4°C. The amount of radioactivity in the bound DNA and the free DNA were determined with the AMBIS system. The ratio of bound DNA to free DNA was plotted against the bound DNA
Figure 44. Determination of $K_d$ for the IE3 (-108/+27) ICP4 binding site. Double end labeled probe prepared by Bgl II digestion of p-108bend (see Materials and Methods). This probe contains the IE3 promoter (-108/+27). Different concentrations of probe (specific activity = $1.345 \times 10^7$ cpm/μg) ranging from 2.8 pm (0.01 ng) to 5.6 nM (20 ng) was incubated with partially purified ICP4 (2 μg) and 4.0 μg of Poly dA·dT in a total volume of 20 μl for 45 min on ice. Tracking dye was added and the samples fractionated in the cold (4° C) at 20 mA constant current with buffer recirculation. The gel was dried and subject to autoradiography and analysis on the Ambis radioimaging system. Using the Ambis software, the % radioactivity of the complexed and free DNA was determined. These values were then transformed into concentrations of bound probe and bound:free probe. The ratio of bound:free probe versus the concentration of bound probe was plotted and analyzed by Scatchard analysis (Scatchard, 1949). Phase 1 of the biphasic plot is the highest affinity binding portion of the plot and can be seen in the lower portion of the Figure. The slope is equal to $-1/K_d$ and the X intercept is equal to the concentration of active ICP4 in the binding reaction. The autoradiograph of the mobility shift gel is seen in the middle of the Figure. Amount of probe present in each reaction is listed above each lane.
**Figure 44**

- **Phase 1**
- **Phase 2**

### Plot A
- **X intercept = 0.024 nM**
- **R SQUARE = 0.882**
- **Slope = \( 1/K_d \)**
- **K_d = 2.27 \times 10^{-11} M**

### Plot B
- **IE3 Probe**
- **-108/+27 (ng)**
- **Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15**
concentration using the Scatchard analysis. The plot for the WT IE-3 -108/+27 sequence can be seen (Figure 44). The Scatchard plot of the -108/-342 site is shown in Figure 45. Both plots (Figure 44A and Figure 45A) are biphasic. The first phase of each graph (greatest slope) has a lower $K_d$ (higher binding affinity) than the second phase. The slope on the first phase of the (-108/+27) IE-3 fragment was -44.3, the $K_d$ was equal to $2.3 \times 10^{-11}$ M and the X intercept was 0.024 nM. The slope of the first phase on the (-108/-342) IE-3 fragment was -5.8, the $K_d$ was equal to $1.7 \times 10^{-10}$ M and the X intercept was 0.028 nM (Figure 45). The relative binding affinity ($K_r$) of the capsite binding site to the upstream binding site was determined by comparing the $K_d$ for the (-108/+27) and the $K_d$ for the (-108/-342) fragment of WT IE-3. The relative binding affinity of the capsite sequence, which is the ratio of the equilibrium dissociation binding constant for the IE3 (-108/+27) probe divided by the equilibrium dissociation constant of the IE3 (-342/-108) probe, was $(1.7 \times 10^{-10}/ 2.3 \times 10^{-11})$ which results in a $K_d$ of 7.4. The $K_d$ determined by the mixed probe binding reaction was 6.6.
Figure 45. Determination of $K_d$ for the IE3 (-108/-342) ICP4 binding site. Double endlabeled probe prepared from p-342bend by digestion with Bgl II was used in the binding reactions. Different concentrations of probe (specific activity= $1.817 \times 10^7$ cpm/µg) ranging from 0.1 ng (27.8 pM) to 30 ng (8.3 nM) used in the binding reactions identical to those described in Figure 40. The gel was run and treated identically as described in Figure 40. Analysis of the gel was also the same as in Figure 40. Scatchard analysis of the phase 1 portion of the plot can be seen in the lower portion of the Figure. The slope of the phase 1 portion of the plot is equal to $-1/K_d$ and X intercept is equal to the concentration of active ICP4 in the binding reaction.
Figure 45

**Phase 1**

**Phase 2**

**IE3 Probe**
-108/-342 (ng)

**Figure 45**
ICP4 binds to a number of different HSV-1 gene sequences. These sequences are considered to be either consensus binding sites (Faber and Wilcox, 1986) or nonconsensus ICP4 binding sites (Michael et al., 1988). ICP4 appears to bind consensus binding sites with greater affinity than nonconsensus binding sites (Michael et al., 1988). I have determined the equilibrium dissociation constant of ICP4 for the wildtype IE3 consensus binding site and of the nonconsensus ICP4 binding site in the distal IE3 promoter (-211/-108). The relative binding affinity of ICP4 for its binding sites, on the promoters for IE4/5, IEx, IE3 D2 mutant, IE1 and the IE1 promoter mutations pJD101 and pJD102, were also determined.

Relative binding affinities for binding sites located on separate DNA fragments can be determined in mixed binding reactions containing the DNA binding protein in question and both DNA fragments independent of probe specific activity (Fried and Crothers, 1981). Two binding sites for ICP4 in the IE3 promoter were detected. One site is located at the transcriptional start site of IE3 and
another site (a nonconsensus site) is located distally between -108 and -211 of IE3 (Figure 39, lanes 15 through 17). Use of the distal ICP4 binding site as the standard (Kr= 1.0) in the mixed binding reactions allowed Kr's of the IE3 proximal and the IE3 D2 ICP4 binding sites to be determined. The IE3 proximal ICP4 binding site had a relative binding affinity 6.6x greater than that of the distal IE3 binding site (Figure 39, lanes 4 and 5) and 12.7x greater than the D2 (-6A -5T) mutant (Figure 40, lanes 5 and 6) (Table 3).

Comparison of Kr's for the IE1 and its mutants, IE4/5 and the IEx promoters revealed that ICP4 has a very high affinity for the IEx promoter, a high affinity for the IE1 promoter, a weak affinity for the IE4/5 and IE1 pJD101 promoter and a very weak affinity for the IE pJD102 promoter (Figure 42). The Kr of these promoters was determined using the mixed binding reaction described above. Using the IE4/5 ICP4 binding site as the standard (Kr= 1.0) the IE3 consensus binding site had relative binding affinity 13.1 fold greater than IE4/5, IE1 pJD101 was approximately equivalent to IE4/5 and the IE1 pJD102 Kr was 0.02 relative to that of IE4/5 (Table 3). Kr for IE1 was not able to be calculated in this manner due to a lack of resolution of complexes in the IE4/5 and IE1 mixed binding reaction (Figure 43, lane 6). The Kr value was estimated by
comparing the conversion of IE1 A4 complex formation to that of the IE4/5 complex formation. IE1 had a calculated $K_r$ of 27.3x that of IE4/5. This value is an accurate estimation based on the observation that the percent conversion used for IE4/5 was the value determined in three separate mixed binding reactions (IE3 short, IE1 pJD101 and IE1 pJD102 reactions). Additionally, the percent conversion for the IE3 short probe (a strong ICP4 binding site) did not vary more than 2% in value in either the single or mixed binding reactions (Figure 43, lanes 3 and 4), therefore, use of the IE1 conversion percentage should also be valid for determining $K_r$.

The IEx $K_r$ was also estimated but to a much less accurate degree. The relative binding affinity of the IEx ICP4 binding site was not analyzed by the mixed probe method but was instead estimated by visual inspection of many different exposures of Figure 42, comparing lanes 2 and 16 with lanes 11 and 19. It appears that the IEx binding site has a relative binding affinity at least 3 to 4 fold greater than the IE1 promoter.

Utilizing the data obtained from the mixed binding reactions a hierarchy of ICP4 binding sites has been determined based upon the calculated and estimated $K_r$'s. The order of relative binding strength is as follows IEx > IE1 > IE3 > IE3 distal > IE3 D2 mutant > IE4/5 = IE1 mutant
(pJD101) >>> IE1 mutant (pJD102). ICP4 also appears to have a slightly lower relative binding affinity for its binding site in gD (-112) and the Vmw65 (+154) compared to the IE3 +1 site. This conclusion was based upon numerous different mobility shift assays containing the IE3 +1 site and either the gD -112 binding site or the Vmw65 +154 binding site (Figure 16 and data not shown). Identifying ICP4 binding sites, determining the relative binding affinity to these sequences and comparison of the placement of the site to other cis sites will be instrumental in elucidating the overall effect ICP4 binding has on gene expression. This topic will be examined in Chapter 5.

The equilibrium dissociation constant ($K_d$) value ($2.3 \times 10^{-11}$ M), determined for the IE3 proximal promoter (+1) complex formed at 0°C in 30 mM KCl (Figure 44), is two orders of magnitude lower than the value ($1 \times 10^{-9}$ M) determined for ICP4/IE1 promoter complex formed in 100 mM NaCl at 4°C (Kattar-Cooley and Wilcox, 1989). The $K_d$ value of ICP4/IE3 (+1) complex is higher than the $K_d$ value of the lambda repressor ($3 \times 10^{-13}$ M) (Johnson et al., 1980) or E. Coli lac repressor ($1 \times 10^{-11}$ M) (Riggs et al., 1970), but less than $K_d$ values for DNA complexes with SV40 large T antigen ($0.4$ to $4.0 \times 10^{-9}$ M) (Muller et al., 1987) or the E. Coli tryptophan (trp) repressor ($2.6 \times 10^{-9}$ M) (Klig et al., 1987). Comparison of these values is inappropriate
because binding conditions and methods of analysis were not the same for each of these experiments.

The $K_d$ value determined by Kattar-Cooley and Wilcox, (1989) is different from the value determined in this study for a number of reasons. First, the two different binding sites are known to have different relative binding affinities for ICP4 (Table 3). IE1 has a $K_r$ 2x greater than the $K_r$ of IE3, indicating that the $K_d$ of the IE1/ICP4 complex should be 2x less than that of the ICP4/IE3 complex. Secondly, the ICP4/IE1 complex was formed in 100 mM NaCl, 0 mM NaPO$_4$ and 10 mM MgCl$_2$. The ICP4/IE3 complex was formed in 30 mM KCl, 12.5 mM NaPO$_4$ and 0 mM MgCl$_2$. Both monovalent salt conditions are low enough to be comparable and do not effect ICP4 binding differently; however, addition of NaPO$_4$ to the binding reaction stimulates A4 complex formation (Figure 7) and addition of MgCl$_2$ to concentrations greater than 2.0 mM result in a dramatic decrease in A4 complex formation both when partially purified and affinity purified ICP4 was used as a source of protein (Figures 14 and 15). The addition of NaPO$_4$ and elimination of MgCl$_2$ to the binding reactions used in this study could easily account for the discrepancy between $K_d$ values determined here and that observed by Kattar-Cooley and Wilcox (1989). Lastly, it should be noted that $K_d$ determinations made here used the mobility shift assay as opposed to the filter binding
technique employed by Kattar-Cooley and Wilcox. The filter binding technique cannot differentiate non-specific protein/DNA complexes from specific protein/DNA complexes while the mobility shift assay does (Fried and Crothers, 1981). Inclusion of non-specific complexes with specific complexes will erroneously decrease the $K_d$ value using the filter binding method as opposed to the mobility shift assay. Mobility shift assays, intrinsically, do not have this problem. Non-specific complexes will either not survive migration through the gel or will not migrate as the specific complex does. Therefore, use of the filter binding technique may result in lower $K_d$ values as compared to those derived by mobility shift assay. This difference does not play a role here because, the variance in $K_d$ values is so great in favor of IE3 rather than IE1.

The distal IE3 ICP4 binding site's $K_d$ value was determined to be $1.7 \times 10^{-10}$ M (Figure 45). Calculation of $K_r$ between the proximal and distal IE3 ICP4 binding sites was 7.4 based upon the $K_d$ values. This value is in agreement with the value of 6.6 calculated from the $K_r$ of the mixed binding reaction with both probes (Figure 39, lanes 4 and 5). Additional support for the accuracy of the $K_d$ determinations came from analyzing the binding data as a Scatchard plot (Scatchard, 1949). The value of the active protein concentration in the reaction can be determined from...
the X intercept and should be equivalent. This value is not the same for both of the IE3 ICP4 binding sites (Figures 44 and 45) indicating that there is some error in experimental protocol or in measurement. The active ICP4 concentration values are similar therefore, the calculated K_d's appear to be representative.

Multiple binding sites for ICP4, with varying affinity, may allow for the fine tune control for both stimulation and repression of a promoter having multiple sites. This could be accomplished, by varying the affinity of binding to different sites in response to post-translational modification of ICP4. It has been shown, that the various post-translationally phosphorylated forms of ICP4 bind consensus and nonconsensus ICP4 binding sites with apparently different affinities (Michael et al., 1988). It is not unreasonable to envision ICP4 binding to various ICP4 binding sites, with various affinities, depending upon its state of post-translational modification. Once bound, ICP4 could interact with the cellular transcriptional machinery via protein-protein interactions to either stimulate or repress gene expression.

Posttranslational modification may be important for the control of the gene class expression pattern observed during a productive HSV infection. ICP4, at increasing times PI, is post-translationally modified and its migration in SDS-
PAGE gels slowed so that three different forms of the protein are seen at early times PI, but appear as a single band at late times PI (Wilcox et al., 1980; Faber and Wilcox, 1986b). As the infection progresses, only the slowest migrating form of ICP4 is observed (Wilcox et al., 1980) (Figure 2). Post-translational modifications, such as phosphorylation, may not play a role in the ability of ICP4 to bind DNA (Deluca and Schaffer, 1988; Patterson and Everett, 1988). Instead, they may modulate ICP4-protein interactions, as they do in dimer formation of the cyclic AMP response element binding protein (CREB) (Yamamoto et al., 1988).

ICP4 could also influence the binding of other transcription factors, should their cognate cis binding sites be near the ICP4 binding site. This influence may result via DNA conformational changes at the cis sites as a result of ICP4 binding. Examples, of this, have been demonstrated for the TATA region in the IE1 and IE3 promoters (DiDonato and Muller, 1989) and in the IEx promoter (Figure 27, lanes 8 and 10). Binding of ICP4 could also preclude binding of other transcription factors having overlapping binding sites. An example, of this type of regulation, is evidenced by the large T antigen of SV40, which autoregulates its own expression (Rio and Tjian, 1983). ICP4 binding to DNA close to the other cis sites
could interfere with transcription factor interactions between each other or with the transcriptional apparatus.
CHAPTER V
TRANSIENT EXPRESSION ASSAYS OF
IMMEDIATE-EARLY PROMOTER DRIVEN CHIMERAS

INTRODUCTION

It is known that genes contain cis-regulatory elements upstream of the 5' start site of transcription and are frequently positive regulators of gene activity (McKnight and Tjian, 1986). IE gene 3 of HSV-1 encodes a 175 kDa phosphoprotein, designated ICP4, that is a trans-activator of other viral genes and is essential for lytic replication of the virus (Wilcox et al., 1980; Preston, 1979; Watson and Clements, 1980). In addition to being required for expression of the early and late classes of herpes genes, a number of studies suggest that ICP4 regulates its own expression and those of other IE genes negatively (Dixon and Schaffer, 1980; Preston, 1979; Deluca and Schaffer, 1984). The promoter region of IE3 contains multiple SP1 binding sites that have been mapped in vitro (Jones and Tjian, 1985). Additionally, there are several copies of the TAATGARAT sequence, when complexed with the virion
associated trans-activator (65Ktif) (Mackem and Roizman, 1982; Gaffney et al., 1985) and one or more cellular factors (O'Hare and Goding, 1988, Preston et al., 1988, Kristie and Roizman, 1987), elevates the level of expression of IE3. ICP4 is a DNA binding protein and studies in this laboratory (Muller, 1987; DiDonato and Muller, 1989; DiDonato and Muller, 12th International Herpesvirus Workshop, 1987, p293) and others (Faber and Wilcox, 1988; Roberts et al., 1988) have demonstrated that ICP4 binds specifically near its own transcriptional start site.

Transient-expression assays, with the cloned wild-type IE gene 3, revealed that early and late genes are induced by ICP4. IE3 (and other IE genes) are repressed by ICP4 (DeLuca and Schaffer, 1985, Gelman and Silverstein, 1985, O'Hare and Hayward, 1985a, O'Hare and Hayward, 1985b); therefore, the transient-expression assay results reflect those seen in the in vivo situation, where ICP4 levels (transcription) are down regulated within a few hours post infection (Dixon and Schaffer, 1980; Preston, 1979). The details of negative regulation by ICP4 have not been firmly established, although recent studies have strongly indicated an autoregulatory mechanism (Muller, 1987; DeLuca and Schaffer, 1988; Faber and Wilcox, 1988; Roberts et al., 1988; DiDonato and Muller, 1989; DiDonato and Muller, 12th International Herpesvirus Workshop, 1987, p293).
In this chapter, I have addressed this problem in two ways. First, by analyzing the WT IE promoters linked to indicator genes, the basic reaction to ICP4 can be determined. Secondly, alteration of these promoters in the ICP4 binding site by mutation, deletion, insertion of additional sites at different positions or by changing the spacing of the ICP4 binding site to TATA should give insight as to how ICP4 mediates its effect in these genes. To easily visualize the different mutant promoters, a diagram of the relevant constructions is located at the end of the chapter in Figure 60.
RESULTS

Transient expression assay and RNA analysis of pWT-GH.

To investigate the influence of ICP4 binding on expression from the IE3 promoter it was important to establish the validity of the expression system at both the protein and the RNA level. The transient expression system employed in these experiments was the human growth hormone (hGH) assay system (Selden et al., 1986) and the chloramphenicol acetyl transferase (CAT) assay system (Gorman et al., 1982). Assays for hGH, a secreted gene product, are performed on cell culture supernatants. (This reporter gene was selected over the thymidine kinase gene (TK) because the TK gene displayed some low level binding to ICP4 in vitro (Figure 6) and the influence of these cryptic sites on ICP4 transactivation is unknown. Later experiments were performed using CAT as the indicator gene due to its easy manipulation in cloning of the different constructs.

The wild type IE3 promoter from (-333/+27) was fused to a promoterless hGH gene to form pWT-GH. The impact of two effector genes (pMC1, which encodes 65K_tif [also called
Vmw65] and pGX58 which encodes ICP4) on the expression of pWT-GH was evaluated. Co-transfection of pWT-GH with increasing molar gene equivalents (MGE's) of 65Ktif resulted in increased hGH levels (Figure 46, panel A, solid line). In the presence of ICP4 and 65Ktif (dashed line), hGH levels decreased to roughly 50% of basal level hGH expression. This was consistent with the findings of other investigators using IE3 TK or CAT chimeras in similar transient assays (DeLuca and Schaffer, 1985; Everett, 1984; Gelman and Silverstein, 1987a; 1987b; O'Hare and Hayward, 1985; O'Hare and Hayward, 1987; Quinlan and Knipe, 1985; Roberts et al., 1989). Nuclease S-1 protection assays confirm negative regulation is reflected at the RNA level. The levels of hGH mRNA increase from basal expression levels upon addition of 65Ktif (Figure 46, panel B, compare lanes 3 and 4 with 5). When ICP4 (pGX58) is added to the co-transfections, in increasing molar gene equivalents with 65Ktif (pMC1), hGH mRNA decreased to approximately 30% of the levels detected in the absence of ICP4 (compare lanes 1 and 2 with lanes 3 and 4).

ICP4 represses expression of pWT-GH to a level clearly below that of basal level expression (compare lanes 1, 2 with lane 5). These results and others (using CAT reporter genes, data shown below) demonstrate that ICP4 effectively represses basal level expression and 65Ktif enhanced
expression. Additional control experiments that include addition of a plasmid containing multiple copies of the IE3 promoter to the cotransfection, confirm that down regulation cannot be attributed to titration of a required transcription factor(s) or "promoter competition" (data not shown).

Mutations in the ICP4 DNA binding site of IE3 alter negative regulation.

The virion protein, 65Ktif, stimulates expression from IE genes and is also reflected with IE chimeras in transient co-transfections (Post et al., 1981; Mackem and Roizman, 1982a; 1982b; Cordingly et al., 1983; Bzik and Preston, 1986; Campbell et al., 1984; , Gelman and Silverstein, 1986; 1987a; 1987b;, Kristie and Roizman, 1984; O'Hare and Goding, 1988;, Preston et al., 1988, and Figure 47). The wild type (pWT-GH) and mutant (pD2-GH) reporter genes were co-transfected with either 65Ktif or 65Ktif + ICP4 as effectors. Both indicator genes were stimulated by 65Ktif although, basal levels of expression were slightly different between the two (Figure 47, panel C, solid lines). Addition of ICP4 (Figure 47, panel C, dashed lines) resulted in down regulation of the wild type promoter, pWT-GH. The mutant (pD2-GH) was unresponsive to ICP4.
Figure 46. Transient expression assays and S1 analysis of transiently expressed RNA. A. Transfection experiments of BHK cells were performed as in Materials and Methods with 2.5 µg of pWT-GH as the indicator gene per 100cm² plate. The indicator gene was transfected alone or in the presence of 0.5, 1 and 2 molar gene equivalents of the effector plasmid pMC1 which encodes the virion stimulatory protein 65K_{tif} (VP16). Molar gene equivalents are the ratios of the number of effector genes present compared to the number of indicator genes in the transfection. Tissue culture media samples were obtained at 48 h post transfection and assayed for the levels of secreted hGH in the growth media for each different molar gene equivalent of effector studied using the Allegro hGH kit (Nichols institute, San Francisco, CA.) (see Materials and Methods). Cotransfections were performed exactly as described above except that pGX58 which encodes ICP4 was also added as an effector at the designated levels of molar gene equivalents of effector. The hGH levels of each of the cotransfections were also measured using the Allegro hGH kit. B. S1 nuclease analysis was performed on total RNA prepared from each plate of transfected or cotransfected BHK cells as described in Materials and Methods. The probe used in the S1 analysis was the Ava II to Eco RI probe of IE3-GH (+62/-108). RNA from cells transfected with indicator gene only protected the probe in lane 5 from S1 nuclease digestion. Lanes 4 and 3 contain S1 nuclease protected probe from transfection of 1 and 2 MGE of pMC1. RNA obtained from cotransfection of 1 and 2 MGE of pMC1 + pGX58 protected the probe in lanes 2 and 1. The G and G + A sequencing ladders are in lanes 6 and 7. The DNA sequence surrounding the mRNA cap site is shown to the right as is the transcriptional initiation site.
Figure 46

**A.**

- DNA: $\frac{65K_{tff}}{+ICP4}$
- MGE: 2 1 2 1 0 A G

**B.**

- A G A G
- C G T G C C

**Figure 46**
Furthermore, pD2-GH was not activated to the same extent as the wild type promoter by 65K_tif, possibly because the deletion altered some aspect of stereo-specific alignment between TAATGARAT and other cis-acting sites (see also Takahashi et al., 1986). To avoid this complexity (see below), I determined whether ICP0 stimulated the wild type (pWT-GH) and deletion (pD2-GH) promoters equally.

ICP0 is an immediate early gene product (Honess and Roizman, 1974; Periera et al., 1977) and can trans-activate expression from many cellular and viral promoters (Everett, 1984; Gelman and Silverstein, 1985; 1986; 1987a; 1987b; O'Hare and Hayward, 1985a; 1985b; Quinlan and Knipe, 1985). Unlike ICP4 and 65K_tif, a specific DNA target sequence through which ICP0 mediates its activity has not been defined. In transient assays with pWT-GH and pD2-GH, ICP0 stimulated expression of both constructions to the same degree (Figure 47, panel A, solid lines). When ICP4 was added to the co-transfection, expression levels of pWT-GH remained essentially at basal levels (Figure 47, panel A, dashed lines). Thus, ICP4 exerts a negative effect on its own promoter in the presence of ICP0, which is a potent trans-activator. The expression levels of the deletion mutant pD2-GH were not diminished by ICP4 in trans. The response of the mutant (pD2-GH) and wild type (pWT-GH) promoters, to ICP0, were essentially identical. Addition of
ICP4 did not influence the deletion mutant to the extent that WT-GH was (panel A). The -6A/-5T bases in the A4 site were required for negative regulation of IE gene 3 by ICP4 as evidenced by pD2-GH's lessened sensitivity to ICP4 mediated negative regulation.

To examine ICP4 responsive elements further, constructions containing the minimal promoter elements of IE3 (-108/+27) were used in transient transfection experiments (Figure 47, panel D) (Lang et al., 1984; Gelman and Silverstein, 1987). The minimal promoter constructs would discriminate between proximal (negative) regulatory elements and the distal (positive) IE regulatory site contributions to expression such as TAATGARAT and the possible interplay between transcription factors through protein-protein interaction. Note, Figure 47 (panel C) shows that alterations in the A4 binding site exert some influence over the ability of 65K* to trans-activate the promoter. The minimal promoter element is a weak promoter; therefore, I used ICP0 to stimulate expression. When co-transfected with ICP0 the minimal wild type (pEWT-GH) and deletion (pED2-GH) promoters were stimulated to different levels (Figure 47, panel B). ICP4 reduced expression of pEWT-GH to roughly 35% of basal levels indicating the wild type minimal promoter was also down regulated by ICP4. In the presence of ICP4 and ICP0, the mutant minimal promoter
Figure 47. Transient expression using wild type and deletion mutant full and minimal IE3 promoters. BHK cells were transfected with 4 μg of either the full (-333/+27) or minimal (-108/+27) promoter IE3-hGH chimeras and increasing molar gene equivalents (MGEs) of effector. A. The wild type chimera pWT-GH ( ) or the deletion mutant pD2-GH ( ) which contain the full IE3 promoter were cotransfected with increasing MGEs of pMC1 (65Kbif) as the effector gene ( ) or with pMC1 + pGX58 ( ) as the effector genes. The growth media was harvested after 48 hours and assayed for hGH which was secreted into the media. B. The minimal wild type chimera pEWT-GH ( ) or the deletion mutant pED2-GH ( ) were transfected as above. Increasing MGEs of p111 (ICP0) was used as the effector gene ( ) or p111 + pGX58 was used as the effector genes ( ). Growth hormone was assayed 48 h post transfection as in Fig. 9A. C. Increasing MGEs of pMC1 ( ) or pMC1 + pGX58 ( ) were cotransfected with either pWT-GH ( ) or pD2-GH ( ). Growth media samples were harvested at 48 hours post transfection and assayed for hGH secreted into the tissue culture media. D. Diagram of the full and minimal wild type and deletion mutant chimeras used in the transfection experiments.
Cotransfection of either pWT-GH or pD2-GH with the genes encoding ICPO and/or ICP4

A

Cotransfection of pEWT-GH or pED2-GH with the genes encoding ICPO and/or ICP4

B

Cotransfection of pWT-GH or pD2-GH with the genes encoding 65K and/or ICP4

C

Indicator Genes:

D

Figure 47
(pED2-GH) displayed eight fold more activity than basal level. The mutant was stimulated by ICP0 + ICP4 in a synergistic manner. The mechanism of this stimulation is unknown; however, these data reveal that a functional ICP4 binding domain, located near the transcription start site, can offset the synergism.

It is known that ICPO in conjunction with ICP4, can synergistically trans-activate cellular, early and late viral promoters (Everett, 1984; 1986; Gelman and Silverstein, 1985; 1986; 1987a; 1987b; Navromara-Nazos et al., 1986; O'Hare and Hayward, 1985a; 1985b; Quinlan and Knipe 1985; DiDonato and Muller unpublished observations). I conclude that pED2-GH was stimulated by ICP0 + ICP4 in a synergistic fashion with a minimal promoter element but not with the full promoter. The minimal IE3 promoter tested here was very similar to early and late class HSV-1 promoters (reviewed Everett, 1988). Since the synergism was originally demonstrated with early promoters (Everett, 1984; 1986), this may explain these findings. A common theme of the co-transfection data was that an intact ICP4 DNA binding sequence at the transcriptional start in IE3 resulted in negative regulation when ICP4 was present. Once the A4 sequence was disrupted, as in pD2-GH or in pED2-GH, negative regulation by ICP4 was abolished.
Further studies on the effects of ICP4 on IE3 and other IE chimeric genes utilized the chloramphenicol acetyltransferase (CAT) gene as the reporter gene. The change to the CAT reporter gene was done for two reasons. First, as mentioned previously, various chimeras could be constructed more easily if the CAT gene was used instead of the hGH gene. Second, even though levels of correctly initiated IE3 hGH RNA and hGH protein correlate with each other (Figure 46, panels A and B) the differences in levels of RNA vary to a much greater extent than do the levels of protein. To circumvent this, the CAT gene was used as the reporter gene in all subsequent experiments. Previous studies by other investigators have shown a direct relationship between CAT enzyme activity and CAT RNA driven from the IE3 promoter (O'Hare and Hayward, 1985a; Gelman and Silverstein, 1986).

**Effect of viral transactivators on expression of various chimeric IE3 indicator genes in transient transfection assays.**

ICP4's ability to regulate expression of various IE3 promoter constructions fused to CAT was examined in the presence and absence of Vmw65 (the virion transinducing factor) in transient expression assays. The amount of
transiently transfected cell extract used in the CAT assay was adjusted so that basal level expression (the expression due to the indicator gene alone) was appreciable. This allowed a decrease in expression to be more readily observable.

WT IE3 promoter (-333/+27) fused to CAT (designated as pJD10CAT) was transfected into baby hamster kidney cells (BHK) either alone or with 1 MGE of the plasmid encoding Vmw65 (pMC1) or with 1 MGE of Vmw65 (pMC1) + ICP4 (pGX58) using the calcium phosphate precipitation method (Graham and van der Ebb, 1973). Additionally, ICP4 alone was included in the cotransfection. The cells were harvested at 36 - 48 hr post transfection and cell extracts prepared as described in Materials and Methods. Basal activities were defined as having a specific activity of 1.0. The change in specific activity for each experimental permutation, was calculated. Vmw65 was capable of stimulating the WT IE3 promoter (-33/+27) 1.6x that of basal level expression (Figure 48, lanes 3 and 4). When ICP4 was included with Vmw65 in the cotransfection the change in CAT specific activity was reduced 7.2 fold relative to basal level expression (lanes 5 and 6). ICP4 alone resulted in a 10.6 fold reduction in activity (lanes 7 and 8). This result was consistent with the findings of other investigators (Gelman and Silverstein,

The D2 IE3 promoter (-333/+27; -6A/-5T deletion in the core ICP4 consensus binding site) fused to CAT (designated as pJD11CAT) was then evaluated. The D2 chimera was stimulated 3 fold by Vmw65 (Figure 49, lanes 3 and 4). Addition of ICP4 + Vmw65 resulted in a slight (1.4 fold) decrease in activity relative to basal level expression (lanes 5 and 6). Cotransfection with ICP4 resulted in a 2 fold decrease in activity from basal level (lanes 7 and 8). This observation is in agreement with results from another laboratory, which had generated and examined this same mutant (Roberts et al., 1988).

To investigate the effect that deletion of the entire ICP4 binding site plus a few additional bases in the IE3 promoter (-18/+27; the pJD40 construction) the mutant pJD40CAT (see diagram in Figure 50) was used in transient expression assays similar to the ones described immediately above. Vmw65 stimulated expression 6.7 fold relative to basal level expression (Figure 50, lanes 3 and 4). Addition of ICP4 + Vmw65 resulted a 1.3 fold reduction in activity (lanes 5 and 6) and a 1.8 fold increase over basal levels, when ICP4 alone was included in the cotransfection (lanes 7 and 8). Deleting the ICP4 binding site (and replacing it with polylinker sequence) resulted in the slight stimulation
Figure 48. Activity of WT IE3 promoter (Fused to CAT) under the influence of various trans-activators encoded by the virus. WT IE3 CAT (pJD10CAT) (4.0 μg) was used as the indicator gene in this transient transfection experiment, performed in duplicate, examining the effect Vmw65 (pMC1) has on stimulating expression in the presence and absence of ICP4 (pGX58). The ability of ICP4 to negatively regulate the IE3 promoter is also examined. The change in CAT specific activity is indicated above the duplicate transfections. The effector plasmids pMC1 and pGX58 were added to the cotransfections as 1 molar gene equivalent (MGE). The cells were transformed by the calcium phosphate method described in Materials and Methods.
<table>
<thead>
<tr>
<th>Change in CAT specific activity:</th>
<th>+1</th>
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<td></td>
</tr>
<tr>
<td>+Vmw65+ICP4</td>
<td></td>
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</tr>
<tr>
<td>+ICP4</td>
<td></td>
<td></td>
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</tbody>
</table>

Figure 48
Figure 49. Activity of Mutant (AT deleted) IE3 promoter under the influence of viral trans-activators. The -6A, -5T deletion mutant pD2 (pJD11CAT) (4 µg) was examined in a transient transfection experiment exactly as the WT version of IE3 (pJD10CAT) was in Figure 48. Duplicate transfections are labeled as to the effector used and the change in CAT specific activity is listed above each transfection.
Change in CAT specific activity: +1 +3 -1.4 -2
Basal + Vmw65 + Vmw65 + ICP4 + ICP4

Mutated A4 Site: ATCGTC to **CGTC [2bp deletion]

Figure 49
by ICP4 in the transient expression assays (compare lanes 1 and 2 with lanes 7 and 8).

**Effect of additional A4 sites placed 5' of the TATA box on expression of a linked gene.**

It is known that when an ICP4 binding site is located far upstream from TATA, as in the gD promoter, a linked gene can be positively modulated by ICP4 (Everett, 1984; Beard et al., 1986; Tedder and Pizer, 1989; Tedder et al., 1989). When the ICP4 binding site is 5' to TATA but close (< 45 bp), as in IE1, ICP4 can regulate a linked gene driven by this promoter negatively (Gelman and Silverstein, 1986; 1987; Resnick et al., 1989; this study). To further examine the affect that positioning the ICP4 binding site 5' relative to TATA in the IE3 promoter would have on expression, the following constructions were made. The two constructs pJD32 and pJD33 are derivatives of the WT IE3 (-333/+27) and D2 IE3 (-333/+27) promoters respectively. The A4 binding site (-18/+27) of IE3 has been placed in these two promoters at the Eco RI site (-108). Therefore, in pJD32, there are two A4 binding sites. In pJD33 there is one A4 binding, the A4 site at +1 contains the D2 deletion. The CAT gene was fused to these promoter constructions and used in transient transfection experiments with and without
Figure 50. Effects of deletion of the A4 site (ATCGTC) on activity of the IE3 promoter. A deletion taking out sequences between -18 and +27 of IE3 was replaced by vector polylinker sequences to make pJD40CAT. This construct (4 µg) was tested in a duplicate transfection experiment to observe the effect deletion of the A4 sequence had on stimulation by Vmw65 (pMC1) and negative regulation by ICP4 (pGX58). Change in CAT specific activity is listed above the effector genes used.
Change in CAT specific activity: +1  
+6.7  
+1.8  
Basal  
+Vmw65  
+Vmw65+ICP4  
+ICP4  

PJD40CAT  
Deleted  
+27  
+1  
A4  
IE3  
TATA  
TAATGARAT  
TAATGARAT  
CAT  
20 bp deletion  
-110  
-265  
-333  

Figure 50
the addition of ICP4 and Vmw65. ICP4 cotransfected with Vmw65 resulted in an 18 fold decrease in activity of pJD32CAT (Figure 51, lanes 5 and 6) and a 11 fold decrease from basal levels of pJD33CAT (Figure 52, lanes 5 and 6). ICP4, added to the transfection, caused an 18 fold reduction in activity of pJD32DAT (Figure 51, lanes 7 and 8). Similarly, activity of pJD33CAT decreased 7.5 fold relative to basal levels in the presence of ICP4 (Figure 52, lanes 7 and 8). Addition of the second A4 site at -108 resulted in a more pronounced negative regulatory effect by ICP4 in these constructions compared to the parental plasmids (pJD10CAT and pJD11CAT) (compare Figure 48, lanes 7 and 8 with Figure 51, lanes 7 and 8; also, compare Figure 49, lanes 7 and 8 with Figure 52, lanes 7 and 8).

Positional translocation of the A4 site in the IE3 promoter and its effect on gene expression.

The effect of differential positioning of the A4 site, on expression from the IE3 promoter, was examined by assaying various mutants fused to the CAT gene in transient transfection assays. The spacer mutant pJD45CAT contains the WT IE3 promoter (-333/+27) and has 22 bp vector sequence inserted between TATA and the A4 site at the -18 position. This insertion increases the distance between TATA and the
Figure 51. Effects of adding a second A4 site (+27/-18) 5' of the TATA box. WT IE3 CAT (pJD11CAT) was used to insert the +27 to -18 region of IE3 at position -108. This new construct (pJD32CAT) has two complete copies of the A4 sequence. Vmw65 and ICP4 were tested in duplicate transfection experiments to examine the effect ICP4 has when there are two binding sites, one is 5' of TATA. Change in CAT specific activity is listed above the effector genes.
Change in CAT specific activity: +1 +1 -18.2 -18.6 Basal +Vmw65 + Vmw65 + ICP4 + ICP4

pJD32CAT

1st copy of A4 (WT) 2nd copy of A4 (WT)

Figure 51
Figure 52. Effects of a second A4 site at -108 and a mutated site at +1. The D2 mutant CAT construct (pJD11CAT) was used to insert a second copy of the A4 site at -108. This experiment is identical to the experiment described in Figure 51. Change in CAT specific activity is listed above the effector genes.
Change in CAT specific activity: +1 +1.2 -10.8 -7.5

Basal + Vmw65 + Vmw65 + ICP4 + ICP4

pJD33CAT

IE3

CAT

TATA

WT

A4

TAATGARAT

(-6A, -5T) deletion in A4 (D2)

2nd copy of A4 (WT)

Figure 52
A4 site from 26 bp to 48 bp (DiDonato and Muller, 1989). When pJD45CAT expression was analyzed in transient cotransfection assays, basal level expression was similar to basal level expression of WT IE3CAT (pJD10CAT) or D2 IE3CAT (pJD11CAT) (Figure 53, compare lanes 9 and 10 with lanes 1, 2, 5 and 6); however, pJD45CAT was not down regulated by ICP4 (Figure 53, lanes 11 and 12). In contrast, WT IE3CAT was down regulated by ICP4 (6.4 fold from basal level pJD10CAT expression lanes 1 and 2). The D2 mutant (pJD11CAT) was down regulated slightly when ICP4 was included in the transfection (lanes 7 and 8).

A4 position mutants were also examined to determine their effect upon moving the A4 site 5' of TATA. The A4 deletion mutant pJD40CAT which lacks an A4 site (-18/+27) (described in Materials and Methods and in DiDonato and Muller, 1989) was used as the parental plasmid to construct pJD42CAT and pJD43CAT. Both contain one copy of the -18/+27 A4 site of IE3 inserted at the -211 position (NcoI site). The orientation of the A4 insert in PJD42CAT 5' to 3' was -18/+27 and in pJD43CAT the A4 insert was in the opposite orientation. Basal level expression of each construct (pJD40CAT, pJD42CAT and pJD43CAT) in transient expression assays was approximately the same (Figure 53, compare lanes 13, 14, 17, 18 and 19). ICP4 failed to down regulate these three constructs. Expression from all three constructs were
Figure 53. Trans-regulation by ICP4 of WT and Mutant IE3 promoters driving expression of CAT indicator genes. The A4 site placement mutants (pJD42CAT and pJD43CAT) were assayed along with the A4 site spacer mutant pJD45CAT and the total A4 site deletion mutant pJD40CAT. The WT and D2 mutan were assayed as a comparative control. There was 4 μg of indicator used per dish. Transfections were done in duplicate. Change in CAT specific activity from basal levels is listed below the effector gene.
### Figure 53

#### Change in Cat Specific Activity:

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<th>WILD TYPE IE3</th>
<th>IE3 AT DELETION</th>
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<td></td>
<td>pJD10 CAT</td>
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<td>pJD45 CAT</td>
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### A4 Site @+1 Deleted and Moved To:

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<th>-211 (3’-5’)</th>
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<td>pJD42 CAT</td>
<td>pJD43 CAT</td>
</tr>
<tr>
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<td>+1.5</td>
<td>+1.4</td>
<td>+1.2</td>
</tr>
<tr>
<td>BASAL + ICP4</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Trans-regulation of A4 placement mutants by 65K\textsubscript{tif} and ICP4. The constructions pJD40CAT (IE3 -18/+333), pJD42CAT (pJD40CAT with the -18/+27 of IE3 inserted at the -211) and pJD43CAT (same as pJD42CAT only the insert is in the opposite orientation; see Materials and Methods for details of the insert orientation) were assayed for the ability to be trans-activated by 65K\textsubscript{tif} or with ICP4 or in combination. The amount of indicator plasmid per transfection reaction was 2 \textmu g per 60 mm\textsuperscript{2} dish. Effector plasmids were added to 1 MGE.
### TABLE 4.

<table>
<thead>
<tr>
<th>INDICATOR GENE</th>
<th>BASAL</th>
<th>65K&lt;sub&gt;eff&lt;/sub&gt;</th>
<th>65K&lt;sub&gt;eff&lt;/sub&gt; + ICP4</th>
<th>ICP4</th>
<th>EFFECTOR GENE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJD40CAT</td>
<td>4.2</td>
<td>81.3</td>
<td>6.2</td>
<td>5.6</td>
<td>% CONVERSION</td>
</tr>
<tr>
<td></td>
<td>+1</td>
<td>+19.3</td>
<td>+1.5</td>
<td>+1.3</td>
<td>CHANGE IN CAT SPECIFIC ACTIVITY</td>
</tr>
<tr>
<td></td>
<td>4.7</td>
<td>74.0</td>
<td>18.9</td>
<td>11.7</td>
<td>% CONVERSION</td>
</tr>
<tr>
<td>pJD42CAT</td>
<td>+1</td>
<td>+15.7</td>
<td>+4.0</td>
<td>+2.5</td>
<td>CHANGE IN CAT SPECIFIC ACTIVITY</td>
</tr>
<tr>
<td>pJD43CAT</td>
<td>13.9</td>
<td>79.6</td>
<td>44.8</td>
<td>37.6</td>
<td>% CONVERSION</td>
</tr>
<tr>
<td></td>
<td>+1</td>
<td>+5.7</td>
<td>+3.2</td>
<td>+2.7</td>
<td>CHANGE IN CAT SPECIFIC ACTIVITY</td>
</tr>
</tbody>
</table>

Transient expression assays of ICP4 binding site position mutants
stimulated slightly (between 1.2 to 1.5 fold see lanes 15, 16, 19, 20, 23, and 24). These three chimeras were also tested for positive regulation by Vmw65 in transient cotransfections. Vmw65 positively modulated expression of all three constructs (5 to 19 fold above basal level expression of each, see Table 4). Cotransfection with ICP4 + Vmw65 stimulated expression levels 1.5 to 4 fold relative to basal level expression (Table 4). ICP4 alone caused a slight stimulation of these three chimeras (Table 4).

A nonconsensus ICP4 binding site exists in the IE3 promoter between position -108 and -211 (Michael and Roizman, 1989). This region is also a part of the enhancer/UAS portion of the IE3 promoter. The following constructions were analyzed by transient expression assays to ascertain the importance of this nonconsensus site in regulation of IE3. Wild type IE3 and the D2 IE3 promoters were each cut with Eco RI and NcoI, the ends filled (with the large fragment of E. Coli DNA polymerase I) and religated. A 99 bp deletion (between -112 and -211 of IE3) is constructed. The WT version of this deletion was designated as pJD34 and the D2 version was pJD35. The CAT gene was placed at the +27 position (placed identically as in pJD10CAT and pJD11CAT). Basal level expression from both pJD34CAT and pJD35CAT was less than those of the parental constructs. Both mutants were down regulated by the
addition of Vmw65 + ICP4. Addition of ICP4 reduced activity in pJD10CAT 10.6 fold (Figure 54, lanes 7 and 8) and 3.0 fold in pJD34CAT (lanes 15 and 16) relative to the basal activity of each. The D2 mutants were slightly down regulated (1.6 fold relative to basal activity, lanes 23, 24 [pJD35CAT] and 1.1 fold, lanes 27, 28 [pJD11CAT]).

ICP4 negatively regulates expression from the IE1 promoter in transient assays.

The minimal IE1 promoter constructions previously described (pJD100, pJD101, and pJD102) were fused to the CAT gene and used in transient expression assays to determine the effect ICP4 had on expression of a linked indicator gene controlled by these promoters. The minimal promoter element of IE1 was used to simplify the analysis by eliminating any contributions of upstream enhancer sequences on expression (Ebert and Muller, unpublished observations). The A4 site in pJD101CAT and pJD102CAT carry progressively more severe changes in the core ICP4 consensus sequence (see Figure 55 for a diagram of the IE1 constructions). These three chimeras allowed comparison between the effect of ICP4 on expression in transient assays and the integrity of the A4 binding site as it was made progressively worse (pJD101CAT and pJD102CAT). The spatial relationship between
a possible CTF binding site at -79 and an Spl binding site at -59 and its impact on basal level expression was also examined (pJD100CAT and pJD101CAT vs pJD102CAT). Basal level expression of the minimal WT IE1 promoter was 1.3 fold greater than that of pJD101CAT and 4 fold greater than pJD102CAT (Figure 56, compare lanes 1 and 2 with 5, 6, 9 and 10). ICP4 strongly down regulated the WT IE1 chimera 44 fold (lanes 3 and 4), while pJD101CAT and pJD102CAT were down regulated to a lesser degree (8.5 and 4 fold respectively, [compare lanes 11, 12 with lanes 7 and 8]).

**ICP4 trans-regulation of other IECAT chimeras.**

Searching the HSV-1 IE gene sequences for homology to the consensus A4 site revealed that besides the previously identified sites in the IE1 and glycoprotein D genes (Faber and Wilcox, 1986; Kristie and Roizman, 1986; DiDonato and Muller, 1989) and the IE3 gene (Faber and Wilcox, 1986; 1988; Muller, 1987; DiDonato and Muller, 1989; Roberts et al., 1989) a degenerate ICP4 consensus binding site exist in the IE2 promoter at ca. -115 5'ATTCGTCTTGTCTGC3' (Mackem and Roizman, 1982) compared to the consensus ICP4 sequence 5'ATCGTCRNNNYCGR3' [where R= purine, Y= pyrimidine and N= any base](Faber and Wilcox, 1986). An exact match to the ICP4 consensus binding site was also found in a potential
Figure 54. Deletion of the distal ICP4 binding site in IE3 and its effect on Trans-regulation by ICP4. The 99 bp deletion mutants in the -112/-211 region of wild type and D2 mutant IE3 (pJD34CAT and pJD35CAT) were examined in transient transfections to discern the effect that deleting the nonconsensus ICP4 binding site would have on trans-regulation by ICP4. Transient transfections were done in duplicate with 4 μg of indicator per dish being used. Wild type IE3CAT and D2CAT (pJD10CAT and pJD11CAT) were included as a positive and negative control.
| Indicator Gene | CAT Specific Activity:
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>pJD10 CAT</td>
<td>Change in CAT Specific Activity:</td>
</tr>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td></td>
<td>+1</td>
</tr>
</tbody>
</table>

| Indicator Gene | CAT Specific Activity:
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pJD34 CAT</td>
<td>Change in CAT Specific Activity:</td>
</tr>
<tr>
<td></td>
<td>+1</td>
</tr>
</tbody>
</table>

| Indicator Gene | CAT Specific Activity:
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pJD35 CAT</td>
<td>Change in CAT Specific Activity:</td>
</tr>
<tr>
<td></td>
<td>+1</td>
</tr>
</tbody>
</table>

| Indicator Gene | CAT Specific Activity:
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>pJD11 CAT</td>
<td>Change in CAT Specific Activity:</td>
</tr>
<tr>
<td></td>
<td>+1</td>
</tr>
</tbody>
</table>

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Figure 54
Figure 55. The IE1 promoter mutants (pJD100 is WT). The three IE1 mutants, the Wild type sequence (+56/-126) is drawn as the top cartoon (pJD100CAT). The double transition, single transversion mutant in IE1 (pJD101CAT), this mutant promoter is also known as the IE1 -T construct. The 4 bp deletion mutant IE1 dl* is pJD102CAT and is the bottom cartoon in the Figure.
Figure 55
Figure 56. Trans-regulation of the minimal IE1 (WT and Mutant) promoter by ICP4. The IE1 promoter constructions (refer to Figure 55 for identification and descriptions) pJD100CAT and the mutant versions of this construct pJD101CAT and pJD102CAT were assayed for basal level expression and expression in the presence of 1 MGE of ICP4. There was 4 μg of indicator per dish. The transfections were done in duplicate. The minimal promoter element is listed as is the full IE1 promoter as a comparison.
Change in CAT specific activity: +1 -44 +1 -4 +1 -8.5

<table>
<thead>
<tr>
<th>Wild type A4: GAATCGTC</th>
<th>ΔICP4 Mutant: GGG****C</th>
<th>-T Mutant: GGGACGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>BASAL</td>
<td>+ICP4</td>
<td>BASAL</td>
</tr>
</tbody>
</table>

WT IE1 promoter

Minimal IE1 promoter

Figure 56
novel IE promoter (DiDonato, Muller, Silverstein, and Bohenski, unpublished results) designated as IEx (see Chapter 2, Figure 20 and Figure 27). IEx is located 735 bp 5' to the IE1 TATA box and is transcribed in the opposite direction.

The promoter regions of IE2 (-240/+1) and (-84/+1) were fused to the CAT gene and designated as pIGA95 and pIGA98 respectively (see Materials and Methods for a detailed description). The IEx promoter (-910/+35) was also fused to the CAT gene to form IExCAT (pRAB6). The IEx promoter was reversed in orientation and fused to CAT (pRAB8). The IE1 promoter drives expression of the CAT gene in pRAB8 (Figure 57) [for a schematic, see Figure 20 and Materials and Methods for details in its construction].

These IECAT chimeras were used in transient cotransfections with and without ICP4 to ascertain the effect ICP4 had on expression from these promoters. ICP4 decreased IExCAT (RAB6) expression 2.4 fold relative to basal levels (Figure 57, lanes 3 and 4). IE1CAT (pRAB8) was down regulated 17 fold by ICP4. WT IE3CAT (pJD10CAT) and D2 IE3 CAT (pJD11CAT) were also used in cotransfections as a positive and negative control. ICP4 decreased pJD10CAT activity 6 fold from basal levels and D2 IE3CAT was down regulated 2 fold (lanes 1 through 8).
Figure 57. Trans-regulation of IE1 and IEx by ICP4. The complete promoter elements of IE1 and IEx were used to drive CAT expression. These promoters are the same fragment only cloned in different directions (see the diagram below the data). The lanes are indicated as to which vector is driving expression. The WT and D2 mutant of IE3 are included in the transfection data as a comparison. All transfections received 4 μg of indicator gene per 60 mm² dish and the transfections were done in triplicate.
Figure 57
Figure 58. Trans regulation of IE2 and Minimal IE2 by ICP4. Transfections were done in triplicate. There was 4 μg of indicator gene transfected per 60 mm$^2$ plate. Wild type and D2 mutant IE3CAT indicator genes were also transfected as a control.
Change in CAT
Specific activity:

<table>
<thead>
<tr>
<th>BASAL</th>
<th>INDICATOR + ICP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>+1</td>
<td>-5.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BASAL</th>
<th>INDICATOR + ICP4</th>
</tr>
</thead>
<tbody>
<tr>
<td>+1</td>
<td>-1.5</td>
</tr>
</tbody>
</table>

INDICATOR GENE
WT IE3 (pJD10 CAT) IE3 AT deletion (pJD11 CAT)

Change in CAT
Specific activity:

<table>
<thead>
<tr>
<th>+1</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>+1</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>+1</th>
</tr>
</thead>
</table>

| +1.2              |

INDICATOR GENE
WT IE2 CAT (pIGA95) Minimal IE2 CAT (pIGA98)

WT IE2

<table>
<thead>
<tr>
<th>CAAT</th>
<th>TAATTARAT</th>
<th>Degenerate A4 site</th>
<th>CAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>278</td>
<td>237</td>
<td>-169 -84</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Minimal IE2</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>-84</th>
<th>+1</th>
</tr>
</thead>
</table>

Apa I  Eag I

Figure 58
WT IE2CAT (pIGA95) failed to be down regulated by ICP4 in cotransfection expression assays (Figure 58, compare lanes 1 and 2 with lanes 3 and 4). The minimal IE2 promoter (-84/+1) (pIGA98) which lacks the degenerate ICP4 consensus binding site was also examined in a transient cotransfection expression assay. Addition of ICP4 to the cotransfection resulted in a slight increase (1.2 fold) in activity relative to basal level pIGA98 expression (compare lanes 10-12 with lanes 7-9).
DISCUSSION

The effect of A4 site mutation, deletion, spacing differences from TATA and alternate A4 site placements on IE1 and IE3 gene expression in the presence of ICP4 was investigated. Like their hGH counterparts, the wild type IE3 (-333/+27) and IE3 D2 mutant CAT constructs were regulated in similar fashion. The WT IE3 promoter was down regulated from basal levels roughly 4 to 8 times more than the D2 promoter construct. These results agree with the transient expression assay results of the other laboratory which have made these same constructions (Robert et al, 1988). The difference in down regulation between these two promoters is 4 to 8 times while the relative binding affinity is 13 fold (Table 3). Gene expression was measured in vivo and binding affinity was determined in vitro. If DNA binding alone determines the magnitude of negative regulation, the following two possibilities exist to explain the discrepancy; first, ICP4 may bind to the D2 site in vivo with a greater affinity than in vitro. Evidence in support of this conclusion was observed in the increased binding affinity for the D2 promoter when anti-
Vmw65 monoclonal antibody containing ascites fluid was added to the binding reaction (Figure 16, lane 9). Second, perhaps ICP4, when bound, can communicate more easily with TATA as a result of the -6A, -5T deletion, thereby enhancing its negative regulatory effect. Construction of shorter spaced mutants in the WT IE3 promoter should uncover which factor is involved.

Elimination of the ICP4 binding site (-18/+27) in IE3 and replacement with vector sequences results in slight stimulation of gene expression by ICP4 (Figure 50). This implies sequences 5' to TATA do not down regulate expression via ICP4. This would then suggest that the nonconsensus ICP4 binding site (between -108/-211) plays no role in down regulating basal level expression when ICP4 is bound. ICP4 is known to be a weak positive trans-active protein (Everett, 1984; O'Hare and Hayward, 1985). The slight stimulation by ICP4 on pJD40CAT may be a result of binding at the nonconsensus site and weakly stimulating expression.

Effect of differential A4 site placement in the IE3 promoter

A4 sites were placed at the -108 position in the WT and D2 mutant IE3 promoters, resulting in duplicate copies of the A4 site. Both constructs were more strongly down regulated by ICP4 (an additional 8 fold, Figures 48 and 49.
compared to the wild type construct). ICP4 binding to the additional site is not synergystic but instead is additive. This result can be explained a number of ways. First, ICP4 could bind to the -108 A4 site and affect the transcriptional apparatus via protein-protein interaction or by altering the conformation of cis sites. A second possibility is that binding of ICP4 interferes with the communication of upstream cis bound factors and the transcriptional machinery. The first alternative does not appear likely based upon several observations: i) the distance between TATA and -108 is >48 bp which was shown not to result in TATA helix conformational alteration when ICP4 was bound at the A4 site (Figure 26) and not to result in negative regulation (Figure 53, lanes 11 and 12); ii) the placement of the A4 site at -108 is similar to that of gD which also does not exhibit TATA hypersensitivity (Figure 26); iii) and is not down regulated (Beard et al., 1986; Tedder et al., 1989b). When an ICP4 binding site is placed this far from TATA (ca. 80-90 bp) as in gD or IE2, the effect of ICP4 on expression is can be slightly positive (Everett, 1984;87; Gelman and Silverstein, 1987; this study).

ICP4 may down regulate genes when bound 5' to TATA by interfering with transcription factor binding or communication between the transcription factor and the
transcriptional machinery. This possibility is favored for the following reasons. Deletion analysis of the IE3 promoter defined various regions between -108 and -333 which, decreased expression stepwise as they were deleted (Bzik and Preston, 1986). These regions are located between -327 to -292, -293 to -275, and -198 to -174. An additional site between -135 to -197 which augments expression of IE3 was also known to exist (Kristie and Roizman, 1984). These sequences are G+C, G+A and A+T rich and correspond to binding sites for SP1 (Jones and Tjian, 1985), enhancer factors and the OCT-1, 65 Ktif complex (TAATGARAT binding) (O'Hare and Goding, 1988; Preston et al., 1988). Binding of ICP4 could act to stearically block and interfere with the (-135 to -197) region's communication with the transcriptional machinery. Important cis binding sites >150 bp away may have enough flexibility in the DNA to loop around the axis of the helix and avoid stearic hindrance effects of the bound ICP4 at the -108 position, but not if the critical cis site(s) were located near nucleotide -108 and located on the same face of the helix as ICP4. Based upon the contribution of the -135 to -197 and the -174 to -198 region of IE3 to promoter activity (Kristie and Roizman, 1984; Bzik and Preston, 1986), their elimination would correspond with the 8 fold additional decrease in IE3 expression for the -108 A4 site insertion mutants in the
presence of ICP4. This is a type of promoter occlusion repression and could be a common feature in IE2 (in some cell types but not BHK) and IE-1 gene repression observed with ICP4, as a result of competition of ICP4 and transcription factor (CTF in particular) binding to essential cis sites which overlap the ICP4 binding site or are extremely close to it. This possibility will be discussed further in the next section.

Placement of the A4 site (-18/+27) in either orientation at the -211 position in the A4 site deletion mutant (pJD40CAT) resulted in only a slight stimulation, in gene expression, over basal expression levels. These constructs have a nonconsensus ICP4 binding site between (-108 and -211) and a consensus ICP4 binding site at -211. Placement of the A4 sites at -211 was expected to increase stimulation compared to the parental construction's expression level much like that seen for gD (this was not observed). Multiple tandem A4 site insertions (3 copies or more) in the gD promoter resulted in enhanced stimulation by ICP4 when compared to single site constructs (Tedder et al., 1989b). Tandem duplicate A4 site insertions resulted in only a slight stimulation in expression compared to the single site construction (Tedder et al., 1988b). The lack of strongly enhanced stimulation in the -211 insertion mutants may result from ICP4 binding to one site or the
other but not both, perhaps (again) due to stearic hindrance (this is a reasonable explanation and is supported by experimental results seen in Figure 53 and Table 4). The nonconsensus ICP4 binding site was recently reported to be located at position -195 from the IE3 capsite (Michael and Roizman, 1989) lending further strength to this argument. This observation could explain the lack of enhanced expression in the -211 insertion constructions.

A spacer mutation which increases the distance between the A4 site and TATA in IE3 to 48 bp was not down regulated by ICP4 (Figure 53). Its TATA helical conformation was also not altered by binding of ICP4 to the A4 site in vitro (Figure 26). At a 26 bp separation as in WT IE3CAT (pJD10CAT), the TATA DNA helix conformation is altered by ICP4 binding as evidenced by in vitro footprinting studies and expression from the promoter was regulated negatively by ICP4 in transient expression experiments in vivo. Models, which might explain negative regulation of IE3, include a stearic hindrance model (similar to the prokaryotic operator-repressor model) requiring ICP4 to bind to the A4 site and block RNA polymerase access (Roberts et al, 1988, Faber and Wilcox, 1988). An example, of this, would be the large T antigen of SV40, which, autorepresses its own expression (Rio and Tjian, 1983). Preliminary evidence, with transient transfection assays utilizing a GAL4 fusion
protein, indicate the fusion protein does not down regulate an IE3 promoter mutant containing the GAL4 DNA binding sequence, in the analogous position to the A4 site in the WT IE3 promoter (DiDonato and Muller; DiDonato, Muller and Balsubramanian, unpublished observations-data not shown). If stearic hindrance and the operator-repressor model was valid for ICP4 negative regulation in IE3, the ICP0-GAL4 fusion protein should have down regulated the mutant, but it did not (DiDonato and Muller; DiDonato, Muller and Balsubramanian, unpublished observations-data not shown).

Another model, which was speculative invokes ICP4 interaction with the transcriptional machinery, possibly with TFIID, to alter binding to the TATA region (Patterson and Everett, 1988a; Shepard and Deluca 1988a). A related model, proposed by DiDonato and Muller and based upon the first evidence showing a relationship between TATA and ICP4 binding, suggests that ICP4 binding induces a conformational change of TATA helical geometry directly impacting on TFIID/TATA binding interactions, causing an alteration in gene expression (DiDonato and Muller, 1989). ICP4 binds to the opposite face of the DNA helix than TFIID (separation distance 2.5 helical turns). Protein/protein interaction between ICP4 and TFIID or a component of the transcriptional apparatus may actually change the orientation of TFIID or the transcriptional complex. RNA
polymerase would not be able to form the equivalent of an open transcriptional complex or bind the preinitiation complex. Alternatively, the transcriptional complex may fail to form with TFIID because proper contacts to the helix can not be made due to conformational changes in the DNA resulting from ICP4 binding nearby.

The latter option appears likely because, TATA is conformationally altered by ICP4 which may affect TFIID binding affinity. Protein/protein interactions between ICP4 and TFIID could influence TFIID's position (orientation) which would be detrimental to preinitiation complex formation; therefore, RNA polymerase II binding would not be probable. The pseudorabies virus (PRV) immediate early protein has been shown to stimulate the binding of TFIID to the TATA element in the adenovirus major late promoter in vitro and to facilitate the assembly of stable preinitiation complexes on TATA during in vitro nucleosome assembly (Abmayr et al., 1988; Workman et al., 1988). The results of these investigators imply that the PRV IE protein interacts at least transiently with TFIID. The PRV IE protein and ICP4 share amino acid homology in several different structural domains which are also conserved with the VZV 140K protein (the ICP4 VZV equivalent) (Vlcek et al., 1989).
ICP4 binding to the IE1 promoter and negative regulation.

ICP4 negatively regulates expression from the minimal IE1 promoter (mIE1). IE1 promoter mutations which decrease ICP4 binding affinity, also decrease the magnitude of negative regulation observed in transient expression assays. Basal expression from the mIE1 mutant pJD101CAT (–T mutant) was 15% less than the WT mIE1CAT expression level. The altered sequences were located at the AT of the 5'ATCGTC3' in the IE1 ICP4 consensus binding site. This mutation does not alter the spacing between the CAAT box at nucleotide position -78 (10 bp 5' to the IE1 A4 site) and the Sp1 binding site 10 bp 3' to the A4 site (-58). A mutation (pJD102CAT [dl°]) changing the spacing (-4 bp) between the CAAT box and Sp1 sites of pJD101CAT results in a 67% decrease in basal level expression when compared to that of the wild type construct. The spacing of cis binding sites for the CAAT box, ICP4 and Sp1 are 1 turn of the DNA helix from each other thus, each site is located on the same face of the helix. Impairment of basal promoter function by altering the spacing between sites implicates a required separation distance between the CAAT box and the Sp1 site or a stereo-specific alignment of the sequences 5' to the mutation point with sites 3' to the mutation. Stereo-specific effects on promoter function have been observed in
the SV40 early promoter (Takahashi et al., 1986) and the adenovirus E1B promoter (Wu and Berk, 1988). Perhaps both the CAAT binding protein and Spl interact to activate the linked gene.

ICP4 binding to the promoter at the A4 site produces an alteration in TATA conformation in vitro (DiDonato and Muller, 1989) (Figure 24). The ICP4 binding site in IE1 is 3.5 turns of the helix from TATA. Like IE3, the ICP4 binding site is located on the opposite face of the DNA helix from TATA. The 3' half of the ICP4 binding site overlaps with the Spl binding site; therefore, if ICP4 is bound, Spl will not be able to bind and the opposite is also true. In the process of binding to the A4 site, ICP4 will be on the same face of the helix as the CAAT binding site. Binding of ICP4 to its site in IE1 could also interfere with factor binding to the CAAT binding site at -78. ICP4 may down regulate IE1 via two different pathways: i) the same pathway that negatively regulates IE3 (the TATA conformational alteration model); ii) the promoter occlusion model which implicates ICP4 interference with CAAT box protein communication with Spl or the transcriptional apparatus. The likelihood that both pathways are at work is good based upon the data which show that alteration of alignment of the CAAT box and Spl lowers basal level
expression and the fact that ICP4 severely alters expression (negatively) when present.

The IE1 promoter extending to (ca. -790) from the IE1 transcriptional start site was regulated negatively to a lesser extent (16.5 fold) versus (44 fold) for the mIE1 version, whose promoter element extends only to nucleotide position -126. Use of the longer promoter element enables the interplay of the different transcription factors bound at their cognate sites but it is also a more complex system to analyze. A strong enhancer activity has been observed near the -325 region of IE1 (Ebert and Muller unpublished observations) and may account for lessening ICP4 mediated negative regulation. Other investigators have reported that ICP4 does not down regulate basal expression of IE1 in transient expression experiments (Roberts et al, 1988, Resnick et al, 1989). These investigations were performed using different cell types (than the experiments reported here) for performing the transfection experiments. It is known that cell type will effect the pattern of expression regulation, sometimes to great extents (Gelman and Silverstein, 1987a; Everett, 1988). These fluctuations in regulation are attributed to the differing supplies of transcription factors in different cell types.

Negative regulation of the IE1 promoter by ICP4 has also been reported (Gelman and Silverstein, 1986, 1987a,b).
ICP4 trans-regulation of other genes

IEEx resembles an IE gene promoter. It shares 68% homology in sequence with the IE3 promoter over a 25 bp region beginning at the A of the ATCGTC extending 3'. The A4 sites in both promoters are separated from TATA by 26 bp and 24 bp respectively (middle to middle distance of each site). IEEx is located ca. 750 bp 5' of IE1 and is divergent. The enhancer element of IE1 mentioned above is ca. -400 bp 5' to the IEEx transcription start site. IEEx is expressed as a late gene with the transcriptional start site lying within the ATCGTC A4 core consensus site (Saul Silverstein, personal communication). ICP4 binds to the IEEx A4 site and induces TATA hypersensitivity similar to that observed for IE3 (compare Figure 27 with Figure 22). Unlike IE3, IEEx is down regulated only to a small degree by ICP4 in transient expression assays. There could be several reasons for this. First and most simplistically, IEEx is not an IE gene. Second, multiple Sp1 and CAAT binding protein sites predominate the IEEx promoter 5' of TATA, these additional sites may increase expression by attracting the transcriptional machinery to the promoter. Last, the enhancer element at -400 may play a role in increasing expression similar to the effect observed between the expression levels of the full IE1 promoter versus the mIE1
promoter in the presence of ICP4. To determine the effect sequences 5' to TATA play in potentiating ICP4's effect via down regulation through TATA, the sequences 5' to the IEx TATA should be replaced with the IE3 sequences located 5' to TATA and visa versa. Transient assays could then be performed. Contributions of the sequences 5' to TATA to overcoming down regulation by ICP4 can be evaluated in this manner.

**ICP4 trans-regulation through binding to nonconsensus binding sites.**

The IE gene 2 promoter contains a degenerate ICP4 binding site similar in position to the A4 site located in the gD promoter. ICP4 fails to repress expression from either the full IE2 (-278/+1) or the mIE2 (-84/+1) promoter. The IE2 promoter is differentially regulated by ICP4 in different cell types (Gelman and Silverstein, 1987a,b). The degenerate A4 site in IE2 is located ca. 95 bp 5' of TATA. At this distance, it seems doubtful that ICP4 could regulate expression negatively through the same mechanism proposed for IE3. IE2 like IE1 contains a cis binding site which overlaps with the ICP4 binding site. This sequence, in IE2, is a CAAT box and in IE1, it is a Spl site. Differential down regulation of IE2, in various cell types, may result
from the lack of that particular cell type to contain the specific CAAT box binding protein, in sufficient quantity to activate the promoter from that particular site (-115). HSV IE promoters contain a multitude of various \textit{cis} binding sites. It seems logical that given the chance of infecting a variety of different cell types the assortment of transcription factors present within that cell will be able to activate the promoter using the available assortment of \textit{cis} binding sites.

\textbf{A MODEL: ICP4 REGULATES IE GENE EXPRESSION NEGATIVELY VIA TWO PATHWAYS}

The schematic depicts the two pathways (Figure 59). In the first, ICP4 binds the A4 site in IE3, IEx and IE1 resulting in DNA bending and alteration of TATA helix conformation. Alteration of helical conformation could affect TFIID binding resulting in decreased affinity for TATA. If this were the case, the transcriptional complex may not assemble or if it did, it may be unstable as a result of protein/protein interaction with ICP4, which is located on the opposite face of the helix from TATA. Interaction between TFIID and the PRV IE protein have been implied, resulting in enhanced binding of TFIID to TATA (Abmayr et al., 1988; Workman et al., 1988). ICP4 is known
Figure 59. Model: ICP4 negatively regulates expression from the IE3 and IE1 promoters by two different mechanisms. Panel A depicts the mechanism by which IE3 is down regulated. This mechanism is effected by the hypersensitivity of TATA when ICP4 is bound, signifying a change in helix conformation. This will directly impact on TFIID's ability to bind TATA. Decreased binding affinity will down regulate the promoter - the transcriptional complex will not form or if it does it may have its orientation altered by protein-protein interactions with ICP4, which is rotationally displaced by one-half helical turn from TATA. Panel B depicts the manner in which ICP4 negatively regulates the minimal IE1 promoter. ICP4 binding interferes with the stereo-specific alignment of the CAAT box site and TATA or the SP1 site. Binding of ICP4 may preclude binding of Sp1 because the ICP4 binding site overlaps the Sp1 site at the A4 sites 3' end. ICP4 binding may also interfere with CAAT box binding factor due to its close proximity (10 bp). Occlusion of a necessary transcription factor binding site will have detrimental effects on expression.
ICP4 binds DNA and induces TATA helix conformational changes so that TFIID's binding affinity is altered.

Mechanism 1

ICP4 causes missalignment of TFIID and the transcriptional complex can not bind to the DNA and form.

Mechanism 2

ICP4 interferes with the stereo-specific alignment of other cis-bound factors.

Figure 59
to be elongated in shape and could possibly extend to TATA from its binding site based upon its stokes radius (Metzler and Wilcox, 1985). According to its stokes radius, it can be calculated that ICP4 could cover 52 bp of DNA. The ICP4 footprint covers 15-16 bp of DNA, which leaves 36 to 37 bp unaccounted for. Cis sites within 37 bp of the ICP4 binding site could contact ICP4. This observation is consistent with negative regulation of IE genes containing bound ICP4 within 36 bp of TATA. Alternatively, ICP4 may bind and act to hinder RNA polymerase binding or initiation, reminiscent of the prokaryotic operator-repressor model such as the lac repressor. The latter possibility does not seem likely based on the observation that repressors utilizing this method (for example the lac repressor) bind DNA between +1 to +15 resulting in repression by inhibiting the formation of the RNA polymerase open complex (Straney and Crothers, 1987). As the lac operator is placed further 3' to the +1 site, repression is diminished but not eliminated (Elledge and Davis, 1989). Recently, in mammalian tissue culture cells, the lac operator, when located far downstream of the +1 site, was shown to block the passage of RNA polymerase through the sequence when repressor was bound, thus causing premature termination of the transcript (Deuschle et al., 1990). Additional spacing of 22 bp between TATA and the A4 site in IE3 resulted in loss of negative regulation by ICP4.
This would rule against the stearic hindrance model and argue that ICP4 effects some step prior to the formation of open stable transcription complex. Additional preliminary evidence suggests a GAL4-ICP0 fusion protein, binding to its cognate binding site in an IE3 promoter A4 substitution mutant, with identical spacing relative to TATA as the A4 site in the IE3 promoter, did not result in negative regulation in transient transfection experiments. This is a further indication that ICP4 does not regulate expression negatively from IE3 by blocking RNA polymerase binding or formation of an open stable transcription complex. Perhaps ICP4 interferes with a step prior to RNA polymerase binding, such as TFIID binding to TATA or establishment of the transcriptional complex.

In the second pathway, binding of ICP4 5' to TATA, within the promoter, interferes with the binding of transcription factors, interactions between transcription factors or with interactions between transcription factors and the transcriptional complex. Evidence to support this hypothesis is based on the decrease in basal expression of the IE1 promoter as a result of changing the spacing between the CAAT box and Spl site by a half helical turn placing them on opposite faces of the DNA helix. The A4 site in IE1 covers the Spl site. Like the Spl site, the ICP4 binding site is on the same face of the helix as the CAAT box. ICP4
strongly down regulates this promoter. The binding of ICP4 to its binding site should bend the DNA and alter the stereo-specific alignment of the CAAT box binding site with sites 3' to it, additionally, ICP4 binding would result in occlusion of Sp1 binding. Perhaps, ICP4 binding may also prevent or affect the binding of CAAT box binding factor to the CAAT box site, thus decreasing potential activation of the promoter.

These two pathways explain ICP4 down regulation in IE3 and IE1 and possibly for IE4/5 also (the ICP4 binding site is within 40 bp of TATA). In the IE2 promoter, the proposed ICP4 binding site overlaps a potential CAAT box site. This promoter is regulated differently by ICP4 in various cell types (Gelman and Silverstein, 1987a,b). The requirement to use this CAAT box site instead of an alternate CAAT box sites present in the promoter could explain the cell type variations in trans-regulation. ICP4 fails to down regulate IE genes immediately during the infection (0-2 hr PI). This could be a result of the lack of an essential posttranslational modification which activates an ICP4 negative regulatory domain. Alternatively, the number of strong and moderately strong ICP4 binding sites in the cellular and viral DNA far outnumber the ICP4 binding sites on the IE genes and therefore could titrate available ICP4 until the concentration of ICP4 in the cell is great enough
to saturate these non-IE sites. At this point, ICP4 would be available to bind to its sites in the IE genes.

These studies have elucidated the sequence through which ICP4 binds to and negatively regulates IE gene 3 and IE gene 1 expression. The manner in which and the sequences to which ICP4 binds have also been examined and a more accurate consensus sequence has been derived. The effect on DNA structure ICP4 binding induces has also been determined. All of these observations have led to the genesis of the above model which explains how ICP4 can regulate IE genes via two different pathways.
Figure 60. Various IE3 promoter constructs. This is a diagram of most of the IE3 mutant promoters. Mutants not listed are versions of either their wildtype counterpart, for example pJD35 is listed but pJD34 is not.
Figure 60
ICP4 had been demonstrated to be a component of a protein/DNA complex that formed at the transcriptional start site of its own gene. This \textit{in vitro} finding (Muller 1987) corresponded with those determined genetically (Preston, 1979; Dixon and Schaffer, 1980) to relate ICP4 with negative regulation of IE3. I have extended these initial observations by describing the relationship between ICP4, DNA binding and the ability to negatively regulate expression from the IE gene 3 and IE gene 1 promoters. Furthermore, the relative binding affinity of ICP4 to many ICP4 binding sites has been determined and shown to be related to the conservation in sequence it has to a single ICP4 binding DNA consensus sequence. The consensus sequence was determined by analysis of a data base of ICP4 binding sites using an algorithm (Joseph Spitzner, TEAM Assoc.). Additionally, binding of ICP4 induces a protein-induced bend in the DNA located at or very near the ICP4 site. Finally, binding of ICP4 to consensus ICP4 binding sites located within 36 base pairs of TATA induces an alteration in the DNA helical geometry in TATA \textit{in vitro}. Alteration of TATA
conformation appears be related to regulate negatively the expression from these promoters in vivo. This was the first direct evidence of a relationship between ICP4 and TATA.

ICP4 content increases throughout the infection cycle and reaches a maximum at 12 hours PI. Commensurate with the increase in ICP4 content was the ability to form the A4 complex on DNA probes containing the IE3 (-18/+27) region. During the early and late periods of the infection cycle, A4 complex forming ability increased at a greater rate than did the increase in ICP4 content. Coupled with this observation, was the increase in slower migrating forms of ICP4 (in SDS-PAGE gels) presumed to be posttranslationally modified forms of ICP4 (Wilcox et al, 1980; Preston and Notarrani, 1983, Faber and Wilcox, 1986b). These observations led me to the next set of experiments to identify other proteins that may be present in A4 complexes and to determine the DNA sequence requirements and conditions for A4 complex formation. A4 site DNA affinity chromatography of infected extracts resulted in the substantial purification essentially of ICP4, indicating that no additional proteins are required for A4 complex formation. The sequence elements between -15 to +16 of IE3 were identified as the minimum IE3 sequence required for A4 complex formation. Deletion of the -6A, -5T in the ATCGTC core consensus ICP4 binding site in the IE3 promoter.
greatly diminished A4 complex formation. These mutants were later examined for trans-regulation by ICP4 in transient expression assays (see below). The presentation of this result was the first description of the importance of this sequence in ICP4 binding to the transcriptional start site of IE3 (DiDonato and Muller, 12th International herpes Workshop, 1987, p293).

Affinity purified ICP4 and less purified sources of ICP4 gave rise to multiple A4 complexes that could have been a result of multiple binding of ICP4 to the target DNA, altered binding of ICP4 to the DNA or protein/protein interactions between itself or some other protein. Chemical footprinting, DMS interference and missing base contact analysis on different consensus ICP4 binding site 2°A4 complexes identified no additional base protection patterns or base contacts than had been identified for 1°A4 complexes, indicating multiple complex formation resulted from protein-protein interactions most likely as a result of ICP4 self associations. This is the first evidence of protein/protein interaction of ICP4 with DNA bound ICP4. Supporting this assumption was the isolation of partially purified ICP4 as a homodimer and DNA affinity purified ICP4 (>95% homogeneous) as a mixture of homodimers and tetrameric ICP4 (Metzler and Wilcox, 1985; Kattar-Cooley and Wilcox, 1989).
Missing contact analysis revealed that ICP4 binding to ICP4 consensus sites was impaired by alteration of specified bases in the consensus sequence. Additionally, alteration of some bases within the binding site not specified by the consensus sequence also altered binding affinity. Relative binding affinities were calculated for many of the ICP4 binding sites. Relative binding affinities for a variety of ICP4 binding sites agree with our sequence consensus to predict potential ICP4 binding sites (according to the matrix mean score).

ICP4 binding to the IE-1, IEx, and IE3 A4 sites separated from TATA by 35 bp, 24 bp, and 26 bp respectively, results in a conformation change in the TATA DNA helix based on Cu-OP results. Movement of A4 site in IE3 to 48 bp from TATA and examination of the gD promoter which has a ca. 90 bp separation between the A4 site and TATA exhibited no alteration in TATA helical conformation when ICP4 was bound. This is the first description of ICP4 induced conformational alteration of DNA structure. DNA structural alteration at TATA by ICP4 displayed a distance or stereospecific dependence between the binding site and TATA. Structural alterations in DNA can result from bending of the DNA when protein is bound (Wu and Crothers, 1984; Shuey and Parker, 1986b). This possibility was examined for the WT IE3 promoter. Binding of ICP4 induced bending of the DNA at
or near the ICP4 binding site regardless of the presence of TATA. This is the first report of ICP4 to bend DNA. Evidence for structural alteration of ICP4 was observed as a loss of binding anti-ICP4 antibody to DNA/protein complexes at intermediate and high salt concentrations which was not observed for ICP4 immunoblots. The results of the footprinting experiments and mutant A4 site binding studies led to experiments performed to examine their relationships to trans-regulation of expression from these promoters.

Transient expression experiments were performed to determine a functional relationship between ICP4 binding in vitro and its effect in vivo. Wild type IE1, IErex and IE3 promoter controlled gene chimeras were regulated negatively by ICP4 in transient transfection assays. Promoters containing a single A4 site (48 bp and farther from TATA) failed to be regulated negatively by ICP4. Mutation in the A4 site of WT IE3 severely diminished ICP4 mediated negative regulation. Total elimination of the A4 site frees the promoter from ICP4 negative trans-regulation. Mutation in the A4 site of IE1 diminished both basal level expression and negative trans-regulation by ICP4 in a manner proportional to the severity of the mutation. Additionally, a mutation which effects the stereo-specific alignment of the CAAT box and Spl sites in IE1 severely impairs basal level expression. This study is the first evidence relating
the importance of the stereo-specific alignment of these two sites to IE1 expression and the stereo-specific/distance dependent negative autoregulation of IE3 by ICP4.

Taken together, the results in this study indicate that ICP4 binding to a consensus ICP4 site within 36 bp of TATA results in negative regulation of expression driven from these promoters. Additionally, placement of ICP4 binding sites in close proximity to other important cis sites can also result in negative regulation by ICP4. I have used these observations to propose ICP4 can negatively regulate expression via two separate pathways that are not mutually exclusive.
Appendix A

Table 5. Determination of $K_r$ for the consensus and nonconsensus ICP4 binding site in IE3. Mobility shift gels were analyzed on the AMBIS system detector. Background and total radioactivity was measured and then adjusted to give the net radioactivity for each complex. Background measurements were taken just below each complex.
Table 5

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K_r=7.05

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K_r=6.2

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Average K_r= (7.05 + 6.20)/2
=6.6 +/- 0.4
Table 6. Determination of $K_p$ for the D2 A4 site and the non consensus ICP4 binding site in IE3. Mobility shift gels were analyzed on the AMBIS system detector. Background and total radioactivity was measured and then adjusted to give the net radioactivity for each complex. Background measurements were taken just below each complex.
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Average K_r = (0.55 + 0.48)/2
= 0.52 +/- 0.04
Appendix C

Table 7. Raw and corrected values for determining the $K_d$ for the consensus ICP4 binding site in IE3. The mobility shift gel was dried and autoradiographed and then analyzed on the AMBIS. Radioactivity was measured and expressed as a percentage of radioactivity present in the lane. Background values for each lane were taken from an area just below the complex. B/F is the Bound over Free DNA ratio.
Table 7

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* not used in calculations
Table 8. Raw and corrected values for determining the $K_d$ for the nonconsensus ICP4 binding site in IE3. The mobility shift gel was dried and autoradiographed and then analyzed on the AMBIS. Radioactivity was measured and expressed as a percentage of radioactivity present in the lane. Background values for each lane were taken from an area just below the complex. B/F is the Bound over Free DNA ratio.
Table 8

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The -108 to -342 probe
LIST OF REFERENCES


Johnson, D. C., and P. G. Spear. 1982. Monenism inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. J. Virol. 43:1102-1112.


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