FURTHER CHARACTERIZATION OF THE UL37 PROTEIN OF HERPES SIMPLEX VIRUS TYPE 1 AND ITS INTERACTION WITH ICP8, THE MAJOR DNA-BINDING PROTEIN OF HERPES SIMPLEX VIRUS

1994

ALBRIGHT

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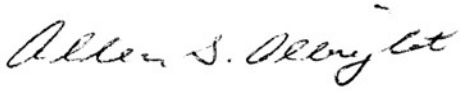

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ABSTRACT

Title of Dissertation:

Further Characterization of the UL37 Protein of Herpes Simplex Virus Type 1 and its Interaction with ICP8, the Major DNA-Binding Protein of Herpes Simplex Virus

Allen G. Albright

Doctor of Philosophy, 1994

Dissertation directed by:

Frank J. Jenkins, Ph.D.

Associate Professor, Department of Microbiology and Immunology

The UL37 protein of herpes simplex virus type 1 has been a focus of study in our laboratory. Previous studies demonstrated that the UL37 open reading frame (ORF) encoded a 120 kDa protein which belonged to the γ1 class of HSV-1 genes in HSV-1-infected cells. In addition, the UL37 protein was found to co-elute from both single-stranded (ss) and double-stranded (ds) DNA columns with IPC8, the major DNA-binding protein of HSV-1. Comparative studies using a vaccinia virus recombinant, V37, and an ICP8 mutant, d21, strongly suggested that the apparent DNA binding exhibited by the UL37 protein from HSV-1-infected cells was due to an association with the ICP8 protein on these columns and not from an intrinsic affinity of the protein for DNA. The UL37 gene product was also classified as a nonstructural protein since

it did not appear to be a component of the virion within the limits of detection (Shelton et al., 1990; Shelton, 1992).

In this study, we demonstrate that the ability of the UL37 protein to bind ssDNA columns is dependent solely upon the presence of a DNA-binding competent ICP8 protein. These results are based on comparative studies using two vaccinia virus recombinants, V37 and V8, which express the UL37 and ICP8 proteins independent of other HSV-1 genes, respectively, and the HSV-1 ICP8 mutant, n10. In addition, results from studies using non-DNA matrices, including blue sepharose and amylose, suggest that the UL37-ICP8 protein:protein interaction may be one that is stabilized or perhaps mediated by the presence of DNA. Furthermore, co-immunoprecipitation of the UL37 and ICP8 proteins from both HSV-1-infected and V37-and V8-coinfected cell lysates as well as from an in vitro translation system was achieved using ICP8-specific reagents which provides additional evidence for a stable and perhaps functional interaction between these two proteins.

Through the development of more sensitive UL37-specific antisera, further characterization of the UL37 protein was performed. Pulse-labeling and pulse-chase studies of HSV-1 infected cells using [^{35}S]methionine and $^{32}P_i$ demonstrated that the UL37 protein is a stably phosphorylated protein which did not have a detectable rate of turnover throughout the viral replicative cycle. The UL37 protein expressed from a vaccinia virus recombinant, V37, was also phosphorylated during infection indicating that interaction with the ICP8 protein is not a prerequisite for UL37 protein phosphorylation and suggesting that the UL37 protein is phosphorylated by a cellular kinase.

Finally, re-examination of purified mature HSV-1 virions and L particles (light particles) by immunoblot analysis demonstrated that the UL37 protein is present

in detectable amounts. To determine the location of the UL37 protein within the virion, trypsin digestion experiments were performed on purified virions in the presence or absence of detergent. The results of these studies demonstrated that the virus envelope was able to protect the UL37 protein from trypsin, since virus-associated UL37 protein was sensitive to digestion only after detergent treatment. In addition, the UL37 protein remained associated with the virus particle when the virion-specific glycoproteins were removed after detergent treatment. Taken together, these data suggest that the UL37 protein is located within the tegument region of the HSV-1 virion. Although the function of the UL37 protein is unknown, we postulate that the UL37 protein may assist or modify the ICP8 protein for a function late in the viral replicative cycle and/or perhaps have a function independent of the ICP8 protein.

FURTHER CHARACTERIZATION OF THE UL37 PROTEIN OF HERPES SIMPLEX VIRUS TYPE 1 AND ITS INTERACTION WITH ICP8, THE MAJOR DNA-BINDING PROTEIN OF HERPES SIMPLEX VIRUS

by

Allen G. Albright

Dissertation submitted to the Faculty of the Department of Microbiology and Immunology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1994

DEDICATION

This dissertation is dedicated to my mother and father. Their love, support, and prayers have been steadfast especially over the last six years during the course of my graduate career. My trips home for holidays and breaks during this time, in particular, have provided much comfort, rest, and reprieve from an otherwise hectic schedule for which I am truly grateful. Mom and Dad, thanks for the example you have set by being there for me in so many ways.

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Robert Long and Arthur Hays- for their understanding and support as roommates during the last couple years of my studies.

"Whom have I in heaven but Thee? And besides Thee, I desire nothing on earth. My flesh and my heart may fail, but God is the strength of my heart and my portion forever."

Psalm 73:25-26

"You have made us for Yourself, O Lord, and our hearts are restless till they rest in You."

Augustine

"Thou art the Life by which alone we live,
And all our substance and our strength receive;
O comfort us in death's approaching hour,
Strong-hearted then to face it by Thy power."

John Calvin

"It is axiomatic that to perpetuate itself, a virus must disseminate in the host population...To the victim of infection, herpes simplex virus is anathema, an intractable disease that seems to become exacerbated in direct relation to the anxiety it causes. To the student of its structure and manifestations, the virus is an exquisite model of selective adaption to the exigencies of survival. Above all, it is a model of itself; it is also a model of gene expression, regulation and of macromolecular interactions."

Bernard Roizman and Amy Sears

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I. INTRODUCTION

Preface

This introduction consists of four sections. Part one contains an overview of the current understanding of herpes simplex virus (HSV) pathogenesis, replication, and molecular biology. Particular emphasis is placed on the characteristics of infected cell polypeptide 8 (ICP8) since this topic is relevant to the work presented from this project. The second section provides a description of HSV virion maturation which highlights the roles and locations of most of the known HSV structural proteins, a subject associated with some of the results of this study. The third section briefly discusses protein phosphorylation in HSV since this is a newly discovered property of the UL37 protein, also described in this study. The last part of the introduction details the specific aims of this dissertation.

A. Herpes Simplex Viruses

1. Definition

Herpes simplex virus (HSV) belongs to the herpesviridae family of viruses whose membership is based on the architecture of the virion. The virions of the various herpesviruses cannot be differentiated by electron-microscopic examination and are composed of the following structural elements: (1) core, which consists of a fibrillar spool on which the linear double-stranded DNA is wrapped in a toroidal arrangement, (2) capsid, an icosahedral protein shell containing 12 pentameric and 150 hexameric capsomers which encloses the DNA, (3) tegument, which consists of proteinaceous material asymmetrically arranged around the capsid, and (4) envelope, a trilaminar membrane containing surface glycoproteins that surrounds the entire structure (reviewed in Roizman, 1993; Roizman and Sears, 1993) (Figure 1).

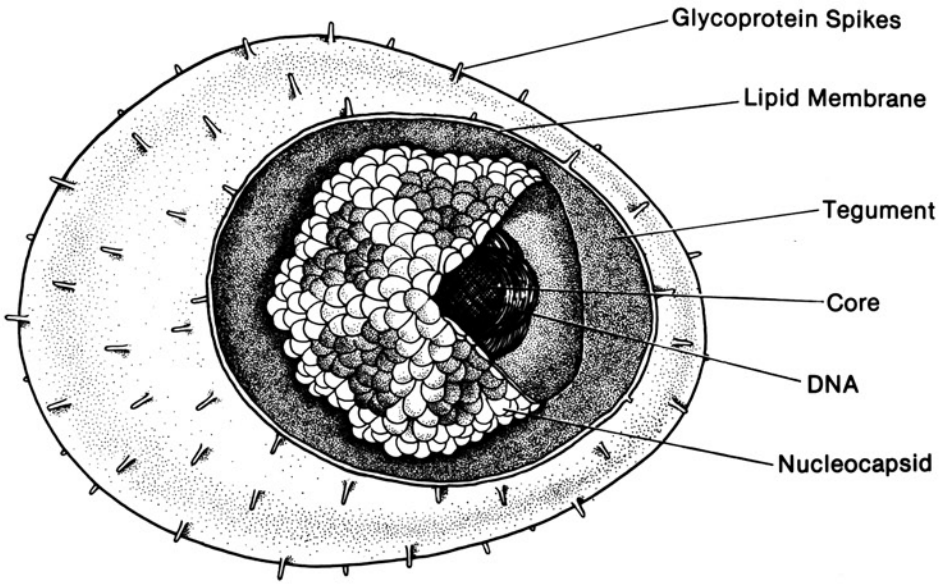


Figure 1

Structural model of the herpesvirus virion. A cutaway diagram of a virion showing the individual structural components. (Reproduced with permission from Hay et al., 1987)

The herpesviruses share many features in their patterns of replication, morphogenesis, and ability to remain latent in their hosts (Roizman and Sears, 1993). Of the seven human herpesviruses isolated to date, five have been formally grouped into various subfamilies and genera on the basis of biologic properties, DNA sequence homology, similarities in genome sequence arrangement, and relatedness of viral proteins demonstrable by immunologic methods. These include herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) as well as varicella-zoster virus (VZV) (Alphaherpesvirinae), cytomegalovirus (CMV; Betaherpesvirinae) and Epstein-Barr virus (EBV; Gammaherpesvirinae). Human herpesviruses 6 and 7 (HHV-6 and HHV-7) remain unclassified. The members of the subfamily Alphaherpesvirinae are classified on the basis of a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infections primarily but not exclusively in sensory ganglia (Roizman and Sears, 1993).

2. Infections

HSV infections produce a variety of clinical manifestations ranging from asymptomatic and mild illnesses in the majority of cases to sporadic, severe, and life-threatening disease in a small percentage of individuals infected. The severity of disease is dependent primarily on the route of infection and the immunologic status of the host (reviewed in Whitley, 1985; Whitley, 1990).

HSV spreads principally through direct contact of a susceptible individual (namely, one who is seronegative) with infected secretions from someone excreting herpes simplex virus. HSV-1 and HSV-2 infections, commonly referred to as "above the waist" and "below the waist" infections, respectively, are usually transmitted by different routes and involve different areas of the body; however, both serotypes are capable of infecting the same anatomical sites with similar pathogenic consequences

(Whitley, 1985). Generally, infection with HSV-1 is limited to the lips, oral cavity, and the oropharynx and is transmitted by saliva or respiratory droplets while HSV-2 infections are transmitted venereally through genital routes. Neonates can be infected with HSV during passage through an infected birth canal.

Following an initial infection, herpesviruses are capable of producing both a primary infection as well as recurrent infections (Table 1) (White and Fenner, 1986). Primary infections are often inapparent but when manifested clinically tend to be more severe than subsequent recurrences at the same site. Nearly all recurrent infections result from reactivation of an endogenous latent infection. However, some initial episodes of disease, e.g., genital herpes, may occur in people with partial immunuity from a previous heterologous (or less commonly, homologous) HSV infection; such cases are usually mild (White and Fenner, 1986).

3. HSV Pathogenesis

Current understanding of the pathogenesis of recurrent oral, genital, and ocular HSV infections has resulted from studies on experimental animals and clinical observations of humans (reviewed in Hill, 1985; Whitley, 1985; Stanberry, 1986; Whitley, 1990). Briefly, viral replication at the site of infection such as abraded skin or mucous membranes causes vesicles and ulcerations lasting 2-3 weeks with a concomitant production of cellular and humoral immune responses by the host. Following initial infection and many rounds of viral replication in skin or mucous membranes, virus enters into the peripheral nervous system (PNS) by infectious particle penetration of axon terminals of sensory neurons (reviewed in Stevens, 1989). The virus capsids are then transported in a retrograde manner to neuronal cell bodies within the sensory ganglia which innervate primary sites of HSV infection (trigeminal or sacral

Table 1 **Diseases Produced by Herpes Simplex Viruses**

Disease	Primary (P) or Recurrent (R)	Age	Frequency	Severity	Type
Gingivostomatitis	P	Young Children	Common	Mild	1
Pharyngotonsillitis	P	Adolescents	Common	Mild	1 > 2
Herpes labialis	R	Any	Common	Mild	1 > 2
Genital herpes	P, R	> 15 years	Common	Mild to Moderate	2 > 1
Keratoconjunctivitis	P, R	Any	Common	Moderate	1
Dermatitis[a]	P, R	Any	Rare	Mild	1, 2[b]
Encephalitis	P, R	Any	Rare	Severe[c]	1 > 2[d]
Neonatal herpes	P	Newborn	Rare	Severe[c]	2 > 1
Disseminated herpes	P, R	Any	Rare	Severe[c]	1 > 2

[a]Including HSV infection of burns, eczema herpeticum, etc.
[b]Skin above waist, 1 > 2; below waist, 2 > 1; arms, either.
[c]Often fatal.
[d]HSV-2 in neonates.

(Reproduced with permission from White and Fenner, 1986)

for HSV-1 and HSV-2, respectively [Baringer and Swoveland, 1973; Baringer, 1974]) where the viral genomes are released into the nuclei. There, the viral genome can either replicate resulting in neuronal destruction or remain largely quiescent thereby establishing a state of latency.

A latent HSV infection is maintained for the life of the host, but the virus can be reactivated periodically to produce clinically apparent lesions or asymptomatic shedding of virus at or near the primary site of infection (Baringer, 1976). HSV latency and reactivation are diagrammatically shown in Figure 2. Studies using both animal models and human subjects have shown that viral reactivation can be triggered by a variety of stressful or stress-related stimuli including heat, ultraviolet light, fever, hormonal changes, menstruation, and surgical trauma to the neuron (Carlton et al., 1952; Segal et al., 1974; Pazin et al., 1978; Spruance et al., 1985).

4. HSV Replication Cycle

a. Latency. A hallmark of all herpesviruses is their ability to establish and persist in an apparent inactive state for varying durations of time and then be reactivated by the proper stimulus to produce recrudescent disease (Roizman, 1968; Stevens, 1975; Whitley, 1990). This biologic phenomenon is commonly referred to as latency and has been the subject of extensive review (Roizman, 1968; Roizman, 1971; Stevens, 1975; Hill, 1985; Stevens, 1989).

Studies using animal models including mice, guinea pigs, and rabbits have contributed much of the information known about HSV latency and reactivation. The operational definition of latency was developed in the mouse model and is applicable to all the other model systems (reviewed in Roizman, 1987). Briefly, infection of the mouse by inoculation of tissues innervated by sensory ganglia results in local lesions that heal and ultimately disappear. As long as the peripheral lesions at the site of

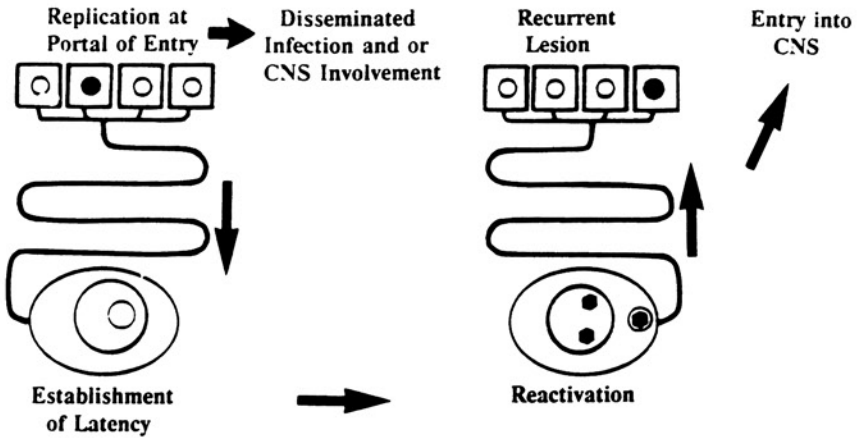


Figure 2

Natural course of HSV infection in vivo. Virus first replicates in epithelial cells (squares) at the portal of entry, then moves through sensory ganglia (curved lines) to establish latent infections in neurons (ovals) from which the virus may periodically reactivate. (Reproduced with permission from Roizman and Sears, 1993)

inoculation persist, infectious virus may also be isolated by maceration of the ganglion and inoculation of susceptible cells in culture (Stevens et al., 1971). After the peripheral lesions have healed, infectious virus may be recovered by explantation of the whole ganglion, minced tissues, or dispersed ganglionic cells in cultures of susceptible cells. During this phase, virus cannot be isolated from macerated ganglionic tissue inoculated into cell cultures. The implicit operational definition is that the latent virus is one that must be induced to multiply (reactivate) and that does not exist in infectious form. More recently, the definition of latency has extended to include viruses that can be detected in sensory ganglia several weeks after infection by in situ hybridation with probes for the latency-associated transcripts (LATs) (described below) or by assays of viral DNA in the ganglia (Roizman and Sears, 1993).

Although the overall mechanisms governing HSV latency remain obscure, extensive studies on ganglia harboring latent HSV have demonstrated several important features of latent infections that relate to the role of virus replication and gene expression. During latency, the viral genome persists as a circular episome (Mellerick et al., 1987) condensed into a chromatin-like structure (Deshmane et al., 1989) and shows a highly restricted and characterisitic pattern of viral gene expression (Hill, 1985). A diverse group of RNAs collectively referred to latency-associated transcripts or LATs accumulate in the nuclei of neurons of latently infected animals and humans and are the only viral transcripts detected (Stevens et al., 1987; Krause et al., 1988). The most abundant or major species is approximately 2 kb in length and is believed to be a stable intron (Farrell et al., 1991) although less abundant 1.5- and 1.45 kb species are also detectable and are presumed to be derived from 2 kb RNA by splicing (Deatly et al., 1988; Wagner et al., 1988). The LATs overlap the HSV ICP0 immediate-early regulatory gene and are transcribed off the opposite DNA strand.

Because it runs in part antisense to the 3' terminus of the ICP0 gene, it has been postulated that the function of LAT is to preclude the expression of ICP0 gene (Stevens et al., 1987); however, studies with LAT mutants suggest that the absence of LAT does not enhance the level of ICP0 mRNA. Confounding the issue are accumulated data indicating that LAT is not required for the establishment or maintenance of latency (Ho et al., 1989; Sederati et al., 1989; Steiner et al., 1989) or for reactivation of latent virus (Block et al., 1990; Hill et al., 1990). In addition, numerous attempts by several laboratories to identify a protein encoded by LAT and expressed during latency in vivo have failed. Although the LAT RNAs are conserved in a very similar form in HSV-2 (Mitchell, 1990), the function of LAT during latency remains elusive.

To date, no virus-encoded determinants, including several essential for lytic viral replication, have been identified as being essential for either establishment and maintenance of latency or reactivation suggesting that these events are primarily mediated by the cellular environment of the neuron rather than by specific virus genes (reviewed in Roizman and Sears, 1993). Moreover, some neuronal factors such as the octomer binding protein (Oct-2) demonstrate an ability to inhibit the trans-activation of HSV immediate-early regulatory proteins (Lillycrop et al., 1993) indicating that their presence in the neuron might contribute to an HSV latent state. In addition, nerve growth factor (NGF) has been shown to decrease HSV pathogenicity in vivo (Aloe, 1987) and upregulate the expression of Oct-2 in neuronal cultures from dorsal root ganglia (Wood et al., 1992) implying that neurotrophic factors may also play a role in HSV latency. An outgrowth of these results has been the development of an in vitro model for HSV latency and reactivation using sensory neurons in culture along with NGF and drugs that inhibit viral DNA synthesis (Wilcox and Johnson, 1988). Removal

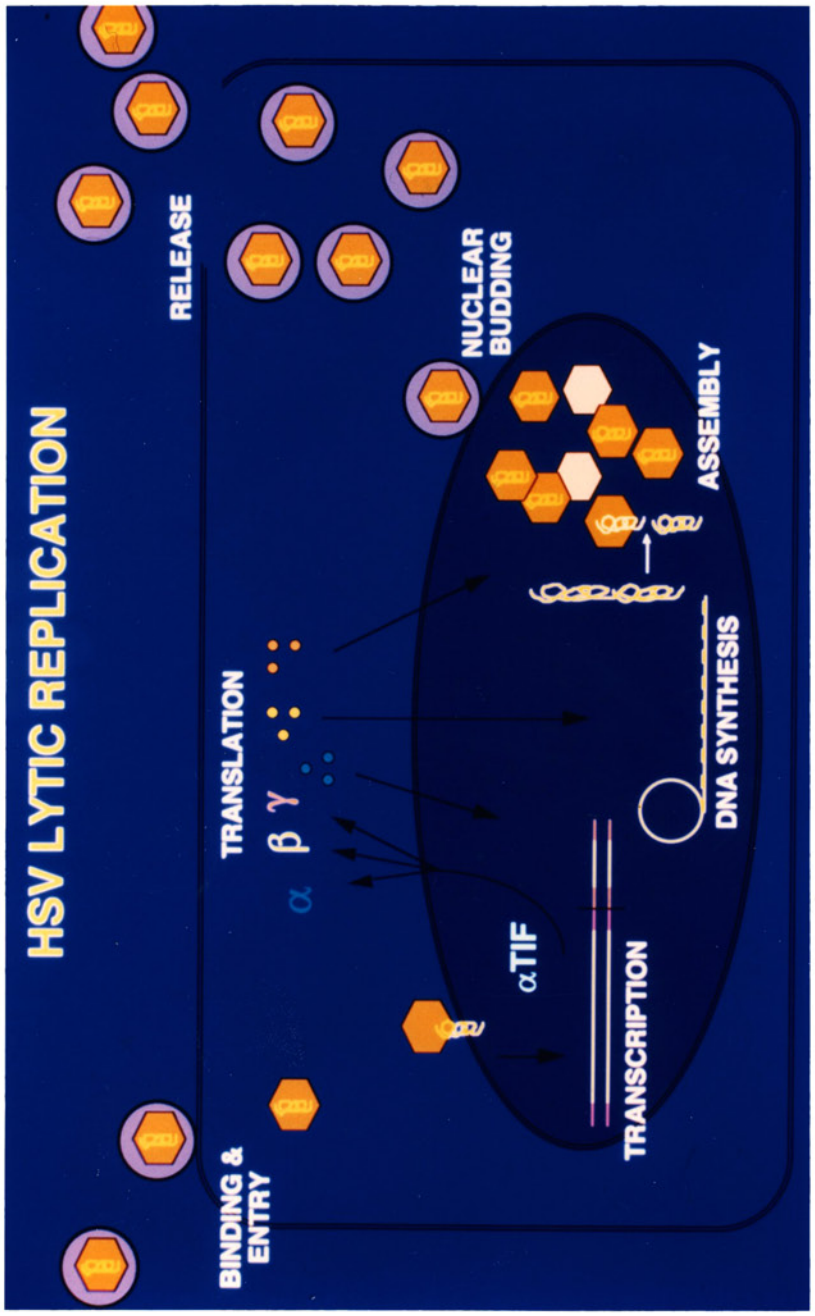
of NGF from these infected cultures results in viral reactivation within 24-48 hours. The role of viral or neuronal gene products in the mechanisms that govern the establishment and maintenance of latency as well as reactivation leading to a productive HSV infection remain to be elucidated.

b. Lytic Replication. Herpes simplex virus replication has been extensively studied in cell culture since HSV exhibits a broad host range in vitro capable of lytically infecting a variety of cells of both human and animal origin (Leetsma et al., 1969). Although HSV displays a tropism for epithelial and neuronal cells in vivo, ironically, attempts to find either cultured mammalian cells lacking receptors or cells that are non-permissive for HSV replication have been unsuccessful.

The replicative cycle of HSV in cell culture is rapid, approximately 12-18 hours in duration, with viral eclipse occurring between 5-8 hours post infection (hpi) (Roizman and Furlong, 1974) (Figure 3). The initial stages of HSV infection involve attachment to the cell surface and fusion of the viral envelope with the plasma membrane. Heparan sulfate has been identified as a major factor mediating the binding of HSV to the cell (WuDunn and Spear, 1989; Shieh et al., 1992); however, removal of heparan sulfate or competition by heparan does not completely abrogate HSV attachment or infectivity (implicating more than one attachment pathway at least in vitro). Of the eleven HSV glycoproteins, gB and gC on the viral envelope are involved in the initial binding to heparan sulfate proteoglycans on the cell surface. (WuDunn and Spear, 1989; Herold et al., 1991; Shieh et al., 1992). Following attachment, fusion and penetration of the virus proceed very rapidly (Huang and Wagner, 1964) by a multi-step process involving at least three HSV glycoproteins (gB, gD, and gH) (Sarmiento et al., 1979; Spear, 1985; Ligas and Johnson, 1988). After penetrating the plasma membrane, the de-enveloped HSV particle is transported across

Figure 3

Herpes simplex virus lytic replication. A diagram showing the various stages of the replicative cycle of HSV in cell culture including attachment and penetration, uncoating, transcription, DNA replication, viral assembly, and egress.


HSV LYTIC REPLICATION
BINDING & ENTRY
TRANSLATION
α β γ
αTIF
TRANSCRIPTION
DNA SYNTHESIS
ASSEMBLY
NUCLEAR BUDDING
RELEASE

the cytoplasm to the nuclear membrane by a mechanism probably mediated by the cellular cytoskeleton (Batterson and Roizman, 1983). The capsid structure is dissociated to release the viral genome into the nucleus most likely through a nuclear pore (Hummeler et al., 1969). Along with viral DNA, some virion components can also translocate to the nucleus upon infection (Batterson and Roizman, 1983; Preston and Notarianni, 1983).

HSV transcription, replication, and assembly occur in the nucleus (Figure 3). Transcription of viral DNA is accomplished by the host-cell RNA polymerase II (Constanzo et al., 1977) but with the participation of viral factors at all stages of infection. HSV-1 genes form at least three transcriptional groups, designated α, β, and γ, (immediate-early [IE], early[E], and late[L], respectively) whose expression is coordinately regulated and sequentially ordered in a cascade fashion (Honess and Roizman, 1974; Honess and Roizman, 1975; reviewed in Roizman and Sears, 1993). The α genes are expressed first and are operationally defined as capable of being transcribed in the absence of de novo viral protein synthesis. The synthesis of α polypeptides reaches peak rates at approximately two to four hpi and continues until late in infection. Most of the α proteins have been shown to have regulatory functions required for the expression of viral genes later in infection and include infected cell polypeptides (ICPs) 0, 4, 22, 27, and 47 (Kit et al., 1978; Knipe et al., 1978; Dixon and Schaffer, 1980; Sacks et al., 1985; Sears et al., 1985) (Table 2). A virion tegument protein designated α-TIF (alpha-transinducing factor) mediates the induction of α gene expression upon infection (Batterson and Roizman, 1983; Campbell et al., 1984; Pellet et al., 1985). The β genes encode proteins necessary for viral DNA synthesis as well as enzymes involved in nucleic acid metabolism (Table 3) and require functional α proteins for their transcription, especially ICP4 (Honess and Roizman,

Table 2 Transactivators

Protein	Gene	Class	^{32}P
ICP0	RL2	α	+
ICP4	RS1	α	+
ICP22	US1	α	+
ICP27	UL54	α	+
αTIF	UL48	γ	+

Table 3 DNA Replication/Metabolism

Protein	Gene	Class	^{32}P
DNA pol	UL30	β	
pol accessory	UL42	β	+
SSB (ICP8)	UL29	β	
Ori-binding	UL9	β	
Helicase	UL5	β	
Primase	UL52	β	
	UL8	β	
TK	UL23	β	
DNase	UL12	β	+
RR (ICP6)	UL39/40	β	+
dUTPase	UL50	β	
Uracil gly.	UL2	β	

Table 4 Cleavage/Encapsidation

Protein	Gene	Class	^{32}P
?	UL6	?	
?	UL25	?	
?	UL28	?	
?	UL32	?	
?	UL33	?	
DNase	UL12	β	+
Lg. Teg	UL36	γ	+

Table 5 Envelopment/Egress

Protein	Gene	Class	^{32}P
gH	UL22	γ	
?	UL20	γ	
Teg.	UL11	?	
DNase	UL12	β	+

^{32}P; Phosphorylated Protein

RR; Ribonucleotide Reductase

gly; Glycosylase

Teg; Tegument Protein

1975; Dixon and Schaffer, 1980). β polypeptides reach peak rates ofsynthesis at about five to seven hpi coinciding with the decline of α gene expression. The γ genes are differentiated from β genes based solely on their requirement for DNA synthesis for maximal expression. The γ genes have been subclassified into two groups designated γ1 and γ2 based on their timing of expression and dependence on viral DNA synthesis. γ1 gene expression is activated by, but not absolutely dependent upon, viral DNA synthesis, while γ2 gene expression is completely dependent on viral DNA replication. All γ proteins examined to date for their presence in virus particles have been shown to be components of the virion (Spear and Roizman, 1968; Morse et al., 1978) (Tables 6-8).

Evidence suggests that HSV DNA replication takes place predominately by a rolling circle mechanism, generating linear concatamers of tandemly repeated viral genomes (Ben Porat et al., 1977; Jacob et al., 1979; reviewed by Challberg, 1989; Roizman and Sears, 1993). Linear viral DNA is circularized shortly after entry into the host cell resulting in a loss of termini (Mocarski and Roizman, 1982). The HSV genome contains three origins (ori) of replication (Frenkel et al., 1976) although the functional significance for this reiteration is not clear. At least seven HSV proteins are required for DNA replication (Olivo and Challberg, 1988). The process of viral DNA synthesis stimulates the expression of γ genes by an unknown mechanism with the resulting γ proteins either localizing to the nucleus to form progeny capsids or associating with the nuclear and plasma membranes (Spear and Roizman, 1968; Gibson and Roizman, 1972; reviewed in Dargan, 1986). Newly replicated DNA is cleaved into unit-length molecules from concatamers and packaged into preformed empty capsids signaled by two cis-acting elements designated pac-1 and pac-2 which direct the cleavage/packaging process (Deiss et al., 1986). Only nucleocapsids containing full

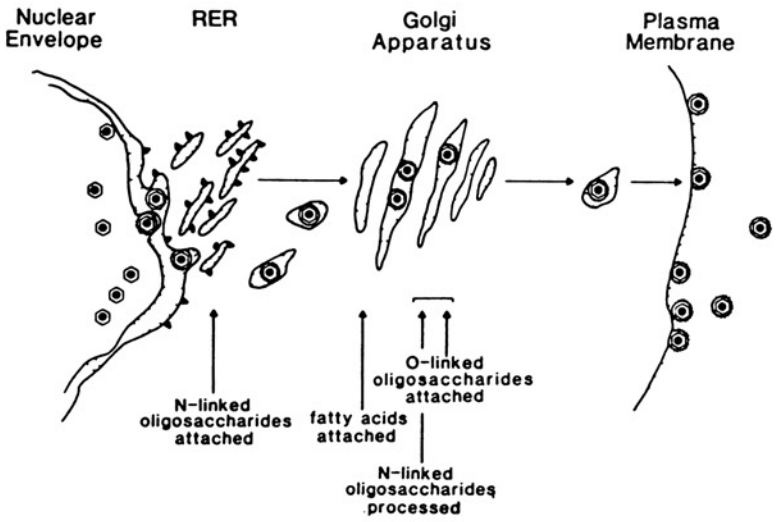


Figure 4

Diagram illustrating HSV envelopment and egress. This diagram shows pathways by which HSV glycoproteins are transported to the cell surface and indicates the intracellular sites at which specific events in posttranslational processing of the glycoproteins occur (Reproduced with permission from Spear, 1985)

length or nearly full-length genomes escape from the nucleus (Vlazny et al., 1982), presumably due to structural changes in the nucleocapsid (Gibson and Roizman, 1972).

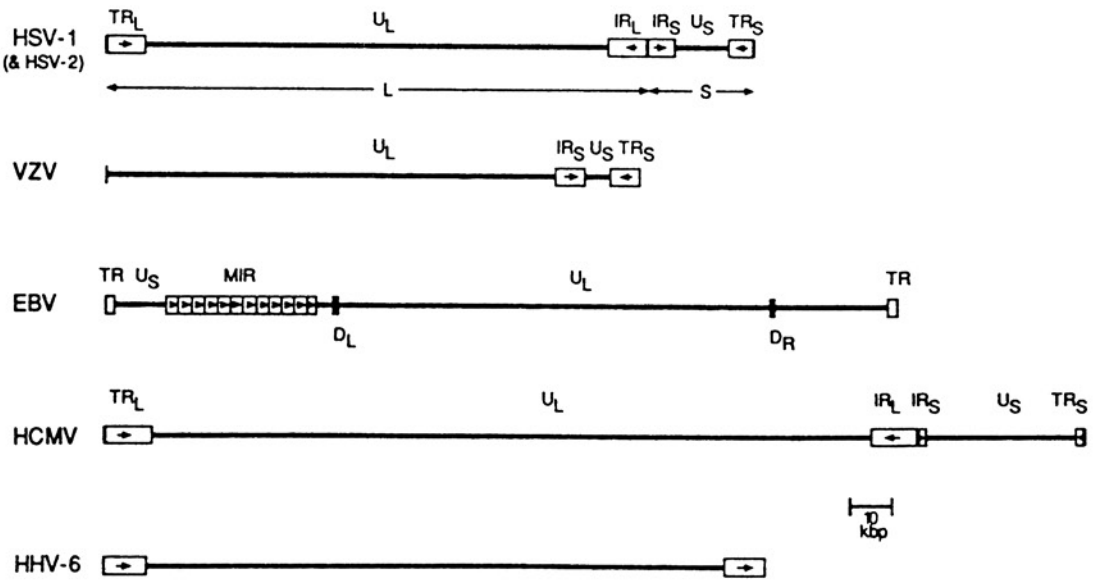
The site of HSV envelopment is generally accepted as the inner nuclear membrane, occurring at dense patches, most likely representing anchorage and tegument proteins on the inside surface and immature HSV glycoproteins on the outside surface (Darlington and Moss, 1968; reviewed in Spear, 1985; Dargan, 1986; Roizman and Sears, 1993) (Figure 4). Such enveloped particles egress from infected cells through the cisternae of the endoplasmic reticulum and Golgi to the plasma membrane; however, there are some reports of HSV transport occurring through a reverse phagocytosis mechanism involving cytoplasmic vacuoles and particles acquiring envelopes from cytoplasmic membranes (Dargan, 1986). Although not essential for virion assembly or infectivity, posttranslational modifications for maturation of HSV glycoproteins and egress are dependent on processing within the Golgi (Johnson and Spear, 1982; Johnson and Spear, 1983). Progeny virions are released either by fusion of transport vesicles with the plasma membrane or cell lysis. Some fusion-inducing HSV (syn) strains can also spread intercellularly by fusion between the plasma membranes of infected cells (Spear, 1985).

5. Genome

The HSV genome is a double-stranded, linear DNA molecule approximately 152 kb in length possessing a 68% G+C base composition (McGeoch et al., 1988). The DNA consists of two covalently linked components, designated as L (long) and S (short). Each segment contains unique sequences (U_L or U_S) bracketed by inverted repeat elements, terminally and internally positioned (TR_L and IR_L, or TR_S and IR_S, respectively) (Sheldrick and Berthelot, 1974; reviewed in McGeoch, 1989) (Figure 5). The sequences of the R_L and R_S elements are distinct, except that there is a 400 bp

Figure 5

Gross structures of the genomes of the human herpesviruses. Each linear genomic DNA is shown with unique sequences as heavy lines and repeat as boxes with relative orientations indicated by arrows. U_L and U_S, long and short (L and S) unique sequences; TR and IR, terminal and internal repeat sequences; MIR, major internal repeat; D_L and D_R (repeat elements) are each approximately 1 kb in length, and their sequences are almost identical to each other. (Reproduced with permission from McGeoch, 1989)


HSV-1
(& HSV-2)
TRL
UL
IRL IRS US TRS
L
S
VZV
UL
IRS US TRS
EBV
TR US
MIR
UL
TR
DL
DR
HCMV
TRL
UL
IRL IRS
US
TRS
10
kbp
HHV-6

direct repeat at the genome termini, termed the a sequence, and at least one other copy of this element is found at the junction of L and S in the opposite orientation to that of the terminal copies (Roizman, 1979; Davidson and Wilke, 1981). The L and S components can invert relative to one another, yielding four different isomeric arrangements of the genome which are found in equimolar amounts in HSV DNA preparations; one isomer is arbitrarily designated as the prototype for purposes of genomic map representations (Roizman, 1979).

The HSV genome demonstrates some similarity with other human herpesviruses in both its structure and genetic content (reviewed in McGeoch, 1989). In general, human herpesvirus genomes consist of linear, double-stranded DNA molecules containing one or two unique segments along with terminal (and sometimes internal) direct repeat elements (Figure 5). Although the genomic size, G+C content, nucleotide sequence, and organization of repeat elements vary among these viruses, by comparisons of the complete genomic sequences for HSV-1 (McGeoch et al., 1988), varicella-zoster virus (VZV; Davison and Scott, 1986), Epstein-Barr virus (EBV; Baer et al., 1984), and human cytomegalovirus (HCMV; Chee et al., 1990), some similarities in sets and layouts of genes are revealed.

For the alphaherpesviruses, HSV-1 and VZV, the layout of genes appears to be largely co-linear, and most of the corresponding pairs of genes in HSV-1 and VZV show recognizable similarities in their predicted amino acid sequences (Davison and McGeoch, 1986; Davidson and Scott, 1986). Comparison of gene content and arrangement between EBV and VZV (subsequently applied to HSV-1) identified 29 pairs of genes homologous by amino acid sequence similarity, and 14 more by their genomic locations, sizes, and hydropathic profiles of encoded polypeptides; however, there are several genes which are unique to each virus (Davison and Taylor, 1987;

McGeoch et al., 1988). Although the HCMV genomic structure is similar to HSV in its layout of repeats and unique segments, it is much larger (230 kb) and contains many distinct genes which show no similarity to genes of other herpesviruses (Chee et al., 1990).

6. Genes and Encoded Functions

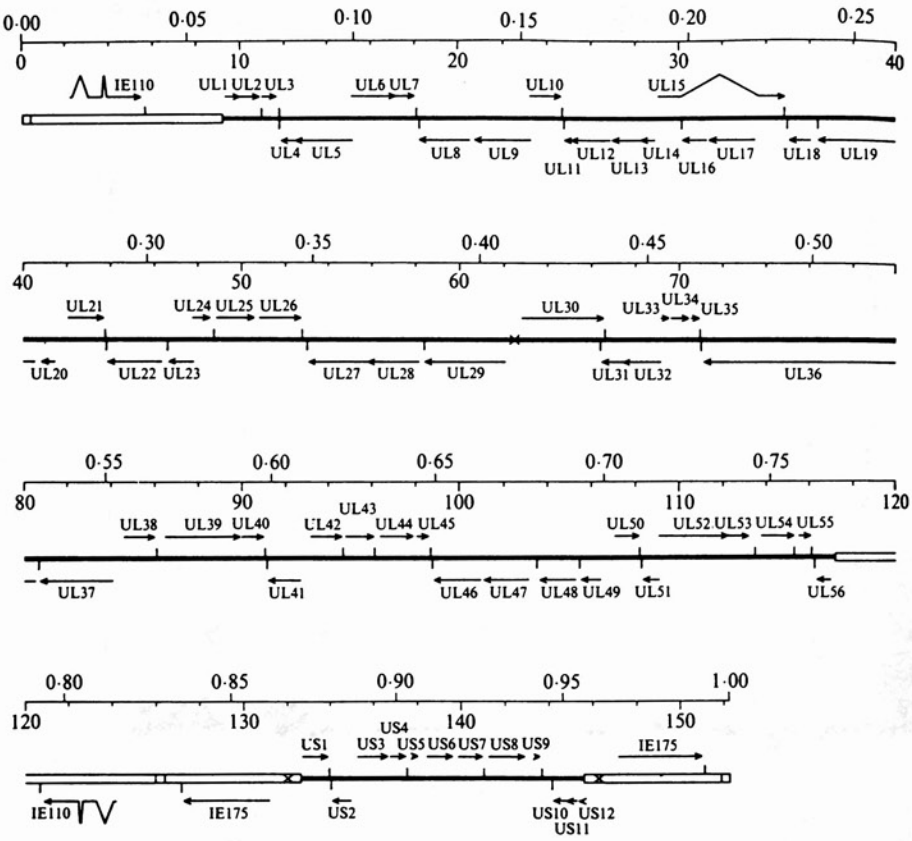
The genome of HSV-1 contains at least 77 open reading frames (ORFs) encoding approximately 75 distinct proteins based on DNA sequence and transcriptional analyses (McGeoch, 1989; reviewed in Roizman and Sears, 1993) (Figure 6). Of the genes known to be encoded by the HSV genome, 58 map in the U_L, 13 map in the U_S, and two copies of 3 genes map in the reiterated sequences which flank U_L and U_S (Baines et al., 1994). Precise functions have been defined for at least 24 HSV-1 proteins; however, the functions of the remaining majority of proteins have been partially characterized or remain unknown.

Identification of the proteins specified by the individual open reading frames has been facilitated by both biochemical and genetic techniques. Formerly, genetic studies of HSV depended largely on the use of temperature-sensitive (ts), conditional-lethal mutants, as well as marker rescue (cotransfection of a mutant viral genome and a wild-type viral DNA fragment); such mutations have been isolated in at least 22 genes. Insertion and deletion mutations have been generated in a number of HSV genes by transferring a DNA fragment containing the mutation and a selectable marker to the viral genome by homologous recombination with flanking DNA sequences.

At least 30 HSV genes have been identified as nonessential for growth in cell culture. However, it should be noted that none of the genes dispensable for replication in cell culture are truly dispensable in nature, since with few exceptions, none of the deletion mutants tested extensively in experimental animal systems are

Figure 6

Layout of genes in the genome of HSV-1. The HSV-1 genome is shown on four successive lines, with unique regions represented by solid lines and major repeat elements as open boxes. The lower scale represents kilobases, numbered from the left terminus, and the upper scale represents fractional map units. The sizes and orientations of proposed functional ORFs are shown by arrows. Locations of proposed transcription polyadenylation sites are indicated as short vertical bars. Locations of origins of DNA replication are shown as X. In the U_L region, on the first three lines, genes UL1 to UL56 are labeled. In the U_S region, on the bottom line, genes US1 US12 are labeled. The locations of introns in the coding regions of gene UL15 and the two copies (TR_L and IR_L) of the IE110 gene are indicated. (Reproduced with permission from McGeoch et al., 1988)


0·00
0·05
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0·15
0·20
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0
10
20
30
40
IE110
UL1 UL2 UL3
UL6 UL7
UL10
UL15
UL4 UL5
UL8 UL9
UL12 UL14 UL17 UL18 UL19
UL11 UL13 UL16
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UL27 UL28 UL29
UL31 UL32
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UL43
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UL44 UL45
UL50
UL52 UL53 UL54 UL55
UL37
UL41
UL46 UL47 UL48 UL49 UL51
UL56
0·80
0·85
0·90
0·95
1·00
120
130
140
150
US4
US1
US3 US5 US6 US7 US8 US9
IE175
IE110
IE175
US2
US10 US12
US11

equivalent to wild-type virus with respect to replication or virulence. Moreover, mutants with deletions in these genes have not been isolated from infected humans (Baines et al., 1994).

Genes essential for HSV replication in cell culture can be mutated (creating a null mutation) and complemented by a cell line carrying an appropriate fragment of HSV DNA which acts as a permissive host (Benjamin, 1970). Such host-range (hr) mutants have been obtained in at least seven HSV genes. Presently, however, no substantial information exists on the essentialness or function for a large number of HSV genes (McGeoch, 1989).

7. Infected Cell Polypeptide 8 (ICP8)

a. General Properties. The major HSV-1 DNA binding protein commonly designated ICP8 was first identified as an abundant infected cell specific polypeptide by Honess and Roizman (1973). The UL29 gene which encodes ICP8 (Quinn and McGeoch, 1985) is initially transcribed and translated after the onset of de novo viral protein synthesis, but before viral DNA replication has commenced, and is thus categorized as a member of the beta (β) or early temporal group of HSV polypeptides (Honess and Roizman, 1974). The nucleotide sequence of the ICP8 gene (Quinn and McGeoch, 1985) indicates that the protein is composed of 1196 amino acids and has a predicted molecular weight of approximatedly 128 kDa which is in good agreement with previous estimates of 120-130 kDa for the molecular weight of ICP8 by SDS-PAGE analysis (Honess and Roizman, 1974). Two electrophoretic species of ICP8 have been observed which appear to be related as precursor-product differing in the extent of reduction or intramolecular disulfide bonds (Knipe et al., 1982). ICP8 is a nonstructural polypeptide (Honess and Roizman, 1973) and does not appear to undergo any posttranslational modification including phosphorylation (Marsden et al., 1978;

Albright and Jenkins, 1993). Recently, ICP8 has been shown to be a zinc metalloprotein with the bound zinc atom appearing to be required for the overall structural stability of the molecule (Gupte et al., 1991). ICP8 plays several important roles in the HSV-1 replication cycle including viral DNA replication and regulation of HSV-1 gene expression and displays properties reminiscent of several single-stranded DNA binding proteins (SSBs) that have been characterized in other systems.

b. DNA Binding. The earliest recognized and best studied property of ICP8 is its ability to bind to DNA. ICP8 binds more tightly to single-strand DNA (ssDNA) than to double-stranded DNA (dsDNA) based on DNA-cellulose chromatography of infected cell extracts (Bayliss et al., 1975; Powell and Purifoy, 1976; Knipe et al., 1982). ICP8 has been isolated from infected cell nuclei in deoxyribonucleoprotein complexes containing viral DNA presumbly reflecting its direct binding to DNA in vivo (Lee and Knipe, 1983; Leinbach and Casto, 1983).

ICP8 has been purified to homogeneity from HSV-infected cells (Powell et al., 1981) and binds to ssDNA preferentially over dsDNA or RNA with no apparent specificity as to either the sequence or base composition of the DNA (Ruyechan and Weir, 1984; Lee and Knipe, 1985). The interaction of ICP8 with ssDNA is cooperative with ICP8 holding ssDNA in an extended conformation and able to stimulate the activity of HSV-1 DNA polymerase (Ruyechan, 1983; Ruyechan and Weir, 1984).

In addition, ICP8 has been shown to have the ability to melt duplexes of complementary DNA homopolymers and to protect bound ssDNA from nuclease digestion (Powell et al., 1981; Wang and Hall, 1990). Recently, ICP8 has been shown to promote homologous pairing and strand exchange (Bortner et al., 1993) as well as promote renaturation of complementary single strands of DNA (Dutch and Lehman, 1993) suggesting that ICP8 may play a role in the high level of recombination that

occurs during HSV-1 replication. The results described above for ICP8 are similar to those observed with procaryotic single-stranded DNA binding proteins, including bacteriophage T4 gene 32 as well as the E. coli SSB, and reflect characteristics of these proteins which may be necessary in DNA replication and recombination.

c. Role in HSV DNA Replication. A requirement for ICP8 in HSV DNA synthesis in infected cells was demonstrated by isolation of viral mutants that are temperature sensitive for growth and DNA replication and encode thermolabile ICP8 molecules (Conley et al., 1981; Weller et al., 1983; Lee and Knipe, 1983). In addition, seven viral genes including UL29 (ICP8) were found to be necessary and sufficient for HSV origin-dependent DNA synthesis using a transient transfection system (Challberg, 1986; Wu et al., 1988; McGeoch et al. 1988, reviewed in Weller, 1991). The six other genes identified through this system encode a DNA polymerase (UL30), polymerase assessory protein (UL42), helicase-primase activity (UL5/UL8/UL52), and an origin-binding protein (UL9). It should be noted that HSV encodes several additional proteins involved in DNA metabolism such as a thymidine kinase (TK; UL23), ribonucleotide reductase (RR; UL39/40), and an alkaline exonuclease (DNase; UL12) which have been shown to be or are presumed necessary for viral DNA replication in growth-arrested cells such as neurons (reviewed in Knipe, 1989) (Table 3). No enzymatic activities have been associated with ICP8, and the precise role of ICP8 in viral DNA replication is not known; however, it is proposed that the function of ICP8 is analogous to other "helix-destabilizing" proteins mentioned above: to bind ssDNA at the replication fork formed by unwinding parental duplex DNA and to facilitate use of these strands by stimulating the activities of DNA replication enzymes and promote the assembly of complexes of DNA replication proteins (Figure 7).

Further evidence of the role of ICP8 in DNA replication has come from

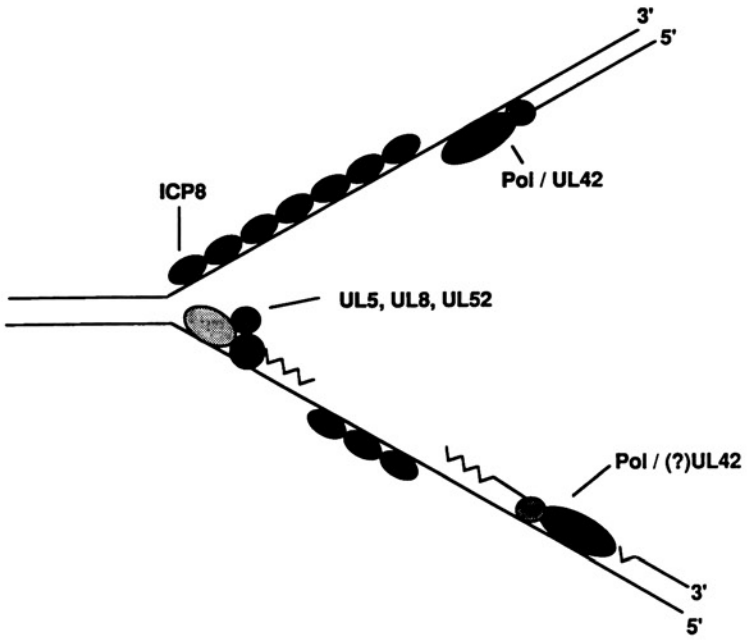


Figure 7

A schematic summary of the biochemical activities of the HSV-encoded replication proteins. This diagram includes six of the seven replication proteins identified by the transient transfection system described by Wu et al. (1988) and highlights the role of the ICP8 protein as discussed in the text. (Reproduced with permission from Olivo and Challberg, 1991)

immunofluorescence microscopy studies which demonstrate that ICP8 is capable of localizing to the nucleus independent of other viral proteins (Quinlan et al., 1984) and that its intranuclear distribution is dependent upon the status of viral DNA replication (Knipe and Spang, 1982). Furthermore, ICP8 acts to facilitate the localization of other viral replication proteins in the nucleus such as the DNA polymerase to "prereplicative sites" and "replication compartments" while promoting the redistribution of the host cell DNA replication apparatus to these sites (de Bruyn Kops and Knipe, 1988; Bush et al., 1991). Thus, one function of ICP8 may be to promote viral DNA replication by organizing the assembly of complexes of viral and cellular DNA replication proteins in the cell nucleus.

d. Interactions with other HSV Proteins. Evidence for the ability of ICP8 to interact with other viral proteins involved in DNA replication including the viral DNA polymerase (UL30) and polymerase assessory protein (UL42) has been obtained from studies utilizing both genetic and physical methods (Powell et al., 1981; Littler et al., 1983; Chiou et al., 1985). Further evidence that these proteins form a specific and probably functional complex has been demonstrated using immunoaffinity columns and coprecipitation techniques (Vaughan et al., 1984; Thomas et al., 1988; Thomas et al., 1992). Interestingly, these studies also demonstrate the ability of ICP8 to associate with the HSV alkaline exonuclease (DNase; UL12), a protein that is not essential for viral DNA replication (Wu et al., 1988; Weller et al., 1990) in cell culture but necessary for the efficient production of progeny virus possibly functioning in the packaging of progeny DNA into virion particles. Recently, a direct physical interaction between ICP8 and the origin-binding protein (UL9) has been demonstrated by protein affinity chromatography (Boehmer and Lehman, 1993).

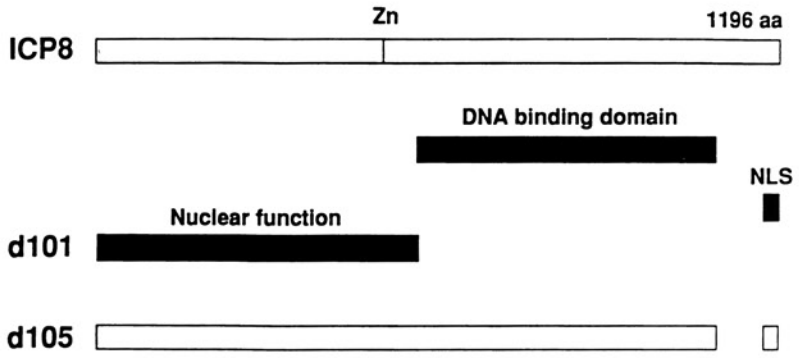
e. Functional Domains. Genetic evidence for functional domains of ICP8 is

based on (1) ts mutants (Lee and Knipe, 1983; Leinbach et al., 1984; Quinlan et al., 1984; Ruyechan et al., 1986; Gao et al., 1988), (2) expression of cloned wild-type, mutant, and truncated versions of the ICP8 gene by transient transfection or in vitro transcription and translation, and analysis of tryptic fragments of ICP8 (Gao et al., 1988; Leinbach and Heath, 1988; Leinbach and Heath, 1989; Wang and Hall, 1990), and (3) hr mutants in permissive cell lines (Gao and Knipe, 1989). These findings are represented in Figure 8 and are summarized as follows: The C-terminal 28 residues are sufficient for nuclear localization while C-terminal amino acids 564-1160 are sufficient for DNA binding. The N-terminus is not absolutely required for binding ssDNA but may be involved in stabilizing the binding activity of the C-terminus; this functional domain exists separately from DNA binding and nuclear localization yet is important for viral growth and DNA replication. A zinc-binding domain (amino acids 499-512), which is common to many DNA binding proteins, can be disrupted by substituting glycine for each cysteine in the zinc finger, and the protein retains some ability to bind DNA.

f. Role in HSV Transcriptional Regulation. ICP8 is required not only for viral DNA replication but also for normal regulation of viral gene expression. A variety of ICP8 mutants show altered expression of α, β, $\gamma 1$, and $\gamma 2$ mRNAs (Conley et al., 1981; Godowski and Knipe, 1983; Godowski and Knipe, 1985; Godowski and Knipe, 1986; Orberg and Schaffer, 1987). However, no common effect, either positive or negative regulation of the different temporal classes, has been consistently reported. Recent studies demonstrate that ICP8 exerts a positive effect on late viral gene expression independent of viral DNA replication (Gao and Knipe, 1991). A mutant ICP8 protein (d105; Figure 8) expressed from a transformed cell line was shown to exhibit a trans-dominant negative effect on γ gene expression while inhibiting viral

Figure 8

Functional domains of the ICP8 protein. Analysis of a variety of ICP8 mutants indicates that the DNA-binding region of the ICP8 protein is located within residues 564-1081 and that the C-terminal 28 residues of the ICP8 protein can function as a nuclear localization signal (NLS). Mutant d101 which lacks residues 17-563 localizes to the nucleus and binds to ssDNA but fails to promote viral DNA replication (Gao and Knipe, 1989); this indicates that the N-terminal half of the ICP8 protein has a nuclear function other than DNA binding. The d105 mutant has a deletion of residues 1082-1169 and exhibits a trans-dominant negative phenotype (Gao and Knipe, 1991).


Zn
1196 aa
ICP8
DNA binding domain
NLS
Nuclear function
d101
d105

DNA synthesis to a very limited degree (3-5 fold) during an HSV infection. These results strongly suggest that ICP8 may play a role late in infection in stimulating γ gene expression. It is proposed that late in infection, ICP8 may optimize transcription by binding single-stranded regions of progeny DNA, keeping promoter regions open for transcription, and that ICP8 may require interactions with other proteins to facilitate this modified ICP8 function and potentially a specific recognition of late gene promoters (Gao and Knipe, 1991).

B. HSV Virion Maturation and Associated Polypeptides

The assembly of herpesviruses is a complex process involving many viral proteins and intricate interactions with the host cell and is as yet not understood in detail (reviewed in Dargan, 1986; Roizman and Sears, 1993; Rixon, 1993). The complexity of the virion is highlighted by the fact that over half of the 75 protein-coding genes of HSV-1 are known or suspected to encode proteins which are present in the virion or involved in virion assembly (Rixon, 1993) (Tables 4-8). Proteins found in the virion are by definition structural; however, their presence alone does not necessarily indicate a fundamental role in structure. Virion proteins may have a variety of roles in addition to that of forming the particle.

Much of the current understanding of virion maturation has resulted from electron microscopy studies of wild-type and mutant herpesviruses. In addition, the use of expression systems such as vaccinia virus and baculovirus has facilitated further dissection of assembly mechanisms by permitting analysis of the roles of individual viral gene products. The following is a brief overview of what is currently known about the assembly process and includes a description of the viral gene products involved at each stage where known.

Table 6 Capsid Proteins

Protein	Gene	Class	Phosphorylated
VP5	UL19	γ	+
VP19C	UL38	γ	
VP21	UL26	γ	
VP22a	UL26.5	γ	+
VP23	UL18	γ	
VP24	UL26	γ	
VP26	UL35	γ	+

Table 7 Tegument Proteins

Protein	Gene	Class	^{32}P
ICP0	RL2	α	+
ICP4	RS1	α	+
	UL11	γ	+
Kinase	UL13	β	+
	UL21	γ	
	UL25	γ	
	UL36	γ	+
	UL37	γ	+
VHS	UL41	γ	+
	UL46	γ	
	UL47	γ	+
αTIF	UL48	γ	+
	UL49	γ	+
	US9	γ	+
	US11	γ	

Table 8 Envelope /Membrane

Protein	Gene	Class	^{32}P
gB	UL27	γ	+
gC	UL44	γ	
gD	US6	γ	
gE	US8	γ	+
gG	US4	γ	
gH	UL22	γ	
gI	US7	γ	
gJ	US5	γ	
gK	UL53	γ	
gL	UL1	γ	
gM	UL10	γ	
	UL20	γ	
	UL34	γ	+
	UL43	γ	
	UL45	?	

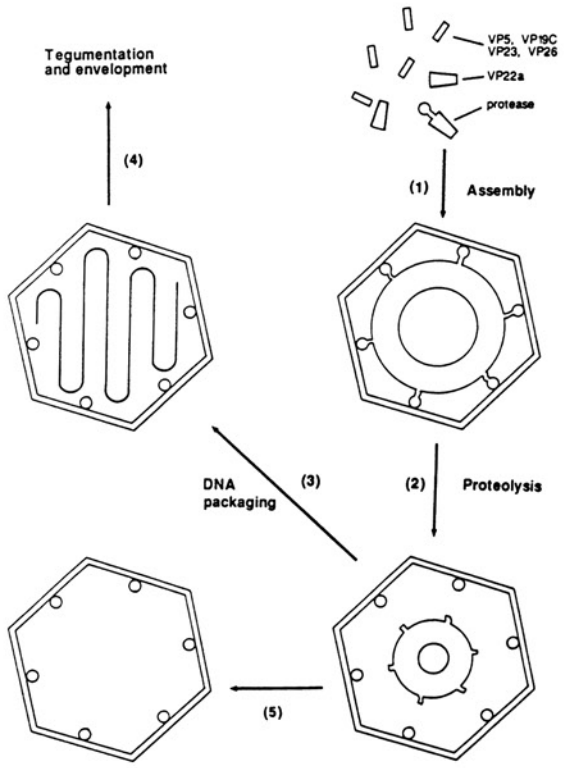
^{32}P; Phosphorylated Protein

Herpesvirus virion maturation can essentially be divided into four sequential steps which includes (1) assembly of nucleocapsids (2) cleavage and encapsidation of the DNA (3) tegumentation and envelopment and (4) egress (Figure 9). Empty viral capsids are first assembled in the nuclei of virus-infected cells. Three types of capsid structures designated A, B, and C, or empty, intermediate, and full, can be isolated by sucrose gradient centrifugation (Gibson and Roizman, 1972). Both A and B capsids lack DNA and are found in the nuclei of infected cells; however, B capsids possess a proteinaceous internal scaffold. C capsids contain DNA and mature into infectious virions (Perdue et al., 1975). Pulse-chase experiments have shown that B capsids are the precursors to both A and C capsids while A capsids are considered to result from abortive attempts at packaging DNA into B capsids (Sherman and Bachenheimer, 1988; Preston et al., 1992). Analysis by SDS-PAGE has shown that HSV-1 capsids are composed of seven proteins, VP5, VP19C, VP21, VP22a, VP23, VP24, and VP26 (Spear and Roizman, 1972; Cassai et al., 1975; Cohen et al., 1980) (Table 6), which are the products of six genes including UL19, UL38, UL26, UL26.5, UL18, UL26, and UL35, respectively (Preston et al., 1983; McGeoch et al., 1988; Pertuiset et al., 1989; Rixon et al., 1990; Liu and Roizman, 1991; Davidson et al., 1992; McNabb and Courtney, 1992a; Person et al., 1993). The major capsid protein, VP5, accounts for approximately 70% of the mass of the icosahedral shell, while VP19C and VP23 make up the remainder of the shell (Steven et al., 1986; Newcomb and Brown, 1989; Newcomb and Brown, 1991; Trus et al., 1992; Newcomb et al., 1993). VP5 is the structural subunit of both the hexons and the pentons, while the intercapsomeric fibrils or triplexes that link the capsomers together are made up of VP19C and VP23.

Packaging of DNA into B capsids results in the concomitant loss of VP22a and VP21. The ability of VP22a to self-assemble into 60 nm structures (toroids) in

Figure 9

Possible scheme for HSV-1 capsid maturation. In step 1, the six capsid proteins assemble via unknown intermediates into large-cored type B capsids. The outer icosahedral shell is formed from VP5, VP19C, VP23, and VP26, while the protease (UL26) and VP22a make up the internal scaffold (Table 6). The unique N-terminal portion of the protease is portrayed as linking the two structures. In step 2, VP22a and the protease undergo proteolytic cleavage which alters the morphology of the scaffold, producing small-cored B capsids, and generates VP21 and VP24. Type C capsids are formed following the insertion of the viral genome with the concomitant removal of the scaffolding proteins VP21 and VP22a (step 3). In step 4, mature virions are produced by tegumentation and envelopment of C capsids. Type A capsids appear to be the result of abortive DNA packaging which causes loss of the scaffold (step 5). VP24 (indicated by circles) is retained in both A and C capsids. (Reproduced with permission from Rixon, 1993)


Tegumentation
and envelopment
(4)
VP5, VP19C
VP23, VP26
VP22a
protease
(1)
Assembly
DNA
packaging
(3)
(2)
Proteolysis
(5)

vitro suggests that VP22a functions as a scaffold in the inner capsid shell around which VP5, VP19C, and VP23 condense to form the capsid shell (Newcomb and Brown, 1991). The location in the capsid of VP24 and VP26 is not known although it is believed VP26 is located in the icosahedral shell, since it binds to capsomers in a 1:1 complex with VP5 (Newcomb and Brown, 1989; Newcomb and Brown, 1991). Recent work from two different groups using recombinant baculoviruses which express the capsid genes independently of other HSV-1 genes demonstrate that HSV-1 capsid proteins alone contain all the information necessary to direct capsid assembly (Thomsen et al., 1994; Tatman et al., 1994).

Newly synthesized viral DNA is processed and packaged into preformed capsids. Processing involves amplification of a sequences (which also contain the signals [pac1 and pac2] for cleavage and packaging [Deiss et al., 1986]) and cleavage of viral DNA lacking free ends (e.g., circular or head-to-tail concatemeric molecules). The cleavage process is specific such that unit length genomes are packaged. At least five HSV-1 genes that are required for cleavage and encapsidation of viral DNA have been identified through studies with HSV-1 temperature sensitive (ts) mutants (Table 4). Results from these studies have shown that the products of the UL6, UL25, UL28, UL32, and UL33 genes are required for formation of full capsids and indicate that cleavage of viral DNA and its encapsidation are tightly coupled events (Addison et al., 1984; Sherman and Bachenheimer, 1987; Sherman and Bachenheimer, 1988; Addison et al., 1990; Al-Kobaisi et al., 1991; Tengelsen et al., 1993).

The model that has emerged for the cleavage/packaging of the HSV-1 genome involves the site specific recognition of the pac1 and pac2 elements by viral and/or cellular proteins, one or more of which must contain a nuclease activity (reviewed in Fujisawa and Hearing, 1994). In addition to the gene products mentioned

above, two distinct protein complexes (V2 and V4) which bind viral DNA at separate signals (pac1 and pac2, respectively) within the a sequence and are potentially significant for the cleavage/packaging event have been reported (Chou and Roizman, 1989). The proteins in one set (V4) were identified as the product of the UL36 gene (a tegument protein) and an as yet unidentified 140 kDa viral protein which are capable of forming a sequence-specific complex with DNA containing the pac2 element. A single protein in the V2 complex was purified and identified as the alkaline exonuclease (DNase; UL12) by the use of a monoclonal antibody; however, the alkaline exonuclease appeared to bind DNA in a sequence-independent manner and therefore, the authors suggested that a sequence-specific activity may be directed by interaction with another protein(s) which was not present within the purified complex (Table 4). It is also believed that capsid proteins may play a role in genome maturation since both VP5 and the UL38 protein exhibit viral DNA binding activity (Powell and Purifoy, 1976; Braun et al., 1984).

Mature nucleocapsids must leave the nucleus and acquire tegument and envelope before exiting from the cell. Far less is known about the structure and organization of the tegument and envelope than the capsid. Nuclear DNA-containing capsids attach to patches of modified inner lamella of the nuclear membrane and become enveloped in the process; envelopment of empty capsids rarely occurs (reviewed in Roizman and Sears, 1993). Because the enveloped virions do not contain detectable amounts of host membrane proteins, it is likely that the patches represent aggregations of viral membrane proteins, presumably including the viral glycoproteins on the outside surface and anchorage and tegument proteins on the inside surface.

The factor(s) that provide the signal(s) for condensation of tegument and envelope proteins onto nucleocapsids is unknown; however both tegumentation and

envelopment are believed to occur through initial interactions or "trigger events" between capsid proteins and proteins in the tegument and envelope (reviewed in Rixon, 1993). Interestingly, studies on HSV L particles (light particles) which consist of tegument proteins surrounded by an envelope, but lack virus capsid and DNA and consequently are non-infectious, suggest L particles contain all the signals necessary for self-assembly of intact tegument and envelope proteins in a process that is not dependent on capsid maturation. Furthermore, following removal of the envelope from L particles, the remaining tegument material largely retained its structural integrity (Rixon et al., 1992; McLauchlan and Rixon, 1992). The uniformity and consistency of the composition of the tegument indicates that it is assembled in a regulated fashion.

At present, the factors that influence tegument assembly are not known, but the isolation of mutants which do not express certain tegument protein genes suggest that these proteins do not play an essential role in determining the structure of the tegument. These proteins would include the virion-host shutoff (vhs; UL41 [Read and Frenkel, 1983] as well as the gene products of UL46 and UL47 [Zhang et al., 1991]). In addition, none of the HSV-1 glycoproteins (including gB, gD, and gH, which are required for virion infectivity) has yet been shown to be necessary for virion assembly or envelopment (Rixon, 1993).

If a structural protein is not a known component of the capsid or envelope, it is generally assigned to the tegument. Although the fact that purified herpesvirus DNA is capable of initiating infection (Sheldrick et al., 1973) proves that none of the virion proteins are required for infectivity, it has become increasingly clear that certain tegument proteins have the ability to influence the process of infection (coupled with the fact that naked nucleocapsids in the cytoplasm of the infected cell are unstable [Roizman and Sears, 1993]). The clearest example of this is provided by the abundant

HSV-1 tegument protein αTIF, encoded by the UL48 gene. αTIF is a transcriptional activator which is required for efficient expression of immediate-early (IE) genes and enhances the ability of virions to initiate infection at low multiplicities (Ace et al., 1989). αTIF also appears to serve an essential structural role in the assembly of mature HSV particles (Weinheimer et al., 1992). Other well characterized components of the tegument include a protein involved in shutting off translation of host cell mRNAs (Strom and Frenkel, 1987) and at least one protein kinase (LeMaster and Roizman, 1980). Recently, the IE proteins ICP0 and ICP4, which are transcriptional activators, have been reported to be constituents of the tegument; however, their role as virion components is unknown (Yao and Courtney, 1989; Yao and Courtney, 1992). In addition, the product encoded by the UL36 gene (ICP1/2) is also a constituent of the tegument (McNabb and Courtney, 1992b) which may be involved in the cleavage and/or packaging of viral DNA. Studies by Chou and Roizman (1989) have demonstrated that the UL36 protein forms a hetero-oligomeric complex with a 140 kDa virus-specific protein, and this protein complex binds in a sequence-specific manner to the a sequences of HSV-1. The UL36 gene product has also been implicated in viral uncoating (Batterson et al., 1983). The product of the UL37 gene which is the focus of this study is a newly identified tegument protein whose function is unknown. The dispensability of many tegument proteins for virus growth in cell culture supports a view of their roles as auxiliary helper functions rather than as essential structural proteins (Rixon, 1993).

Finally, opinions differ as to the route by which enveloped virions leave the perinuclear cisterna and translocate across the cytoplasm (egress) to the extracellular space. However, most models propose that the Golgi complex is involved in maturation of the virion by processing viral glycoproteins on the virion surface and that

the mature virion finally leaves the cell by exocytosis from Golgi-derived vesicles (Rixon, 1993; Roizman and Sears, 1993). Presently, there are at least eleven known antigenically and functionally distinct species of glycoproteins in HSV-1 virions: gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM (reviewed in Spear, 1985; Roizman and Sears, 1993). Studies with temperature-sensitive mutants in gH and deletion mutants in UL20, two genes which encode structural proteins, suggest that HSV-1 may employ different gene products to ensure full processing of glycoproteins and efficient transport across the cytoplasm (Roizman and Sears, 1993) (Table 5).

C. HSV Protein Phosphorylation

Phosphorylation of proteins is an important posttranslational modification that has been shown to modulate a variety of macromolecular events, including transcription, translation, and viral transformation (reviewed in Rubin and Rosen, 1975; Leader and Katan, 1988). Most transcription factors are phosphorylated, and the phosphorylation of specific amino acid residues has been shown to (i) prevent nuclear localization, (ii) regulate protein binding to DNA sequences, and (iii) regulate the trans-activation and trans-repression activities of transcription factors (Gonzalez et al., 1989; Luscher et al., 1990; Moll et al., 1991). Phosphorylation also modulates protein function by inducing allosteric conformational changes and by creating electrostatic repulsive effects on protein domains (Sprang et al., 1988; Hurley et al., 1990).

Analysis of HSV-induced phosphoproteins in infected cells has detected at least eleven separate species (Pereira et al., 1977; Marsden et al., 1978). HSV phosphoproteins that have been identified to date include transcriptional regulatory proteins, proteins involved in DNA replication and nucleotide metabolism, and structural proteins (Tables 2-8). The phosphorylated regulatory proteins include the α proteins

ICP0, ICP4, ICP22, and ICP27 and the alpha-transinducing factor (αTIF) (Pereira et al., 1977; Marsden, 1978; Ackermann et al., 1984; Marsden et al., 1987). It has been demonstrated that the phosphorylation state of ICP4 modulates the ability of the protein to interact with different viral promoters (Papavassiliou et al., 1991).

HSV-encoded phosphoproteins with enzymatic functions include the large subunit of ribonucleotide reductase (UL39), alkaline exonuclease (UL12), and an additional protein kinase which is the product of the US3 gene (Wilcox et al., 1980; Banks et al., 1985; Frame et al., 1987). In addition, the 65 kDa dsDNA binding protein (UL42), which serves as an accessory protein for the HSV DNA polymerase, is also heavily phosphorylated (Marsden et al., 1987). Pulse-chase studies have shown that phosphate cycles on and off several of these proteins during viral replication (Wilcox et al., 1980).

The phosphorylated HSV structural proteins include the gB and gE glycoproteins and most of the tegument proteins including the virion-host shutoff (vhs; UL41) and a virion-associated kinase (UL13) (Edson et al., 1987; Overton et al., 1992; Smibert et al., 1992). The UL37 gene product which is the focus of this study is a newly recognized HSV phosphorylated tegument protein (Albright and Jenkins, 1993). The functional role of phosphorylation for most of these proteins remains unknown.

D. Specific Aims

Protein-protein and DNA-protein interactions mediate a number of important biological and viral processes such as DNA replication, gene regulation, and virus assembly. Knowledge about such interactions can enhance understanding of these processes as well as provide clues about individual protein function. Moreover, an elucidation of all HSV gene functions is necessary to better understand the complex

process of HSV replication and ultimately its pathogenesis. Therefore, the major objectives of the research constituting this dissertation were to analyze the interaction between the HSV UL37 and ICP8 proteins and to further characterize the UL37 gene product as an extension of studies that were initiated in this laboratory (Shelton et al., 1990; Shelton, 1992).

The first specific aim of this project was to produce additional UL37-and ICP8-specific reagents to facilitate studies investigating the nature of the UL37 and ICP8 protein interaction. As a result of the development of more sensitive UL37 reagents, further characterization of the UL37 protein was attempted including an analysis of its ability to undergo phosphorylation as well as its stability in infected cells. In addition, the generation of these UL37-specific reagents prompted a re-examination of purified HSV virions for the presence of the UL37 protein along with experiments designed to determine its location in virus particles; previous results with less sensitive reagents failed to detect the UL37 protein in HSV virions (Shelton et al., 1990). Further characterization of the UL37 protein in both infected cells and HSV virions constituted the overall goals of the second specific aim.

II. MATERIALS AND METHODS

A. Cells

Vero and CV-1 cells (American Type Culture Collection [ATCC], Rockville, MD), U-47 cells (obtained from Dr. Priscilla Schaffer, Department of Microbiology and Molecular Genetics, Harvard Medical School), and human thymidine kinase negative 143 cells (TK- 143; obtained from Dr. Bernard Moss, Laboratory of Viral Diseases, NIAID) were grown in Eagle's Minimal Essential Medium (EMEM; Gibco, Grand Island, NY) supplemented with 10% (v/v) Serum Plus (SP; JRH Biosciences, Inc., Lenexa, KS). To ensure that thymidine kinase revertants were not passaged, TK- 143 cells were occasionally grown in EMEM-10% SP supplemented with 20 μg/ml 5-bromodeoxyuridine (BUdR; Sigma Chemical Co., St. Louis, MO). Baby hamster kidney cells (BHK) (ATCC, Rockville, MD) were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY). All cells were maintained in a volume of approximately 40 ml in either 150 cm^2 plastic flasks (Costar Corp., Cambridge, MA) or 32 oz glass bottles (Bellco Glass, Inc., Vineland, NJ). BHK cells were grown in roller bottles (850cm^2) for virion purifications. For cell passaging, confluent cell monolayers were washed in 10-20 ml phosphate buffered saline (PBS), trypsinized with 4-5 ml trypsin/EDTA (0.25% in buffered saline/0.02% [v/v]) (JRH Biosciences Inc., Lenexa, KS) resuspended in growth media, and seeded into new flasks. All cells were grown at 37°C in an atmosphere containing approximately 5% CO_2.

B. Viruses

1. Virus Strains

Herpes simplex virus type 1 strain F (HSV-1[F]), herpes virus type 1 strain 17 (HSV-1[17]), and the parent vaccinia virus strain WR (obtained from Dr. Bernard Moss, Laboratory of Viral Diseases, NIAID) were propagated in either Vero or CV-1 cells. HSV-1(F), HSV-1(17), and vaccinia virus WR are all wild-type strains whose properties have been previously described (Ejercito et al., 1968; Chakrabarti et al., 1985). Viral stocks were prepared by infecting 5-10 150 cm^2 flasks of confluent cells at a multiplicity of infection (MOI) of 0.01 plaque forming units per cell (PFU/cell). For viral infections, cells were overlaid with 7 ml/flask of EMEM containing the virus which was allowed to adsorb for one hour at room temperature with gentle rocking. Thirty-five ml/flask of EMEM-10% SP was then added, and the cells were incubated at 37°C until a cytopathic effect (CPE) of 80-100% was reached. Infected cells were either shaken loose or scraped from each flask, pelleted in 50 ml conical tubes, and pooled. The infected cell pellet was resuspended in infected cell supernatant at a volume of 0.5 ml/flask, subjected to three cycles of quick freezing and thawing with a dry ice and ethanol bath to facilitate the release of intracellular virus, and aliquoted into wheaton vials and stored at -70°C.

The HSV-1 ICP8 deletion mutants, n2, n4, n10, d101, d102, d301, and xho (obtained from Dr. David Knipe, Department of Microbiology and Molecular Genetics, Harvard Medical School) were propagated on U-47 cells. U-47 is a Vero cell line containing three copies of the ICP8 gene per haploid genome equivalent (Orberg and Schaffer, 1987) and is capable of complementing ICP8 mutant viruses. Expression of the cellular ICP8 gene is induced upon infection with HSV-1 or an ICP8 mutant virus.

All vaccinia virus recombinants utilized in this study including VSC11, V37, V8, and V29 were propagated on either CV-1 or Vero cells and viral stocks of each prepared as previously described for strain WR. Plaque assays for all vaccinia virus recombinants were performed in the same manner as that of HSV-1 except a low gel temperature agarose (LGA) overlay supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (5 Prime to 3 Prime, Inc., West Chester, PA) was used instead of methylcellulose. A total of two overlays were used with the first containing 1% (w/v) LGA in EMEM-5% SP and the second containing the same components supplemented with X-gal (300 μg/ml). The first overlay was added at 3 ml/well of infected cells and allowed to incubate at 37°C for 48 hours. The next overlay was then added and allowed to incubate for another 24-48 hours. Virus titer was determined by counting the number of blue plaques.

2. Viral Plaque Assays

Wild-type strains of both HSV-1 and vaccinia virus were titered by the plaque assay method of Dulbecco (1952). Serial dilutions of virus ranging from 10^{-1} to 10^{-8} were made in EMEM in a total volume of 1 ml. Dilutions were added to confluent Vero cells which had been grown in six well tissue culture plates (Costar Corp., Cambridge, MA) and gently rocked at room temperature for one hour to facilitate virus adsorption. Unadsorbed virus and media were then aspirated and replaced with a methycellulose overlay (EMEM-5% SP with 1% [w/v] methycellulose) at 3 ml/well. Infected cells were then incubated for 48-72 hours at 37°C. To visualize plaques, the cells were washed carefully with PBS, and stained with 0.1% (w/v) crystal violet in 50% (v/v) methanol for 20 minutes followed by washing with water and air drying.

3. Generation of Vaccinia Virus Recombinants

The construction and characterization of V37, a recombinant vaccinia virus that expresses the HSV-1 UL37 protein, and VSC11, a recombinant vaccinia virus that does not express any HSV-1 gene and serves as a virus control have been previously described (Shelton et al., 1990). A recombinant vaccinia virus (V8) which expresses the ICP8 protein of HSV-1 was constructed in this study essentially as previously ^R described (Mackett et al., 1984; Chakrabarti et al., 1985; Mackett et al., 1985). The pV8 construct containing the ICP8 ORF cloned into the vaccinia virus shuttle vector pSC11 was successfully inserted into vaccinia virus (WR) in the following manner. Confluent CV-1 cells in 60 mm plates (Costar Corp., Lenexa, KS) were infected with 0.5 ml/plate EMEM containing vaccinia virus (WR) at an MOI of 0.05 PFU/cell. Following a two hour adsorption period at 37°C, the cells were washed twice with EMEM and overlaid with 0.5 ml/plate of $CaCl_2$-precipitated pV8 DNA in HEPES-buffered saline (1, 2, 5, or 10 μg pV8 DNA, 250 mM $CaCl_2$). Each well then received 5 ml of EMEM-10% SP and the plates were incubated for another 3.5 hour at 37°C. The medium was replaced and the cells were incubated for an additional 48 hours. Recombinant viruses (TK-/LacZ+) were selected from infected cell lysates by plaque assay on TK- 143 cells grown in medium containing BUdR. The infected cells were scraped into the existing media and disrupted by three cycles of freeze/thawing. The cell lysates were diluted in EMEM-BUdR (25 μg/ml) medium and placed on 60 mm culture dishes of TK- 143 cells (90% confluency) and were allowed to adsorb for 45 minutes at 37°C. The medium was then replaced with a 4 ml/dish overlay containing 1% (w/v) low gel temperature agar (LGA) in EMEM with 25 μg/ml BUdR and incubated at 37°C for 48 hours. A second overlay containing 1% (w/v) LGA in

EMEM with 300 μg/ml X-gal was added and allowed to incubate another 24-48 hours. Blue plaques were then picked by sterile aspiration using a micropipette and added to 1 ml of EMEM-10% SP medium (Figure 10). Each individual plaque was then subjected to three cycles of freeze/thawing and diluted for plaque

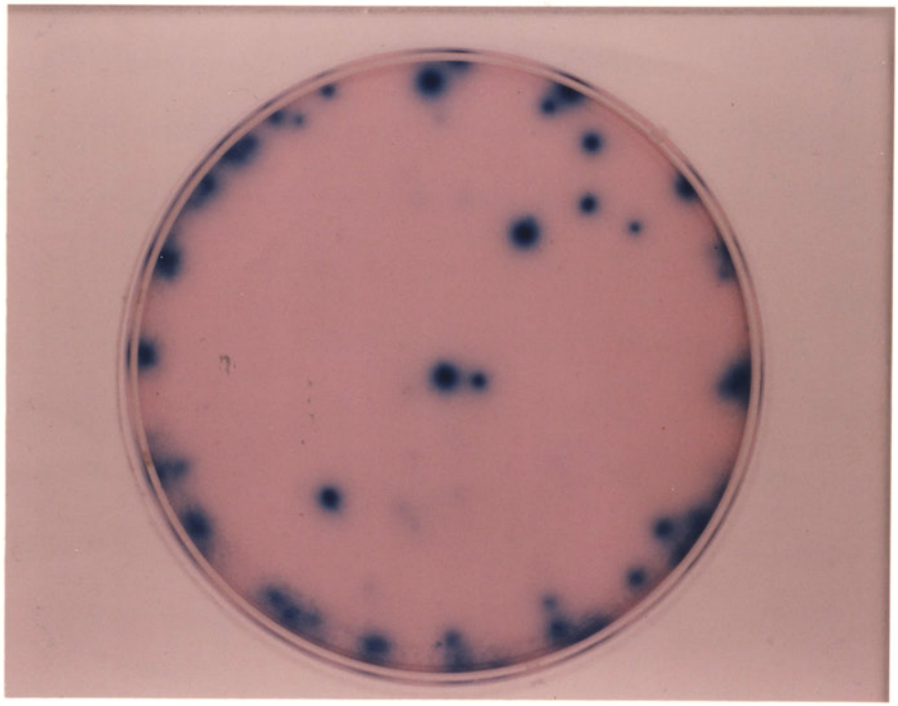


Figure 10

Seletion of recombinant vaccinia viruses. Recombinant vaccinia viruses possess a TK-/lacZ+ phenotype (blue plaques) which provides an efficient method of selection and screening by plaque assay on TK- 143 cells in the presence of BUdR and X-gal (as described in the text).

purification/amplification on TK- cells in the presence of BUdR. Three rounds of the plaque purification/amplication cycle were repeated to ensure the purity of the recombinant virus.

C. Bacteria and Plasmids

1. Bacteria

In this study, only E. coli strains DH5α (Bethesda Research Laboratories, Gaithersburg, MD) and GM119 (dam-/dcm-) were used for transformations and growth of plasmids and constructs.

2. Plasmids

The vaccinia virus shuttle vector pSC11 (Chakrabarti et al., 1985) was obtained from Dr. Bernard Moss (Laboratory of Viral Diseases, NIAID) and used in the construction of the vaccinia virus recombinant, V8. The vector pMAL-c was purchased from New England Biolabs (Beverly, MA), and used to construct the UL37 fusion proteins. The recombinant plasmid pJF67 containing the ICP8 open reading frame (ORF) was constructed in the laboratory of Dr. Frank Jenkins (Shelton et al., 1994) and served as a source of the ICP8 gene for cloning into vaccinia virus. The recombinant plasmid pHC37 containing the UL37 ORF cloned into Bluescript SK+ (Stratagene, La Jolla, CA) was also constructed in this laboratory (Shelton et al., 1990) and served as a source of the UL37 gene for generating fusion protein constructs as well as for in vitro transcription. The plasmid pUC19 was purchased from Bethesda Research Laboratories (Gaithersburg, MD) and used to test competent bacteria for transformation efficiency. A more detailed description of these plasmids as well as the recombinant constructs generated with them is described under Results.

D. DNA Isolation and Analysis

1. Plasmid DNA

Rapid isolation (mini-preparation) and large-scale preparation of plasmid DNA were performed essentially by methods outlined by Maniatis (1989). Bacteria were inoculated into 5-10 ml Luria-Bertani medium (LB) supplemented with the appropriate antibiotic (usually ampicillin [amp] at 200 μg/ml) and incubated overnight at 37°C with vigorous shaking. Overnight cultures were then used for either mini-preparations or inoculated into larger volumes of LB/amp for large-scale plasmid preparations. "Superbroth" which is an enriched derivative of LB supplemented with glucose was often used to enhance the growth of the bacteria.

Mini-preparations of plasmid DNA were performed according to the boiling-lysozyme method. Briefly, 1.5 ml of each bacterial culture was pelleted in Eppendorf tubes and lysed by resuspending in 400 μl STET/lysozyme buffer (8% [w/v] sucrose, 5% [v/v] Triton X-100, 50 mM Tris-HCL [pH 8.0], 50 mM EDTA, lysozyme [10 mg/ml]) and vortex mixing for 15 seconds. Bacterial lysates were then boiled for 55-60 seconds and spun for 10-15 minutes at room temperature in a microfuge. Pellets of insoluble cell components were removed with a toothpick, and plasmid DNA was precipitated by the addition of 400 μl isopropanol/tube, incubation at -20°C for 5 minutes, and centrifugation at 4°C for 5 minutes. Isopropanol was then aspirated and DNA pellets allowed to air dry. DNA pellets were resuspended in 50-100 μl Tris-EDTA (TE) and stored at -20°C.

Large-scale plasmid preparations were performed using the alkaline lysis method (Birnboim and Doly, 1979) as described by Maniatis (1989). Purification of closed circular DNA was achieved by equilibrium centrifugation in cesium chloride

(CsCl)-ethidium bromide density gradients. Plasmid DNA was then phenol/chloroform extracted, ethanol precipitated, resuspended in 50-100 µl Tris-EDTA (TE) and stored at -20°C. Quantity and purity of DNA was determined spectrophotometrically by taking readings at wavelengths of 260 nm and 280 nm.

2. Restriction Enzyme Analysis

Plasmid DNA was diluted in buffers supplied by the manufacturer for restriction enzyme digestions. Enzymes and buffers used in the various cloning procedures were purchased from either Promega Corp. (Madison, WI), Boehringer Mannheim Biochemicals, Inc. (Indianapolis, IN), New England Biolabs, Inc. (Beverly, MA), or Bethesda Research Laboratories (Gaithersburg, MD) and used according to manufacturers' instructions. Following restriction enzyme digestion, reactions were stopped by the addition of 10X loading buffer (0.25% [w/v] bromphenol blue, 0.25% [w/v] xylene cyanol FF, 0.25% [w/v] ficoll) if no further manipulation was required or subjected to phenol/chloroform extraction followed by chloroform extraction, ethanol precipitation, and resuspension in approximately 25 µl TE or water for use in subsequent reactions. If two or more DNA fragments resulted from restriction enzyme digestion, individual fragments were separated by agarose gel electrophoresis as described (see Agarose Gel Electrophoresis). DNA fragments were recovered by first staining the gel with ethidium bromide to visualize fragment location by long wavelength ultraviolet (UV) light followed by excision of the desired fragment(s) with a razor blade. The fragment(s) was then purified from the agarose slice using the Geneclean kit (Bio 101, Inc., La Jolla, CA) as specified by the manufacturer's protocol. The isolated DNA was resuspended in a final volume 20 µl TE or water, and stored at -20°C until needed for cloning.

E. Molecular Cloning of DNA Fragments

1. Construction of Recombinant DNA Molecules

a. General Procedures. The strategy for generation of recombinant plasmids varied depending on the particular construct desired. In most cases, restriction enzyme digestion resulted in a DNA fragment possessing 5' termini that were compatible with unique restriction sites within the vector. If necessary, DNA fragments that had 5' protruding termini were blunt-ended with the Klenow fragment of DNA Polymerase I. A typical fill-in reaction consisted of 4 µl 10X fill-in buffer (500 mM Tris-HCL [pH 7.5], 100 mM $MgCl_2$, 10 mM dithiothreitol [DTT], 500 µg/ml bovine serum albumin [BSA], 2 mM each dNTP, 2 µl Klenow fragment, and 1 µg DNA diluted to 40 µl total volume with water. The reaction was incubated for 30 minutes at room temperature, stopped by the addition of 2 µl 500 mM EDTA, and phenol/chloroform extracted twice to remove proteins followed by a single chloroform extraction. The DNA was precipitated using 95% ethanol and 0.3 M sodium acetate, and the resulting pellet resuspended in 20 µl TE. Occasionally, glycogen (l-2 µl) was added to increase the precipitation efficiency of the DNA.

Dephosphorylation of vector DNA termini was performed in order to suppress self-ligation. The dephosphorylation reaction consisted of 1-2 µg vector DNA, 0.1 U calf intestinal phosphotase (CIP), 5 µl 10X CIP buffer, and water in a total volume of 50 µl. The reaction was incubated at 56°C for 30 minutes and stopped by adding 2 µl 0.5M EDTA. Dephosphorylated DNA was then purified with phenol/chloroform extraction followed by ethanol precipitation and was resuspended in a final volume of 20 µl TE. Prior to ligation reactions, a small amount of both fragment and vector DNA were analyzed by agarose gel electrophoresis as described

to confirm integrity and approximate concentration. Typical ligation reactions included 80-100 ng prepared vector DNA, varying amounts of fragment ranging from 1:2-1:20 molar ratio (vector:insert), 4 µl 5X ligase buffer, 1-2 µl ligase (3 U/µl), and water in a total volume of 20 µl which was incubated overnight at 15°C.

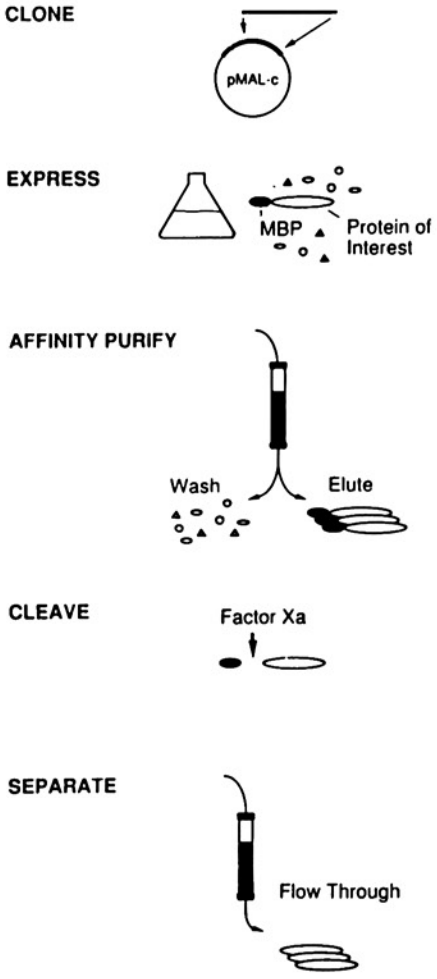
b. Construction of malE-UL37 fusion molecules. Fusion proteins consisting of the E. coli malE gene and regions of the UL37 gene were generated with a protein fusion and purification system (pMAL) purchased from New England Biolabs, Inc. (Beverly, MA) (Figure 11). The utilization of the cloning vector, pMAL-c, and the techniques of the system were performed according to manufacturer's instructions.

Fusions between the E. coli malE gene (maltose binding protein [MBP]) and sequences containing the terminal one-third and two-thirds portions of the UL37 gene as well as the entire UL37 open reading frame (ORF) were constructed. The sequences encoding the different UL37 protein domains were subcloned into the pMAL-c vector's multiple cloning site (MCS) and ligation reactions transformed into competent DH5α bacteria as previously described (see Bacterial Transformation). White transformants were then picked from LB/amp/IPTG/X-gal plates and screened for the presence of the desired insert by DNA restriction enzyme analysis (see Identification of Recombinant Plasmids). Positive colonies were tested for the expression of the desired fusion protein by performing a small scale induction experiment.

For small scale induction, medium containing 5 ml LB/amp was inoculated with a single colony containing the fusion plasmid and grown to OD_{600} = 0.5 (approximately 2 x 10^8 cells/ml). A 1 ml sample (uninduced cells) was then removed from the culture, microfuged for two minutes, and the pellet resuspended in 50 µl 1X SDS-PAGE sample buffer. To the remaining culture, IPTG was added to a final

Figure 11

Protein fusion and purification system. The cloning vector pMAL-c contains the inducible P_{tac} promoter positioned to transcribe a malE-lacZα gene fusion. The polylinker provides restriction endonuclease sites to insert the gene of interest, fusing it to the malE gene and interrupting the lacZα gene allowing a blue-to-white screen for inserts on X-gal plates. Upon induction with 0.3 mM IPTG, a MBP-fusion protein is expressed which can be purified from bacterial extracts by taking advantage of the affinity of MBP for amylose; fusion proteins are eluted from the resin with 10 mM maltose. The cloning vector also contains a sequence coding for the recognition site of the protease factor Xa located within the polylinker allowing the protein of interest to be cleaved from MBP after purification, without adding any vector-derived residues to the protein. The cleaved fusion proteins are then passed over an amylose column a second time to purify the protein of interest from the MBP domain.


CLONE
pMAL-c
EXPRESS
MBP
Protein of Interest
AFFINITY PURIFY
Wash
Elute
CLEAVE
Factor Xa
SEPARATE
Flow Through

concentration of 0.3 mM to induce expression of the fusion protein. The culture was incubated at 37°C with shaking for two hours. A 0.5 ml sample was removed from the culture (induced cells), microfuged, and resuspended in 100 µl 1X sample buffer. Equal volumes of both samples were then analyzed on SDS-polyacrylamide gels along with a purified source of maltose binding protein and high molecular weight protein standards. Induced proteins were then evaluated visually for correct size (i.e., migrating to a position corresponding to the predicted molecular weight of the fusion protein) by Coomassie blue staining. Proteins on a duplicate gel were transferred to nitrocellulose and immunoblotted with an antibody directed against the maltose binding protein to confirm the identity of the fusion protein. Glycerol stocks were prepared for confirmed positive colonies and stored at -70°C.

2. Bacterial Transformation

Preparation and transformation of competent E. coli strains were achieved with the method described by Hanahan (1983). Competent cells were aliquoted into 1 ml volumes and flash frozen using a dry ice-ethanol bath and stored at -70°C. Aliquots were thawed slowly on ice prior to use. Competency of the bacteria was tested by transformation with a closed circular plasmid (e.g., pUC19) to determine transformation efficiency.

A 20 µl ligation reaction was diluted in transformation buffer (87.5 mM $CaCl_2$, 51 µM NaCl, 56.1 µM sodium citrate, pH 7.0) to a volume of 200 µl just prior to transformation. Bacterial transformation was performed by the addition of either purified plasmid DNA or 100 µl of the diluted ligation reaction to 200 µl of competent cells and incubation on ice for thirty minutes. The cells were then subjected to heat shock at 42°C for two minutes and immediately cooled on ice for two minutes. The 300 µl transformation mix was then added to 800 µl of pre-warmed Luria-Bertani (LB)

medium supplemented with 10 mM $MgCl_2$ and 10 mM $MgSO_4$ (to enhance transformation efficiency) and incubated at 37°C with shaking for 1.5 hours. An aliquot of the culture (100 µl) was spread on LB/agar plates supplemented with the appropriate antibiotic (usually ampicillin [amp, 200 µg/ml]) and incubated overnight at 37°C. For α-complementation screening, agar plates were supplemented with 2% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (5 Prime to 3 Prime, Inc., West Chester, PA) and 25 mg/ml isopropylthio-β-D-galactoside (IPTG) (Sigma Chemical Co., St. Louis, MO). On these plates, bacteria which are β-galactosidase positive (LacZ+) appear blue while those that are negative (LacZ-) appear white.

3. Identification of Recombinant Plasmids

Bacterial colonies containing recombinant plasmids were identified by either restriction analysis of plasmid DNA or colony hybridization using specific DNA probes (Grunstein and Hogness, 1975) as described by Maniatis (1989). In cases where insertion of the desired DNA fragment resulted in inactivation of the β-Galactosidase gene in the vector (white colonies), restriction analysis of plasmid DNA for each transformant was performed.

Colony hybridization was performed to identify transformants when the vector did not use the β-galactosidase screening system. Transformants along with a positive control were replica-plated on LB/amp plates in duplicate and incubated overnight at 37°C to allow growth. The following day, one set of plates was designated as master plates and stored at 4°C and the other set used for colony blots. Biodyne nylon membranes (Pall Corp., Glen Cove, NY) were carefully laid across each plate, marked for orientation, and allowed to sit for 15 minutes in order for bacterial colonies to adhere. The membranes containing adherent colonies were then peeled from the plate and sequentially passed through a series of saturated Whatman 3MM

paper: (1) 0.5M NaOH, (2) 1M Tris [pH 7.4] (twice) and (3) 1.5M NaCl, 0.5M Tris (pH 7.4). Blots were soaked in each buffer for five minutes. Following these incubations, the blots were washed gently in 2X SSC (sodium chloride, sodium citrate) for five minutes and air dried. To fix the DNA to the membranes, blots were baked for two hours at 80°C in a vacuum oven.

The blots containing immobilized DNA were prehybridized overnight in a heat-sealable plastic bag (Dazey Corp., Industrial Airport, KS) in 10 ml hybridization buffer (30% [v/v] formamide, 6X SSC (0.99M), and 1X Denhardt's reagent ((1% [w/v] Ficoll, 1% [w/v] polyvinylpyrrolidone, 1% [w/v] BSA)) at 68°C. The next day, the blots were hybridized in fresh hybridization buffer containing a radiolabeled DNA probe (5×10^5 to 1×10^6 cpm/ml hybridization buffer). The ^{32}P-labeled double-stranded DNA probe was prepared by the random hexamer method (Feinberg and Vogelstein, 1983) and separated from free label by a G-50 spin column as described by Maniatis (1989). The probe was denatured by boiling for two minutes and chilled rapidly on ice prior to being added to the hybridization buffer. After overnight hybridization, the blots were washed four times in 2X SSC/0.1% (w/v) SDS (sodium dodecyl sulfate) for 15 minutes with rocking at room temperature, and then twice in 0.1X SSC/0.1% (w/v) SDS for two hours total in heat-sealable bags at 68°C. Hybridizations and washes were sometimes performed at 45°C without compromising stringency by increasing the formamide concentration (50%) and lowering the salt concentration (0.825 M) in the buffer. The blots were air dried, wrapped in Saran wrap, and subjected to autoradiography on Kodak XAR-5 film with an intensifying screen or exposed on a phosphor screen for PhosphorImager (Molecular Dynamics Corp., Sunnyvale, CA) analysis. Positive hybridization signals were aligned with the master plates to identify positive colonies which were then picked for DNA restriction

analysis as described to confirm the presence of the desired DNA insert.

F. Preparation of Protein Extracts

1. Total Cell Lysates for Immunoblots

Confluent Vero cells grown in 150 cm^2 flasks (1.4 x 10^7 cells/flask) were infected with appropriate virus at a multiplicity of infection (MOI) of five plaque forming units per cell (5 PFU/cell). Cells were harvested at either 24 or 48 hpi for HSV-1, ICP8 deletion mutants, and Mock, or VSC11, V8, V37, and V29, respectively. For harvest, infected cells were shaken or scraped from the flask, pelleted, and washed once with phosphate buffered saline (PBS). The cell pellets were then solubilized in 1X SDS-PAGE sample buffer (2% [w/v] SDS, 4% [v/v] glycerol, 720 mM β-mercaptoethanol, 50 mM Tris [pH 6.8], 400 μg/ml bromphenol blue) in a total of 2 ml/flask and aliquoted into 1.5 ml Eppendorf tubes and stored at -20°C. Protein extracts were sonicated and then boiled for two minutes prior to electrophoresis.

2. High-Salt Extracts for Column Chromatography

For column chromatography studies, infected cell protein extracts were prepared essentially as previously described (Powell and Purifoy, 1976). For each preparation, 10-20 150 cm^2 flasks of confluent Vero cells were infected at an MOI of 5 PFU/cell with appropriate virus. Cells were harvested at either 24 or 48 hpi for HSV-1 and ICP8 deletion mutants or vaccinia virus recombinants, respectively, by scraping and pelleting the cells. Cell pellets were frozen at -70°C. To prepare lysates, pellets were lysed gently on ice in 5-10 ml high salt buffer (50 mM Tris-HCl [pH 7.6], 5 mM EDTA, 0.5 mM DTT, 0.12 mM phenylmethanesulfonyl fluoride [PMSF], 1.7 M KCl). Nucleic acids were removed by centrifugation at 35,000 rpm in a Beckman Ti50 rotor for two hours at 4°C. The remaining supernatant was dialysed

overnight two times against two liters of TEDGP buffer (50 mM Tris-HCl [pH 7.6], 5 mM EDTA, 0.5 mM DTT, 20% [v/v] glycerol, 0.12 mM PMSF, and 50-150 mM KCl). The potassium chloride concentration in the buffer varied and was equal to the salt concentration used for equilibrating the column prior to column chromatography. The insoluble fraction in the dialysate was pelleted by centrifugation in a Beckman SS34 rotor at 19,000 rpm and the soluble fraction (supernatant) stored on ice until the column was loaded. For two experiments, infected cell proteins were radiolabeled with a total of 8 mCi $^{32}P_i$ (carrier free; New England Nuclear Corp., Boston, MA) from 6-24 hpi and harvested as described.

3. Cytoplasmic Lysates for Immunoprecipitations

Confluents Vero cells grown in 150 cm^2 flasks were infected or coinfected with the appropriate virus(es) at an MOI of 5 PFU/cell and harvested at 24 or 48 hpi for HSV-1 or vaccinia virus recombinants, respectively. For harvest, cells were washed carefully with PBS and then lysed in 2 ml/flask RIPA buffer (150 mM NaCl, 1% Nonidet P-40 [NP-40],0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl [pH 7.5]) supplemented with protease inhibitors (0.01 mM TPCK [N-tosyl-L-phenylalanine chloromethyl ketone], 0.01 mM TLCK [N-tosyl-L-lysine chloromethyl ketone] [Boehringer Mannheim Corp., Indianapolis, IN], and aprotinin [1:100] [Sigma Chemical Co., St. Louis, MO]). Cell nuclei were removed by centrifugation at 12,000 x g for five minutes at 4°C. The cytoplasmic fraction (supernatant) was then stored at -70°C.

4. Nuclear Lysates for Immunoprecipitations

Nuclear extracts for immunoprecipitation studies were prepared using a modified method of Lorenz (1991). Confluent Vero cells grown in 150 cm^2 flasks were infected with HSV-1 at an MOI of 5 PFU/cell and harvested at 24 hpi. For harvest, cells were washed twice with PBS and then treated with hypotonic buffer (10

mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF) supplemented with 0.5% NP-40 for 15 minutes on ice and disrupted by Dounce homogenization. The resulting nuclei were washed twice with hypotonic buffer and resuspended in cold low salt extraction buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 10 mM DTT, 0.5% NP-40, 1 mM PMSF). The nuclear extract was then clarified at 14,000 rpm using a Beckman SS34 rotor and the remaining pellet resuspended in PBS (0.5 ml/flask) and stored at -70°C.

5. malE-UL37 Fusion Proteins

Large scale purification of fusion proteins used for column chromatography studies or as antigens for immunization of animals for the production of polyclonal antisera were prepared according to the following protocol (New England Biolabs, Inc., Beverly, MA) (Figure 11). Three liters rich broth plus glucose (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose) and ampicillin (200 μg/ml) were inoculated with 30 ml of an overnight culture of cells containing the fusion plasmid of interest. The cells were grown to OD_{600} = 0.5 (approximately 2 x 10^8 cells/ml) by shaking with good aeration at 37°C followed by the addition of IPTG to a final concentration of 0.3 mM to induce expression of the fusion protein. The cells were then incubated at 37°C with shaking for another two hours. A one ml sample was sometimes removed prior to and following the induction period, pelleted, resuspended in sample buffer, and analyzed on an SDS-polyacrylamide gel to check the efficiency of induction of the fusion protein. The cells were harvested by centrifugation at 4000 x g for 20 minutes, resuspended in lysis buffer (10 mM sodium phosphate, 30 mM NaCl, 0.25% Tween 20, 10 mM β-mercaptoethanol, 10 mM EDTA, [pH7.0]), and stored at -70°C. All buffers in this protocol were supplemented with the protease inhibitors TPCK (0.01 mM), TLCK (0.01 mM), and aprotinin (1:100).

Prior to use, the cells were thawed slowly in cold water followed by pulsed sonication for two minutes. Sodium chloride was added to 0.5 M final concentration and the cells centrifuged at 9000 x g for 30 minutes. Solubilized proteins (supernatant) were then diluted 1:2 with column buffer (10 mM sodium phosphate [pH 7.2], 0.5 M NaCl, 1 mM sodium azide, 10 mM β-mercaptoethanol, [pH 7.0]), added to equilibrated amylose resin (5 ml column volume), and mixed end-over-end at 4°C in a 250 ml centrifuge tube for 18 hours. The following day, the resin was washed with three column volumes of column buffer containing 0.25% Tween 20 followed by five column volumes of column buffer without Tween 20. The resin with bound fusion protein was then poured into a 10 ml column and allowed to pack for several minutes at 4°C. Fusion protein was eluted from the column using column buffer supplemented with 10 mM maltose (Sigma Chemical Co., St. Louis, MO) (Figure 11) and collected in 10-15 1 ml fractions. The individual fractions were assayed for the presence of protein by the method of Bradford (1976) and protein-containing fractions pooled. Fractions were concentrated, if necessary, using centricon or centriprep microconcentrators (Amicon Corp., Danvers, MA) to approximatedly 1 mg/ml and stored at 4°C until needed.

G. Radiolabeling of Infected Cell Proteins

Radiolabeling studies were conducted in a similar manner to procedures previously described (Honess and Roizman, 1973; Wilcox et al., 1980). Vero cells were grown to confluency in 60 mm culture dishes (Costar Corp., Cambridge, MA) in media purchased from GIBCO (Grand Island, NY). The radioisotopes $^{32}P_i$ (carrier free) and [^{35}S]methionine were purchased from New England Nuclear (Boston, MA).

1. ^{32}P-orthophosphate

Confluent monolayers of Vero cells were incubated in phosphate-free medium for two hours before and after infection with either HSV-1, V37, or V8 using an MOI of 5 PFU/cell. Phosphate labeling was performed by the addition of 50 μCi/ml $^{32}P_i$ from 6 to 12, or 12 to 24 hpi and harvested at the end of each labeling period. For harvest, the monolayers were rinsed with PBS and scraped into 1 ml per dish of RIPA buffer supplemented with protease inhibitors. Lysates were stored at -70°C.

2. Pulse-chase Labeling

a. [^{35}S]methionine. Confluent monolayers of Vero cells were incubated in EMEM containing one-tenth the normal concentration of methionine for one hour prior to and following infection with HSV-1 using an MOI of 5 PFU/cell. Pulse-labeling was performed by incubating the cells in EMEM containing reduced methionine plus 37.5 μCi/ml [^{35}S]methionine for 30 minutes. Following the pulse, a cold chase was performed by incubating the cells in EMEM containing the normal concentration of methionine. Mock-infected cells were labeled in the same way.

b. ^{32}P-orthophosphate. Confluent monolayers of Vero cells were incubated in phosphate-free EMEM for one hour prior to and following infection with the appropriate virus using an MOI of 5 PFU/cell. Phosphate labeling was performed by incubating the cells in phosphate-free medium containing 62.5 μCi/ml $^{32}P_i$ for 30 minutes (pulse) followed by rinsing and incubation with normal EMEM (chase). Cells were harvested at various times post chase in 1 ml RIPA buffer as described in the preceding section.

H. Gel Electrophoresis

1. Agarose Gel Electrophoresis

DNA fragments greater than 0.5 kilobase (kb) in size were separated using horizontal 0.8% (w/v) agarose slab gels in 1X TBE running buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). DNA samples containing 1X loading buffer (0.25% [w/v] bromphenol blue, 0.25% [w/v] xylene cyanol, 25% [w/v] ficoll) were electrophoresed at constant voltage. Following electrophoresis, the gel was immersed in 1X TBE buffer containing 50 μg/ml ethidium bromide for 15 to 30 minutes to stain the DNA and then destained in water for at least ten minutes to remove excess ethidium bromide. Alternatively, ethidium bromide (0.5 μg/ml) was added to the agarose solution prior to boiling which eliminated the stain/destain steps. The DNA was visualized by placing the gel directly on an UV light box emitting at 254 nm and photographed using Polaroid type 55 or 70 film with a Polaroid MP-4 camera. If the DNA fragments were to be excised from the gel and purified for cloning, visualization of the DNA was achieved by using a hand-held long wavelength UV light to minimize damage to the nucleic acids.

2. SDS-Polyacrylamide Gel Electrophoresis

Proteins were separated by one dimensional denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Large vertical slab gels (16 cm x 20 cm) and minigels were run on Protean II and Mini-Protean II gel rigs, respectively (Bio-Rad Laboratories, Melville, NY). Gels were prepared from a 30% (w/v) acrylamide stock containing 29.2% (w/v) acrylamide and 0.8% (w/v) N,N'-methylene-bisacrylamide (bis) in distilled water. The lower gel buffer (LGB) consisted of 0.1% (w/v) SDS in 375 mM Tris-HCl (pH 8.8)

and the upper gel buffer (UGB) contained 0.1% (w/v) SDS in 125 mM Tris-HCl (pH 6.8). A stock solution of 10% (w/v) ammonium persulfate (APS) in water along with N,N,N',N'-tetramethylethylenediamine (TEMED) was used to catalyze the polymerization of the acrylamide. Running buffer consisted of 25 mM Tris, 192 mM glycine, and 0.3 mM SDS. For large gels, a 2-3 mm acrylamide plug consisting of 22.5% (w/v) acrylamide in LGB with 7.5 µl/ml APS and 7.5 µl/ml TEMED was poured to prevent leakage. The separating gel was usually 10% (w/v) acrylamide in LGB with 3.75 µl/ml APS and 0.75 µl/ml TEMED. The stacking gel consisted of 4.5% (w/v) acrylamide in UGB with 5 µl/ml APS and 2.5 µl/ml TEMED.

Protein samples diluted with sample buffer (2% [w/v] SDS, 4% [v/v] glycerol, 720 mM β-mercaptoethanol, 50 mM Tris [pH 6.8], 400 µg/ml bromphenol blue) were boiled for two minutes to solubilize proteins prior to loading. At least one well per gel was loaded with prestained high molecular weight protein standards (BRL, Gaithersburg, MD). Large gels were run at 160 mAMP-hour/gel, constant current, until the dye front moved into the plug (usually 6-8 hours). To prevent overheating, rigs containing large gels were connected to a water-cooling apparatus (Lauda RM6). Minigels were run at a constant 200 volts (V) until the dye front reached the bottom of the gel. At the end of a run, rigs were disassembled and gels either stained with Coomassie brilliant blue or transferred to nitrocellulose for immunoblot analysis. Mini-Protean II Ready Gels (12% Pre-cast) were also purchased (Bio-Rad Laboratories, Melville, NY) and used in an identical fashion as the hand-poured minigels previously described.

3. Nondenaturing Polyacrylamide Gel Electrophoresis

To resolve DNA fragments of one kb or less, vertical polyacrylamide slab gels were employed as described (see Polyacrylamide Gel Electrophoresis) with the

following modifications. Gels (12% separating) were poured and run in 1X TBE at low voltage (5V/cm) in the absence of sodium dodecyl sulfate (SDS) to prevent denaturation of small fragments of DNA. These gels were then stained and analyzed as described for agarose gels.

I. Detection of Proteins on SDS-Polyacrylamide Gels

1. Coomassie Staining

Proteins separated on SDS-polyacrylamide gels were simultaneously fixed and stained in a Coomassie brilliant blue solution (50% [v/v] methanol, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie brilliant blue). The gel was immersed in staining solution for 15-30 minutes at room temperature with rocking followed by destaining in destain solution (5% [v/v] methanol, 7% [v/v] acetic acid) for at least three hours or until protein bands were clearly visualized. After destaining, the gel was soaked in 10% (v/v) glycerol, sandwiched between two sheets of wetted drying film, and placed in a drying frame (Promega Corp., Madison, WI) until dry.

2. Immunoblotting

To identify proteins separated by SDS-polyacrylamide gels, proteins were transferred to 0.45 μm nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) for immunoblot analysis. Transfer of proteins was achieved with a semi-dry blotting apparatus (Bio-Rad Laboratories, Melville, NY) for large gels or a Bio-Rad Mini-Transblot II for minigels. Prior to transfer, gels were soaked in transfer buffer WB-1 (219 mM glycine, 25 mM Tris-base, 20% [v/v] methanol) and sandwiched next to wetted nitrocellulose membranes between WB-1 saturated Whatman 3MM filter paper and Scotch-brite pads for assembly in transblot cassettes. Semi-dry transfer was achieved by running at 20 volts, limiting current to 0.77 Amp (0.3 Amp x area of gel

[in cm^2]), for one hour. Mini-transblot rigs were filled with WB-1 and run at 100 volts for one hour with ice-pack cooling or 30 volts overnight. Following transfer, the nitrocellulose membranes were blocked with 10% (w/v) nonfat milk in binding buffer WB-2 (4 mM EDTA, 10 mM Tris-HCl [pH 7.6], 0.15 M NaCl, 0.05% [v/v] Tween 20, 0.02% [w/v] sodium azide) at 4°C overnight or until ready to probe with antibody. If proteins were radiolabeled, the nitrocellulose membranes were exposed on a phosphor screen and proteins detected directly by scanning with a phosphorimager (Molecular Dynamics Corp., Sunnyvale, CA) prior to blocking and immunoblotting.

Typically, blots were probed by incubation with the primary antibody diluted 1:50 in 1% (w/v) nonfat milk/WB-2 buffer. Blots and antibody were placed in heat-sealable plastic bags (Dazey Corp., Industrial Airport, KS) and incubated for one to two hours at room temperature with rocking followed by extensive washing in WB-2 buffer (four times 10 minutes each wash). Bound antibody was detected by sealing the washed blots in a plastic bag with WB-2 buffer containing 1 µl/ml ^{125}I-labeled Staphlococcus aureus protein A (127 nCi/µl) and incubating at room temperature for one hour with rocking. Blots were then washed in WB-2 buffer as before to remove unbound protein A, air dried, wrapped in Saran wrap, and analyzed by autoradioagraphy using Kodak XAR-5 film with an intensifying screen or exposed on a phosphor screen for phosphorimager analysis.

To identify radiolabeled proteins, blots were probed with an alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit IgG; Boehringer Mannheim Corp., Indianapolis, IN) in a similar fashion as described above. Phosphate buffered saline (PBS) was used for both washing and secondary antibody dilution since sodium azide interferes with the substrate-enzyme reaction. Chromogenic substrate for alkaline phosphatase was prepared just prior to use by adding 44 µl NBT (nitro blue

tetrazolium, 75 mg/ml in dimethylformamide) and 33 µl BCIP (bromochloroindolyl phosphate, 50 mg/ml in dimethylformamide) to 10 ml alkaline phosphatase buffer (100 mM Tris [pH 9.5], 5 mM $MgCl_2$, 100 mM NaCl). After incubation of the secondary antibody and PBS washes, the blots were transferred to a shallow tray, substrate added, and incubated at room temperature with gentle agitation. When protein bands of desired intensity appeared, the blots were rinsed with PBS containing 20 mM EDTA to stop the reaction, air dried, and wrapped in aluminum foil to eliminate exposure to light.

J. Antisera

1. Antibody Production in Animals

Polyclonal antisera in mice were generated by intraperitoneal (ip) inoculation of BALB/c mice (National Cancer Institute, NIH) with the appropriate purified malE-UL37 fusion protein (50 µg/ml) (see Preparation of Protein Extracts [Fusion Proteins]) emulsified in Freund's complete adjuvant and subsequently boosting at biweekly intervals with 50 µg/ml fusion protein emulsified in Freund's incomplete adjuvant. Blood was collected from the retro-orbital sinus of each mouse two weeks after the second and subsequent injections and resulting sera tested for reactivity. Serum samples were screened for reactivity to the UL37 protein in HSV-1-infected cells by immunoblot analysis. Rabbit inoculations and serum collections were performed by Duncroft, Inc. (Lovettsville, VA). Serum was screened for reactivity in a similar manner as outlined above. Rabbit polyclonal antiserum directed against the 1.1 fusion protein was designated 780 and antiserum against the full length (FL) fusion protein, 779.

2. Other Antisera

Rabbit polyclonal antisera directed against the HSV-1 ICP8 protein (designated ICP8 prep antibody and C-terminal ICP8 antibody) were obtained from Dr. William Ruyechan (Department of Microbiology, SUNY, Buffalo). The monoclonal antibody (ascitic fluid; 11/12c) directed against the ICP8 protein and rabbit polyclonal antiserum (Q1) directed against the HSV-1 alkaline exonuclease protein were obtained from Dr. Kenneth Powell (Wellcome Research Laboratories, Great Britain). The rabbit polyclonal antiserum (487) directed against the HSV-1 UL37 protein (generated against UL37 in vitro translation products) was obtained from Lisa Shelton (Department of Microbiology and Immunology, USUHS). The rabbit polyclonal antiserum (anti-ORF 29) directed against ORF 29 protein of varicella-zoster virus (VZV) was obtained from Dr. Iain Hay (Department of Microbiology, SUNY, Buffalo). The rabbit polyclonal antiserum (anti-α-TIF) directed against the HSV-1 alpha trans-inducing factor (αTIF) was obtained from Dr. Jennifer McKnight (Department of Infectious Diseases and Microbiology, University of Pittsburgh). The rabbit polyclonal antiserum (anti-gB) directed against the HSV-1 glycoprotein B (gB) was obtained from Dr. Richard Courtney (Department of Microbiology and Immunology, Penn State University). The monoclonal antiserum (48s) directed against the HSV-1 ribonucleotide reductase protein (ICP6) as well as the monoclonal antiserum 39S directed against the HSV-1 ICP8 protein were obtained from Dr. Martin Zweig (National Cancer Institute, Frederick, MD). Rabbit polyclonal antiserum (anti-MBP) directed against the maltose binding protein (MBP) of E. coli was purchased from New England Biolabs, Inc. (Beverly, MA). Control mouse ascitic fluid was obtained from Dr. Kathryn Holmes (Department of Pathology, USUHS).

K. Column Chromatography

Column chromatography studies were performed essentially according to methods previously described by Purifoy and Powell, (1976). Column matrices including single-stranded DNA (ssDNA) agarose (Bethesda Research Laboratories, Gaithersburg, MD), blue sepharose CL-6B (Pharmacia, Uppsala, Sweden), amylose (New England Biolabs, Inc., Beverly, MA), phosphocellulose P11 (Whatman Corp., Maidstone, England), and ATP agarose (Sigma Chemical Co., St. Louis, MO) were equilibrated according to manufacturers' instructions and packed into 5-10 ml columns. Columns were re-equilibrated with 10 column volumes TEDGP (usually containing 50-150 mM KCl) prior to each experiment. All chromatography was performed in a 4°C cold room and all buffers supplemented with protease inhibitors to help prevent protein degradation. The columns were loaded with the appropriate protein extract (see Preparation of Protein Extracts) and the flow-through material collected and reloaded at least three times to maximize column binding capacity. For ssDNA agarose, blue sepharose, and phosphocellulose columns, the columns were washed with 5-10 column volumes TEDGP buffer (containing 50-150 mM KCl) and bound proteins eluted using a potassium chloride (KCl) step or linear gradient, ranging from 50 mM-150 mM to 1 M KCl in TEDGP buffer. For amylose and ATP agarose columns, elution of proteins was achieved using 150 mM KCL-TEDGP buffer supplemented with 10 mM maltose and 20 mM ATP (Sigma Chemical Co., St. Louis, MO), respectively. For some experiments, two different protein samples were analyzed on the same column by mixing the two extracts prior to loading or sequentially loading the two preparations with washing steps in between each loading. Fractions (1 ml/each) were collected continuously. A small aliquot of each fraction was combined with SDS-PAGE sample

buffer for immunoblot analysis. The remaining fractions were sometimes concentrated using microconcentrators (Amicon Corp., Danvers, MA) to enhance protein detection and aliquots prepared and analyzed as described.

L. Virion Purification

Purified HSV-1 virions and L particles (light particles) were kindly provided by Dr. Paul Kinchington (Department of Ophthalmology, University of Pittsburgh) and isolated essentially as described by Szilagyi and Cunningham (1991). Confluent monolayers of baby hamster kidney (BHK) cells grown in DMEM-10% FBS using roller bottles (850 cm^2) were infected with HSV-1 strain 17 (HSV-1[17]) at an MOI of 1 PFU/cell in a total volume of 20 ml at 35°C. After one hour adsorption, infection media was replaced with 150 ml fresh DMEM-10% FBS and cells incubated at 35°C for 72 hours. To harvest extracellular virions, cellular debris was removed from the media by low speed centrifugation. The clarified media was centrifuged at 17,000 rpm in a Sorvall SS34 rotor for two hours at 4°C to pellet the virions. The virion pellet was resuspended in 2-5 ml fresh media and incubated overnight at 4°C. The following day, the preparation was mixed by vortexing for 20 seconds to disrupt any virus clumps, layered onto a preformed 5-15% ficoll gradient (prepared in 36 ml PBS), and centrifuged at 14,000 rpm in a SW29 rotor for two hours at 4°C. Visible bands were harvested and re-isolated on a second ficoll gradient. Following the second gradient, two visible bands (upper band representing HSV-1 light particles and lower band representing HSV-1 mature virions) were harvested separately, pelleted at 25,000 rpm, resuspended in 1 ml PBS, and incubated overnight at 4°C. The two suspensions were briefly sonicated and layered onto two additonal 5-15% ficol gradients, centrifuged, and then harvested as described above. Recovered viral suspensions were diluted in a final

volume of 250-500 µl PBS and stored at 4°C.

M. Detergent and Trypsin Treatment of Purified Virions

For detergent studies, purified HSV-1 virions were treated with 1% Nonidet P-40 (NP-40) in PBS for 15 minutes at 37°C followed by microcentrifugation at 16,000 rpm for one hour at 4°C. The supernatant containing solubilized proteins was then carefully transfered to a new tube and the pellet overlayed with PBS, centrifuged, and PBS removed. The pellet and supernatant fractions were resuspended in equivalent volumes of PBS, diluted in sample buffer and subjected to SDS-PAGE for immunoblot analyses.

Trypsin protection studies on purified virions were performed in a similar manner as that described above with the following modifications (Kinchington et al., 1992). Briefly, virion preparations were resuspended in PBS supplemented with 1 µM TPCK and divided into four fractions. One fraction was treated with 1% NP-40 only and a second fraction with PBS only. A third fraction was treated with 1% NP-40 and 0.1 mg/ml trypsin (3055 U/mg) (twice recrystallized; Gibco, Grand Island, NY) and a forth fraction treated with 0.1 mg/ml trypsin without NP-40. Fractions were then incubated at 37°C for five minutes followed by the addition of TLCK [10 µM], PMSF [1 µM], and 1 mg/ml chicken eggwhite trypsin inhibitor to terminate the protease reactions. Sample buffer was added to each of the fractions followed by boiling for 10 minutes and samples subjected to SDS-PAGE for immunoblot analyses.

N. In Vitro Transcription

The recombinant plasmid pHC37 which contains the entire UL37 open reading frame (ORF) under the control of the T7 promoter served as template DNA for in vitro transcription of the UL37 gene. In vitro transcription was performed using

the Riboprobe system (Promega Corp., Madison, WI) according to the manufacturer's protocol. The pHC37 plasmid DNA (20 µg) was linearized by digestion with HindIII and purified using the Geneclean kit as described (see Restriction Enzyme Digestion). The reaction mixture included 5 µg linearized pHC37 DNA, 20 µl 5X transcription buffer, 10 µl 0.1 M DTT, 2.5 µl RNasin (100 u), 2.5 mM each rNTP (rATP, rCTP, rGTP, rUTP), 3 µl T7 RNA polymerase (15 u/µl), and diethylpyrocarbonate (DEPC)-treated water in a total volume of 100 µl and was incubated at 37°C for two hours. Following the transcription reaction, 5 µl DNase (1 u/µg DNA) was added and incubated for 15 minutes at 37°C to remove the DNA template. The reaction was then phenol/chloroform extracted once followed by a single chloroform extraction. The aqueous phase containing the RNA was removed to a new tube and precipitated by the addition of 0.5 volume of 7.5 M ammonium acetate and 2.5 volume of 100% ethanol and placed at -70°C for 30 minutes. The RNA was pelleted by centrifugation in a microfuge, dried under vacuum, resuspended in 20 µl TE, and stored at -20°C until needed for translation.

O. In Vitro Translation

RNA templates generated from in vitro transcription reactions were in vitro translated using a cell free rabbit reticulocyte lysate system (Promega Corp., Madison, WI) according to the manufacturer's instructions. Template mRNA was first heated at 68°C for ten minutes and immediately cooled on ice to denature secondary structure and increase the efficiency of translation. Reaction components were then assembled in a 1.5 ml Eppendorf tube and consisted of the following: 10 µl RNA (1 µg), 70 µl nuclease treated reticulocyte lysate, 2 µl RNasin (40 u/µl), 2 µl amino acid mixture (1 mM) minus methionine and leucine, 8 µl ^{35}S-methionine (10 µCi/µl), and water in a

final volume of 100 µl. Approximately 10 µg of a purified ICP8 protein preparation was sometimes included in the reaction mixture for experimental purposes.

Purified ICP8 protein, obtained from Dr. William Ruyechan (Department of Microbiology, SUNY, Buffalo), was isolated using a protocol similar to previously published methods (Ruyechan, 1988; Gupte et al., 1991). Briefly, U-35 cells were infected with HSV-1 at an MOI of 1 PFU/cell and maintained in the presence of the DNA synthesis inhibitor phosphonoacetic acid (PAA; 300 µg/ml). The U-35 cell line is stably transformed and contains multiple copies of the ICP8 gene integrated into the cellular genome. Under PAA conditions, the integrated copies of the ICP8 gene are overexpressed upon HSV-1 infection, while expression of late genes from the infecting viral genome is minimized. The ICP8 protein from these cells was purified by ssDNA agarose chromatography and heparin sepharose chromatography as previously described (Ruyechan, 1988).

In addition, reactions containing no mRNA and brome mosaic virus (BMV) RNA were set up as negative and positive controls, respectively. The reactions were then incubated at 30°C for one hour. Translation products were detected on SDS-polyacrylamide gels as described (see Detection of Proteins) or immunoprecipitated with specific antiserum (see Immunoprecipitations) prior to SDS-PAGE and immunoblot analysis.

P. Immunoprecipitations

Immunoprecipitation was performed by the addition of 1-10 µl of appropriate antiserum or ascitic fluid to 100 µl of an infected cell lysate (see Preparation of Infected Cell Extracts) and incubation for 18 hours at 4°C with end-over-end rotation. A reaction using normal rabbit serum or control ascitic fluid was

included to serve as a negative control. The resulting complexes were then precipitated by addition of 100 µl of 10% (w/v) protein A-Sepharose beads (Sigma Chemical Co., St. Louis, MO) in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl [pH 7.5]). The immune complexes were pelleted by centrifugation and washed 4-5 times with RIPA buffer (200 µl/wash) supplemented with protease inhibitors (0.01 mM TPCK [N-tosyl-L-phenylalanine chloromethyl ketone], 0.01 TLCK [N-tosyl-L-lysine chloromethyl ketone] [Boehringer Mannheim Biochemicals, Indianapolis, IN], aprotinin [1:100] [Sigma Chemical Co., St. Louis, MO]). The final pellet was resuspended in SDS-PAGE sample buffer, boiled for two minutes, and subjected to SDS-PAGE for analysis as described (see Detection of Proteins).

III. RESULTS

A. Production of UL37- and ICP8-Specific Reagents

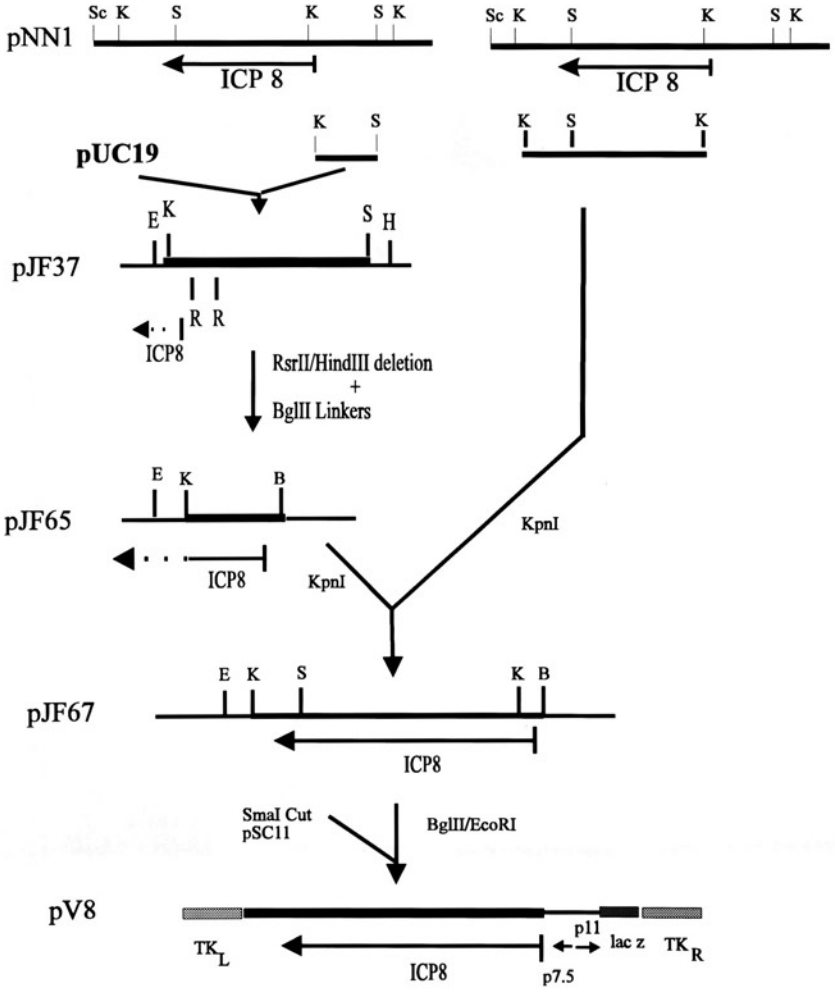
1. ICP8 Expression in Vaccinia Virus

A vaccinia virus recombinant expressing the HSV-1 ICP8 protein independent from other HSV-1 proteins was constructed to facilitate investigation of the ICP8 and UL37 protein interaction as well as to confirm specificity of antisera directed against either the ICP8 or UL37 proteins. The plasmid pJF67 containing the ICP8 ORF was generated by Jonathan Hirsch in this laboratory and was used as a source of the ICP8 gene for cloning into the vaccinia virus shuttle vector, pSC11. A schematic drawing showing the construction of the pJF67 plasmid is shown in Figure 12.

The vector pSC11 contains two vaccinia virus promoters, p7.5 and p11, which direct expression of the inserted foreign gene and the E. coli lacZ gene, respectively. Flanking vaccinia virus thymidine kinase (TK) gene sequences on the vector serve as sites for homologous recombination to mobilize the plasmid DNA into the vaccinia virus genome at the TK locus. Recombinant vaccinia viruses possess a TK-/lacZ+ phenotype providing an efficient method of selection and screening by plaque assay on TK- 143 cells in the presence of BUdR and X-gal (blue plaques) (Figure 10). The entire ORF of ICP8 was excised from pJF67 as a BglII-EcoRI fragment, blunt-ended, and cloned into the SmaI site of pSC11 creating the recombinant plasmid pV8 (Figure 12). CV-1 cells infected with wild-type vaccinia virus (WR) were then transfected with calcium phosphate-precipitated pV8 DNA and recombinants selected and screened for as outlined above. Plaques were purified three times in succession and ICP8 protein expression by the vaccinia virus recombinants confirmed

Figure 12

Generation of the recombinant plasmid pV8. The plasmid pV8 contains the HSV-1 ICP8 gene cloned into the vaccinia virus shuttle vector pSC11; the plasmid pJF67 containing the ICP8 ORF was used as source of the ICP8 gene. The plasmid pJF67 was constructed in the following manner. A 1.6 kbp SalI-KpnI fragment from pNN1 containing the 5' end of the ICP8 ORF was cloned into pUC19, creating the plasmid pJF37. An RsrII-HindIII collapse of pJF37 followed by the addition of BglII linkers created the plasmid pJF65. This placed the BglII site approximately 6 bp upstream of the ICP8 ATG translation start codon. A 4.3 kbp KpnI fragment from pNN1 which contained the remainder of the ICP8 gene was then inserted at the KpnI site of pJF65, creating the plasmid pJF67. The entire ORF of ICP8 was excised from pJF67 as a BglII-EcoRI fragment and cloned into the vaccinia virus shuttle vector pSC11, creating the plasmid pV8. Plasmid pV8 DNA was mobilized into the wild-type vaccinia virus (WR) genome by transfection of WR-infected CV-1 cells and recombinants selected as discussed in the text.


pNN1
Sc K S K S K
ICP 8
Sc K S K S K
ICP 8
pUC19
K S
K S K
E K S H
pJF37
R R
ICP8
RsrII/HindIII deletion
+
BglII Linkers
E K B
pJF65
ICP8
KpnI
KpnI
E K S K B
pJF67
ICP8
SmaI Cut
pSC11
BglII/EcoRI
pV8
TK L
ICP8
p11
lac z
TK R
p7.5

by immunoblot analysis using an ICP8 antiserum. The vaccinia virus recombinant V8 was found to express an ICP8 protein (V8 protein) indistinquishable from the ICP8 protein of HSV-1 infected cells (Figure 13, lanes 1-4). In addition, the V8 protein exhibited an affinity for single-stranded (SS) DNA similar to that of the HSV-1 ICP8 protein as discussed in section B1.

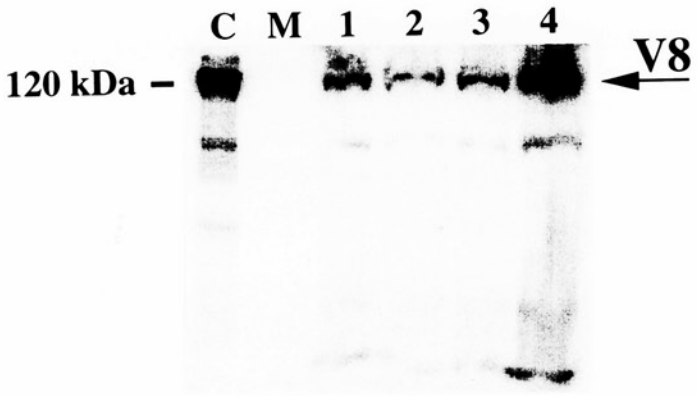
2. Maltose Binding Protein-UL37 Fusion Proteins

To facilitate studies of the UL37 protein independently of other HSV-1 proteins and to examine possible interactions between the UL37 and ICP8 proteins, malE-UL37 gene fusions were constructed to produce corresponding fusion proteins in E. coli. The cloning vector pMAL-c contains the inducible P_{tac} promoter positioned to transcribe a malE-lacZα gene fusion which contains a polylinker between the malE gene and the lacZα domain. The polylinker provides restriction endonuclease sites to insert the gene of interest, fusing it to the malE gene and interrupting the lacZα gene allowing a blue-to-white screen for inserts on LB/X-gal agar plates. In addition, the $lacI^q$ gene on the vector which encodes the lac repressor turns off transcription from P_{tac} until 0.3 mM IPTG is added. Upon induction with IPTG, a maltose binding protein (MBP) fusion is expressed which can be purified from bacterial extracts by taking advantage of the affinity of MBP for amylose. The vector also contains a sequence coding for the recognition site of the protease factor Xa located within the polylinker allowing the protein of interest to be cleaved from MBP after purification, without adding any vector-derived residues to the protein. The protein fusion and purification system is depicted in Figure 11.

Plasmid pMAL-37 was constructed by cloning a 3.5 kb HindIII-SalI fragment from pHC37 into pMAL-c. This created a fusion protein consisting of the E. coli maltose binding protein (MBP) fused to the entire UL37 protein. Plasmids

Figure 13

Expression of the ICP8 protein by the V8 vaccinia virus recombinant. V8-infected CV-1 cell proteins from four different isolates (lanes 1-4) were harvested at 24 hpi, separated on SDS-9% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with an ICP8-specific antiserum. Antibody binding was detected with ^{125}I-labeled protein A followed by phosphorimage analysis. Apparent molecular size based on relative migration to high molecular weight protein standards (not shown) is indicated. C, HSV control extract; M, mock-infected.


C
M
1
2
3
4
120 kDa
V8

pMAL-1.1 and pMAL-2.1 were constructed by cloning 1.1 kb and 2.1 kb HindIII-PstI fragments (which were generated by partial restriction enzyme digestion of pHC37) into pMal-c. This created fusion proteins consisting of the E. coli MBP fused to the carboxy terminal one-third and two-thirds portion of the UL37 protein, respectively. The cloning strategy and respective molecular weights of the UL37 fusion proteins generated is diagrammed in Figure 14.

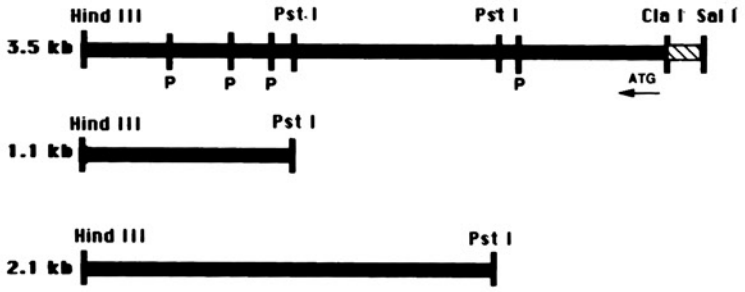
Expression and identification of the three fusion proteins was confirmed by Coomassie blue staining and immunoblot analysis of uninduced and induced bacterial extracts shown in Figures 15 and 16. Induced proteins migrating at 80, 120, and 160 kDa can be seen (indicated by arrows) in the Coomassie stained gels in Figures 15A and 16A marked (+) for IPTG induction; no corresponding proteins are found in the uninduced lanes (-). To identify the induced proteins as MBP-fusions, immunoblot analysis of duplicate gels probed with an antiserum directed against the maltose binding protein (αMBP) was performed, the results of which are shown in Figures 15B and 16B. The αMBP antiserum reacted against the corresponding induced proteins (designated with arrows) from the Coomassie stain as well as purified MBP (lane labeled MBP), which served as a control, but did not react with the uninduced bacterial extracts from each fusion. The fusions proteins are labeled 1.1, 2.1, and FL (full-length) and migrate at the predicted molecular weights of 80, 120, and 160 kDa, respectively; purified MBP migrates at approximately 42 kDa. The lower molecular weight proteins detected in the induced lanes by αMBP most likely represent break down products from each respective fusion protein since no corresponding products are detected in the uninduced lanes. Taken together, these results confirm both the expression and identity of each of the three MBP-UL37 fusion proteins constructed.

Cleavage attempts with protease factor Xa were not successful due to a

Figure 14

A schematic of the cloning strategy and respective molecular weights of the UL37 fusion proteins. The plasmid pHC37 served as source of the UL37 gene for cloning various portions of the UL37 ORF (shown here) into the pMAL-c vector creating MBP-UL37 fusion proteins (as outlined in the text). The predicted molecular weight of each UL37 domain and its respective fusion protein are indicated. MBP, maltose binding protein.

UL37 PROTEIN FUSIONS



~ MOLECULAR WEIGHTS

	Domain	*Fusion
1.1 kb	40 kd	80 kd
2.1 kb	80 kd	120 kd
3.5 kb	120 kd	160 kd

* MBP molecular weight = 42 kd

Figure 15

Expression and identification of the 1.1 and 2.1 MBP-UL37 fusion proteins. Uninduced and induced bacterial extracts (- or + IPTG, respectively) transformed with either pMAL-1.1 or pMAL-2.1 were harvested after a small-scale induction experiment (as described under Materials and Methods). Proteins were then separated on SDS-9% polyacrylamide gels followed by (A) direct staining with Coomassie blue or (B) immunoblot analysis using an antiserum directed against the maltose binding protein (MBP) and ^{125}I-labeled protein A followed by autoradiography. Arrows indicate the position of the induced fusion proteins (A and B). M, high molecular weight protein standards are indicated for comparision; MBP, purified maltose binding protein migrates at approximately 42 kDa.

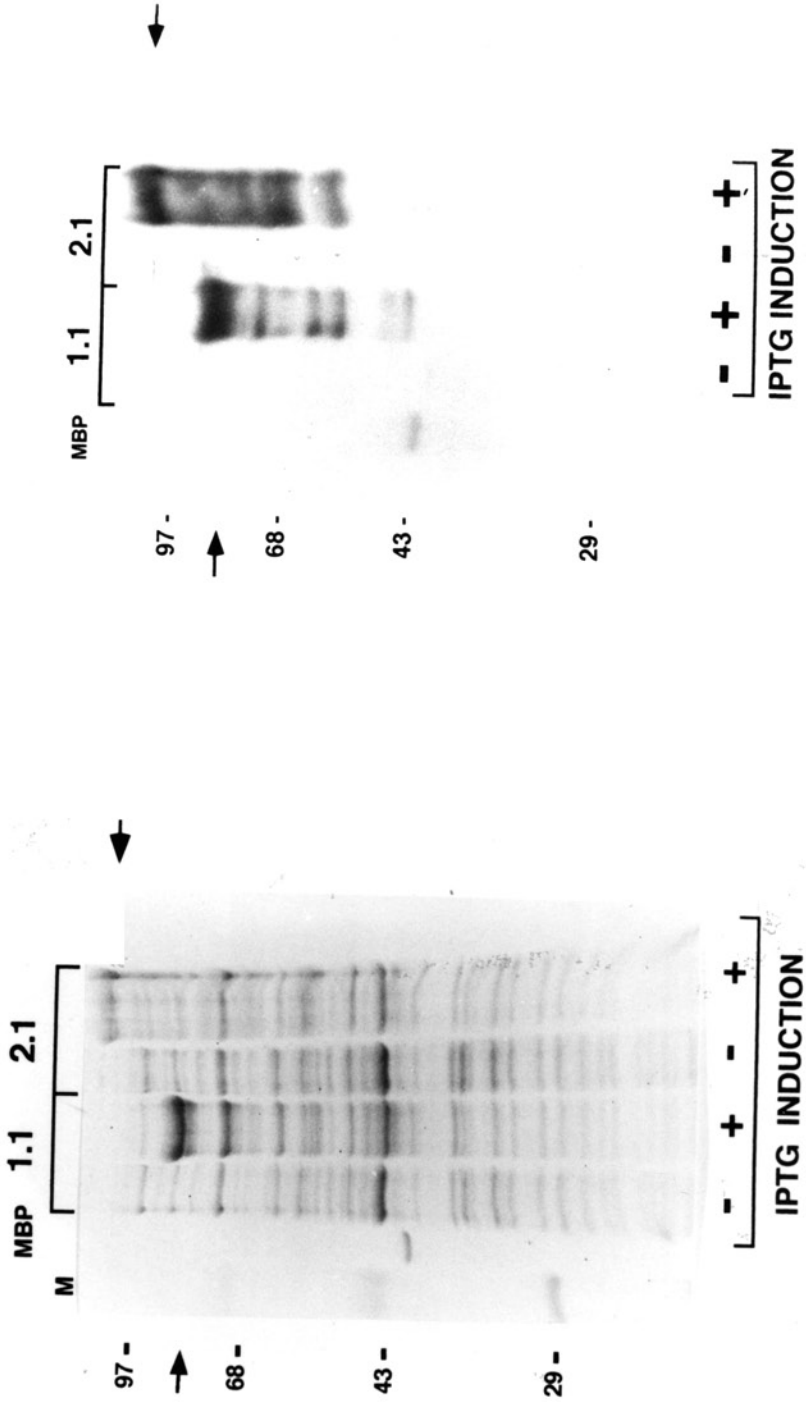
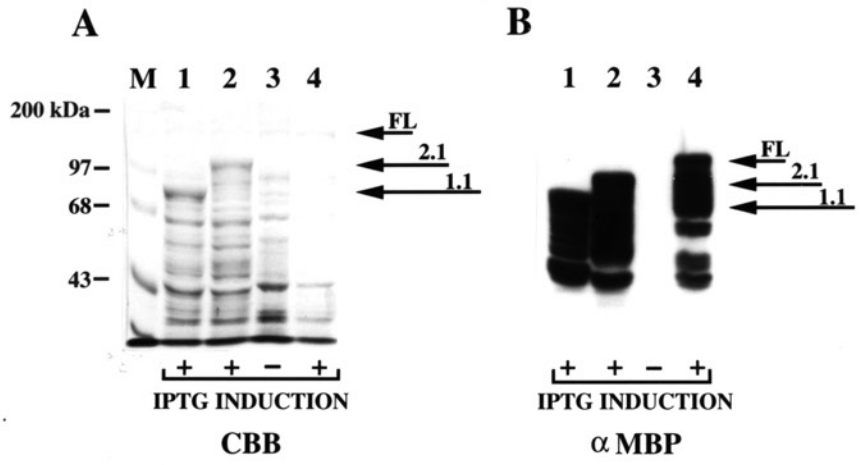

MBP
1.1
2.1
M
97 -
68 -
43 -
29 -
- + - +
IPTG INDUCTION
MBP
1.1
2.1
97 -
68 -
43 -
29 -
- + - +
IPTG INDUCTION

Figure 16

Expression and identification of the full-length (FL) MBP-UL37 fusion protein. Uninduced and induced bacterial extracts (- and + IPTG, respectively) transformed with pMAL-37 were harvested after a small-scale induction experiment (as described under Materials and Methods). Proteins were then separated on SDS-9% polyacrylamide gels followed by (A) direct staining with Coomassie blue or (B) immunoblot analysis using an antiserum directed against the maltose binding protein (αMBP) and ^{125}I-labeled protein A followed by autoradiography. Arrows indicate the position of the induced fusion proteins (A and B); fusion proteins 2.1 and 1.1 are included for comparison (lanes 1 and 2). M, high molecular weight standards (indicated for comparison); CBB, Coomassie brilliant blue.


A
M 1 2 3 4
200 kDa
97
68
43
FL
2.1
1.1
+ + − +
IPTG INDUCTION
CBB
B
1 2 3 4
FL
2.1
1.1
+ + − +
IPTG INDUCTION
α MBP

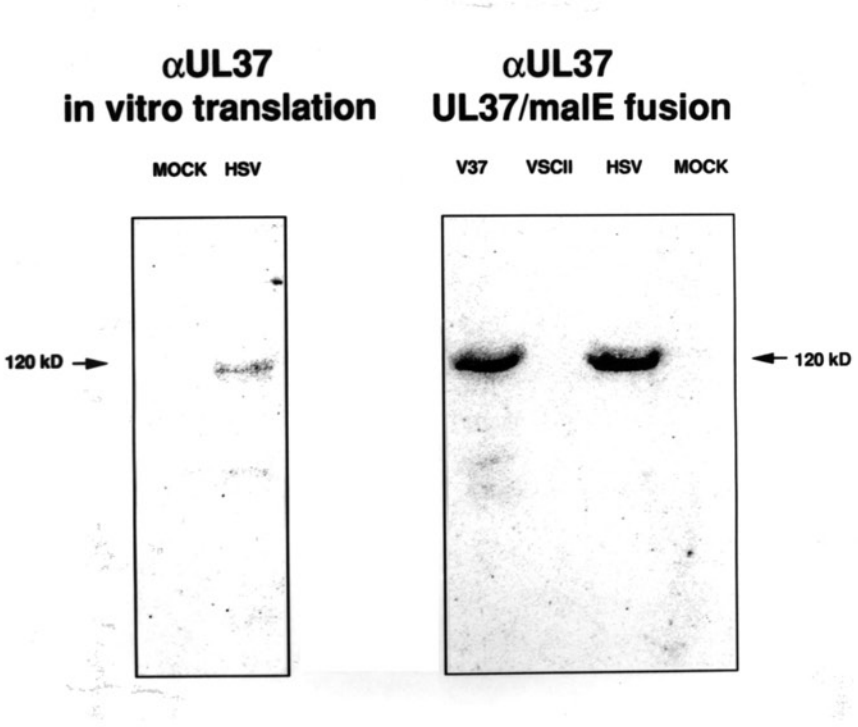
defect in the original design of the pMAL-c vector which precluded factor Xa recognition of the cleavage site. Specifically, a proline residue located adjacent to the factor Xa cleavage site on the pMal-c vector resulted in an inability of the protease to cleave efficiently most likely due to steric hindrance induced by the proline amino acid.

3. Generation of UL37-Specific Polyclonal Antiserum

Due to the ability to overexpress and purify the UL37 fusion proteins in E. coli combined with the fact that these proteins are often excellent antigens, production of high affinity polyclonal antisera directed against the UL37 protein was attempted. The malE-UL37 fusion protein 1.1 consists of the carboxy-terminal one-third domain of the UL37 protein fused in frame to the maltose binding protein (MBP) of E. coli while FL consists of the full-length UL37 protein fused to MBP. Immunogenicity of the 1.1 and full-length (FL) UL37 fusion proteins was first tested in mice. Large scale purifications of both 1.1 and FL fusion proteins were prepared (as described under Materials and Methods) and injected into BALB/c mice at bi-weekly intervals with subsequent testing of sera starting two weeks after the second injection. Once reactivity of the sera (and therefore the antigenicity) against the HSV-1 UL37 protein was confirmed, fusion protein preparations were used to immunize rabbits for large scale polyclonal antisera production. Polyclonal antiserum directed against the 1.1 fusion protein was designated 780 and antiserum against the FL protein, 779. By comparative immunoblotting, both 780 and 779 antisera demonstrated stronger affinity for the UL37 protein in HSV-1 and V37-infected cells and less cross-reactivity with background proteins than that of a previous antiserum (generated in this laboratory) that was directed against UL37 in vitro translation products (487; Shelton et al., 1990). A comparison of the affinities of these antibodies is shown in Figure 17. In addition, the

Figure 17

Comparison of the UL37-specific antisera. HSV-1-, VSC11-, V37-, and mock-infected cell proteins were harvested at 24 hpi, separated on SDS-9% polyacrylamide gels, transferred to nitrocellulose, and analyzed by immunoblot using UL37-specific polyclonal rabbit antiserum. Antibody binding was detected with ^{125}I-labeled protein A followed by phosphorimage analysis. Panel on left was probed with antiserum 487. Panel on right was probed with antiserum 780. Arrows indicate the position of the UL37 protein.


αUL37
in vitro translation
MOCK HSV
120 kD
αUL37
UL37/malE fusion
V37 VSCII HSV MOCK
120 kD

780 and 779 antisera were capable of immunoprecipitating the UL37 protein from both HSV-1 and V37-infected cell lysates.

The specificity of the antisera raised against the UL37 and ICP8 proteins was demonstrated by immunoblot analysis of HSV-1(F)-, V37-, V8-, and VSC11-infected cell proteins harvested at 24 hpi (Figure 18). The antisera directed against ICP8 and its C-terminal peptide (as labeled above respective blots) reacted with a 120 kDa protein from cells infected with either HSV-1 or the vaccinia virus recombinant V8 (lanes designated HSV and V8, respectively) but not with proteins from cells infected with the vaccinia virus recombinant V37 or the VSC11 vaccinia virus control (lanes designated V37 and VSC11, respectively). Similarly, the UL37-specific antisera 780 and 487 (as labeled above respective blots) reacted with a 120 kDa protein from cells infected with either HSV-1 or the vaccinia virus recombinant V37 (lanes designated HSV and V37, respectively) but not with proteins from cells infected with the vaccinia virus recombinants V8 or VSC11 (lanes designated V8 and VSC11, respectivley). Results similar to those obtained with the 780 and 487 antisera were obtained with the 779 antiserum (data not shown). These results demonstrate the specificities of the UL37 and ICP8 antisera and show that the UL37 and ICP8 proteins co-migrate on SDS-polyacylamide gels.

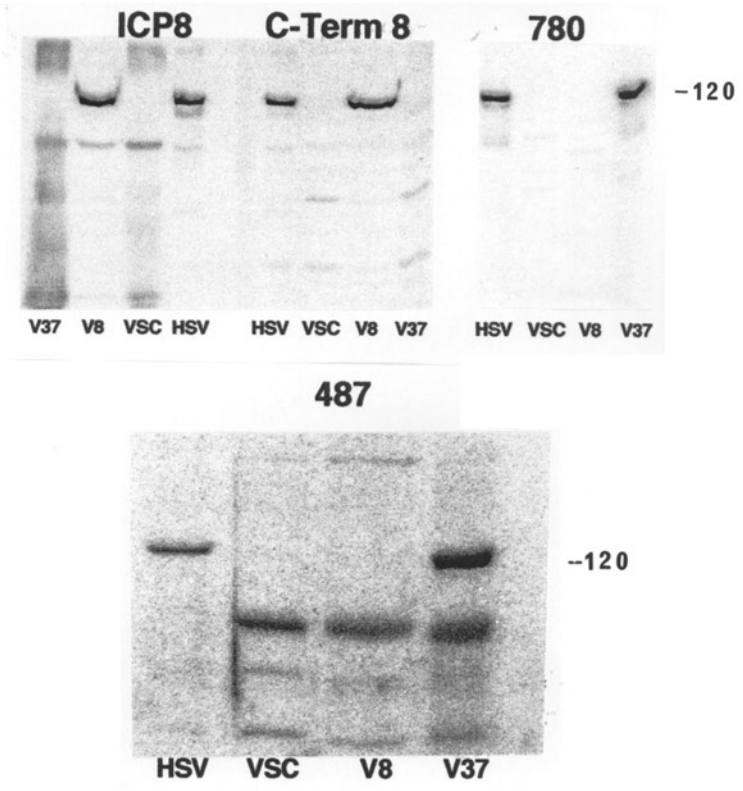
B. UL37 and ICP8 Protein Interaction Studies

1. Single-Stranded DNA Agarose Affinity Column Chromatography

a. V8-Infected Cell Proteins. One characteristic property of the HSV-1 ICP8 protein is its ability to bind tightly to single-stranded DNA immobilized on column matrices or nitrocellulose filters (Powell et al., 1981; Ruyechan and Weir, 1984). The use of vaccinia virus as a vector system for expressing heterologous genes

Figure 18

Specificities of the UL37 and ICP8 antisera. HSV-1(HSV)-, VSC11(VSC)-, V37-, and V8-infected cell proteins were harvested at 24 hpi, separated on SDS-9% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with polyclonal rabbit antiserum directed against either wild-type ICP8 protein (ICP8), ICP8 carboxy-terminal peptide (C-term 8), malE-1.1 UL37 fusion protein (780), or in vitro-translated UL37 protein (487). Antibody binding was detected with ^{125}I-labeled protein A followed by phosphorimage analysis.

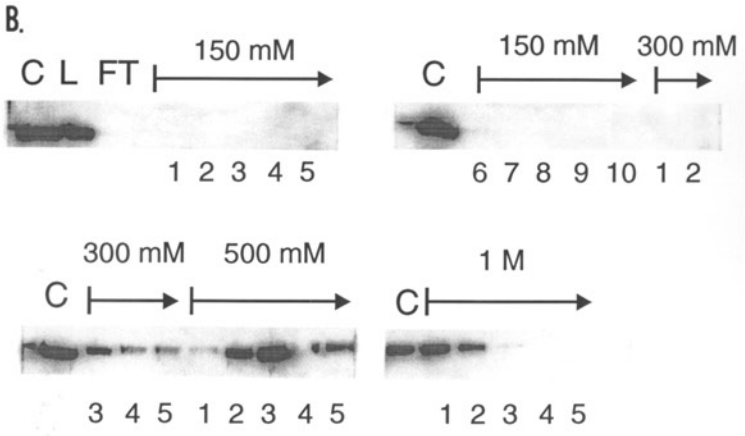

ICP8
C-Term 8
780
-120
V37 V8 VSC HSV
HSV VSC V8 V37
HSV VSC V8 V37
487
-120
HSV VSC V8 V37

is well documented, with expressed proteins typically appearing to be indistinguishable from native proteins in size, posttranslational modification and cleavage, and cellular localization (Mackett et al., 1985; Rice et al., 1985; Mackett and Smith, 1986). As mentioned in the previous section, a vaccinia virus recombinant (V8) was constructed which expressed an ICP8 protein of HSV-1 (V8 protein) which appeared indistinguishable from the native protein by immunoblot analysis (Figure 13). To determine whether the V8 protein has an affinity for single-stranded DNA, protein extracts were prepared from V8-infected CV-1 cells at 48 hpi and separated on a ssDNA agarose matrix using a KCl step gradient (as described under ssDNA agarose chromatography in the Materials and Methods section). Individual fractions were collected and analyzed by immunoblot for the presence of the V8 protein (Figure 19). The majority of the V8 protein was detected in fractions eluted under high-salt (500 mM - 1 M KCl) but not in flowthrough or wash fractions. This profile is similar to that exhibited by the HSV-1 ICP8 protein as previously reported (Bayliss et al., 1975; Purifoy and Powell, 1976; Knipe et al., 1982; Ruyechan and Weir, 1984; Shelton et al., 1990).

b. V37- and V8-Coinfected Cell Proteins. Our laboratory previously reported that the UL37 and ICP8 proteins from HSV-1 infected cells co-eluted from ssDNA-agarose columns (Shelton et al., 1990). The observation that the UL37 protein from HSV-1 infected cells could be reproducibly eluted from ssDNA columns indicated that the UL37 protein either bound this DNA substrate directly or was interacting with other proteins, such as ICP8, which themselves bound DNA. To investigate these possibilities, the DNA binding activity of the UL37 protein in the absence of other HSV proteins was determined by ssDNA chromatography of V37-infected cell extracts. The UL37 protein encoded by V37 did not exhibit any apparent affinity for ssDNA

Figure 19

Analysis of V8-infected cell proteins by ssDNA column chromatography. V8-infected cell proteins were harvested at 48 hpi and separated on a ssDNA-agarose column by using step elutions of 150, 300, 500, and 1000 mM KCl as described. Proteins in each fraction were then separated on SDS-9% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with ICP8-specific antiserum. Antibody binding was detected using ^{125}I-labeled protein A followed by phosphorimage analysis. C, control; L, load; FT, flow-through.


B.
C L FT
150 mM
1 2 3 4 5
C
150 mM
300 mM
6 7 8 9 10 1 2
C
300 mM
500 mM
3 4 5 1 2 3 4 5
C
1 M
1 2 3 4 5

(Shelton et al., 1994). The lack of binding observed with the vaccinia expressed UL37 protein was probably not due to its expression in vaccinia virus since the vaccinia expressed ICP8 protein retained the ability to bind ssDNA (as discussed above). Together, these results demonstrated the inability of the UL37 protein to bind DNA in the absence of other HSV-1 proteins and suggested that the elution of the UL37 protein from ssDNA columns was a result of interactions with one or more HSV-1 proteins.

To determine whether the UL37 protein could bind DNA in the presence of ICP8, protein extracts were prepared from cells coinfected with both V37 and V8 vaccinia virus recombinants and analyzed by ssDNA agarose chromatography. Fractions from the linear KCl elution gradient were analyzed by immunoblot for the UL37 and ICP8 proteins. As shown in Figure 20, the UL37 and ICP8 proteins co-eluted in fractions 4 through 12, which encompassed KCl concentrations of 400 to 600 mM. The lower molecular weight bands seen in the flow-through (FT) and initial gradient lanes (wash), which did not bind to the column, represent a mixture of ICP8 and UL37 degradation products as well as background bands present in vaccinia virus-infected cells. These data strongly suggest that the HSV-1 ICP8 protein is required for the apparent DNA-binding properties of the UL37 protein.

To determine whether the UL37 protein expressed in the absence of the ICP8 protein could be retained on the DNA column in the presence of the ICP8 protein, infected cell protein extracts were prepared from cells infected with either the V37 or V8 recombinant vaccinia virus. These protein extracts were either mixed prior to loading and analysis by ssDNA agarose chromatography or sequentially loaded (as described in Materials and Methods). Fractions from the step KCl elution gradients were collected and analyzed by immunoblot for the presence of the UL37 and ICP8 proteins. As shown in Figure 21 (A and B), the V37 protein co-eluted with the V8

Figure 20

Analysis of V37- and V8-coinfected cell proteins by ssDNA column chromatography. CV-1 cells were coinfected with V37 and V8 and protein extracts harvested at 48 hpi and separated on a ssDNA agarose column by using a linear KCL gradient. Proteins in each fraction were separated on SDS-9% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with either ICP8 (α8) or 487 (α37) antiserum. Bound antibody was detected using ^{125}I-labeled protein A followed by phosphorimage analysis. C, control; L, load; F.T., flow-through fractions.

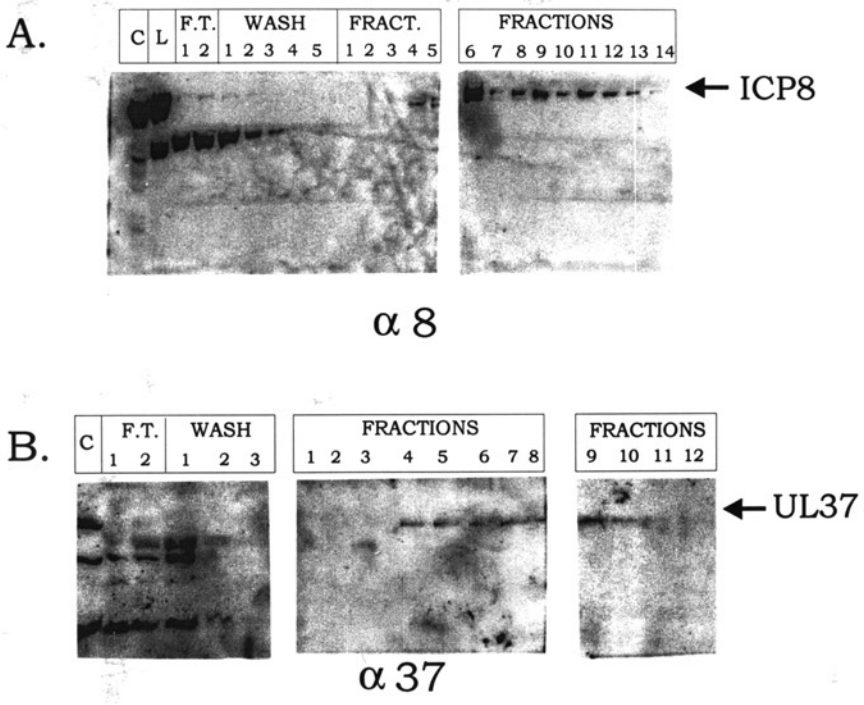
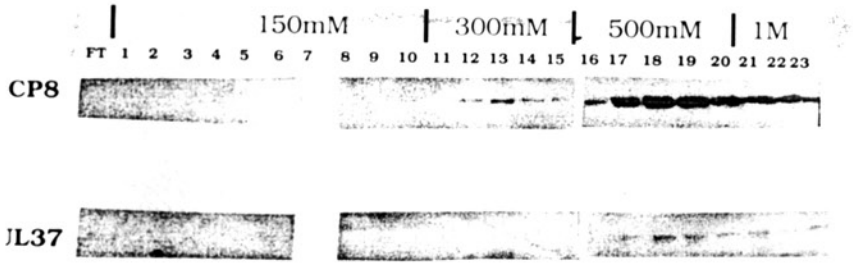

A.
C
L
F.T.
1 2
WASH
1 2 3 4 5
FRACT.
1 2 3 4 5
FRACTIONS
6 7 8 9 10 11 12 13 14
ICP8
α 8
B.
C
F.T.
1 2
WASH
1 2 3
FRACTIONS
1 2 3 4 5 6 7 8
FRACTIONS
9 10 11 12
UL37
α 37

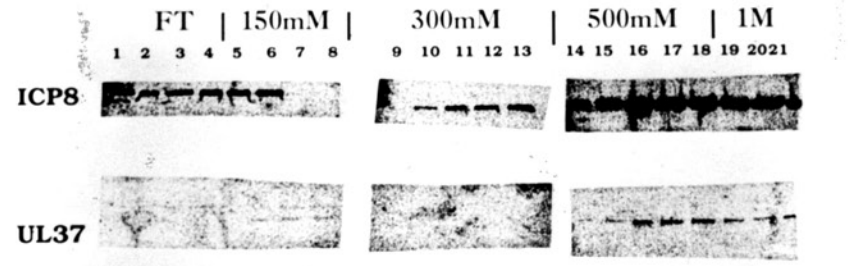
Figure 21

Analysis of V37- and V8-infected cell proteins (mixed or sequentially loaded) by ssDNA column chromatography. V37-infected and V8-infected cell proteins were harvested at 48 hpi and extracts either combined (mixed) prior to loading and analysis by ssDNA agarose chromatography using a step KCl elution gradient or sequentially loaded, V8 proteins followed by V37 proteins (as described under Materials and Methods). Proteins in each fraction were separated on SDS-9% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with either ICP8 or UL37-specific antiserum as indicated. Bound antibody was detected using ^{125}I-labeled protein A followed by phosphorimage analysis. The results of the mixed extract experiment is shown in panel A; the sequentially loaded extracts, panel B. FT, flow-through fraction(s).

V8/V37 MIXED EXTRACTS



V8/V37 SEQUENTIAL LOADS



protein in fractions corresponding to 500 mM - 1 M KCl concentration similar to the results obtained with the coinfected cells. The presence of V8 protein in the 150 mM and 300 mM KCl fractions from the sequentially loaded column (Figure 21B) was most likely due to an overloading effect as the two extracts were added to the column one after the other. In the case of mixed extracts, these results suggest that the UL37 and ICP8 proteins may be capable of forming a complex in the extracts following dialysis while similar results from sequentially loading the two extracts suggest that the two proteins can associate with one another on the ssDNA column; it cannot be ruled out that this was also the reason for the mixed extract results. In both cases, it is difficult to conclude at what point the UL37 and ICP8 proteins formed a complex; however, these results do demonstrate that the UL37 and ICP8 proteins can form an association outside of the infected cell, in the presence of ssDNA.

c. n10-Infected Cell Proteins. Previous studies (as well as those described above) in our laboratory suggested that a functional ICP8 protein might be required for the UL37 protein to bind DNA (Shelton, 1992). For these studies, the ability of the UL37 protein to bind ssDNA in the absence of a functional ICP8 protein was assessed by using an ICP8 deletion mutant, d21. The mutant d21 is an HSV-1 recombinant virus that has 260 internal amino acids deleted from the ICP8 gene, including the entire potential zinc-binding domain (Gao et al., 1988; Gupte et al., 1991) and 23 amino acids into the putative DNA-binding region (Gao and Knipe, 1989; Gao and Knipe, 1991; Wang and Hall, 1990). Extracts from d21-infected CV-1 cells were used as a source of the HSV-1 UL37 and defective ICP8 proteins for ssDNA agarose chromatography. In this study, neither the UL37 nor the defective ICP8 protein bound ssDNA suggesting that an ICP8-UL37 protein interaction might be responsible for the binding of the UL37 protein to ssDNA which is observed with wild-type HSV-1-infected cell proteins

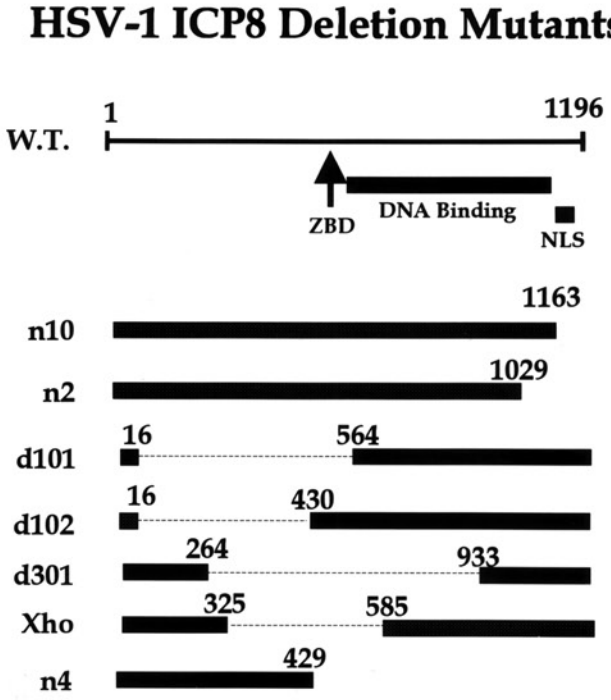
(Shelton et al., 1994).

To extend these studies and investigate which domains of the ICP8 protein might play a role in such an interaction, additional ICP8 deletion mutants including n2, n4, n10, d101, d102, d301, and xho (shown schematically in Figure 22) were obtained from Dr. David Knipe (Department of Microbiology and Molecular Genetics, Harvard Medical School) and infected cell protein extracts prepared for each of the mutants for analysis by ssDNA agarose chromatography. The solubility of most of the defective ICP8 proteins was poor when subjected to high-salt extraction as previously reported (Gao and Knipe, 1989). Upon repeated attempts to isolate a soluble fraction of mutant ICP8 molecules using the respective viruses, only the mutant n10 virus produced enough soluble ICP8 protein in noncomplementing cells for examination on ssDNA agarose columns.

The mutant n10 virus was originally isolated from a complementing ICP8-expressing cell line (Gao and Knipe, 1989; Bush et al., 1991). The n10 genome contains a nonsense codon (TAG) after codon 1160 of the ICP8-coding sequences and produces a protein which lacks the C-terminal 36 amino acids including the nuclear localization signal (NLS) (Figure 22). When this mutant is grown in noncomplementing cells such as CV-1 or Vero, an HSV-1 DNA-negative phenotype results, and the defective ICP8 molecules remain in the cytoplasm (Gao and Knipe, 1989; Orberg and Schaffer, 1987). Since a functional ICP8 molecule is essential for HSV-1 DNA replication (Conley et al., 1981; Powell et al., 1981; Weller et al., 1983; Challberg, 1986; McGeoch et al., 1988a; Wu et al., 1988) and since the UL37 protein demonstrates γ1 kinetics which requires viral DNA replication for full expression, it was first determined whether n10 infection of noncomplementing cells would result in detectable UL37 protein expression thus allowing assessment of correlations between

Figure 22

Schematic of ICP8 deletion mutants. This diagram illustrates the various ICP8 deletion mutants obtained for this study indicating the respective residues that are absent in each mutant ICP8 protein (dotted lines, e.g.). The wild-type ICP8 protein (designated W.T.) consists of 1196 amino acids; functional domains of the molecule are labeled beneath. ZBD, zinc-binding domain; NLS, nuclear localization signal.


HSV-1 ICP8 Deletion Mutants
W.T.
1
1196
ZBD
DNA Binding
NLS
n10
1163
n2
1029
d101
16
564
d102
16
430
d301
264
933
Xho
325
585
n4
429

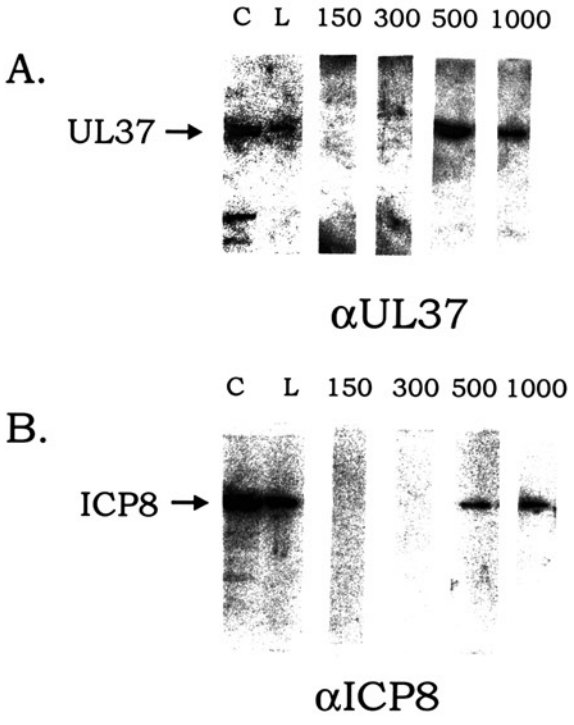
a functional ICP8 protein and the DNA-binding properties of the UL37 protein. Immunoblot analysis of n10-infected CV-1 (noncomplementing) and U-47 (complementing) cells was performed with UL37- and ICP8-specific antisera. The UL37 protein was detected in both CV-1 and U-47 cells infected with n10, with a diminished but clearly detectable level of expression found in the noncomplementing (CV-1) cells. In CV-1 cells, only the faster-migrating mutant ICP8 polypeptide encoded by n10 was detected while in the U-47 cells, both the smaller ICP8 polypeptide encoded by mutant n10 and the native ICP8 protein expressed by the cell line were observed (data not shown).

CV-1 cells infected with mutant n10 virus were harvested at 24 hpi and high-salt extracts prepared and analyzed by ssDNA agarose chromoatography. In contrast to the results obtained with the d21 mutant (in which the mutant ICP8 protein did not bind to ssDNA), the ICP8 protein expressed by the n10 mutant was able to bind ssDNA, which agreed with previous results (Gao and Knipe, 1989). The UL37 protein co-fractionated with the n10 ICP8 mutant protein in 500 mM and 1000 mM KCl step elutions as shown in Figure 23 (panels A and B). This result, together with results obtained from comparative studies using HSV-1-, V37-, and d21-infected cell proteins, demonstrate that the ability of the UL37 protein to bind ssDNA columns is dependent upon the presence of a DNA-binding competent ICP8 protein. The results obtained with the mutant n10 and the coinfection of cells with the V37 and V8 vaccinia virus recombinants also suggest that the ability of the UL37 protein to bind is due solely to the presence of the ICP8 protein and not any other HSV-1 proteins.

d. V37- and V29-Coinfected Cell Proteins. The results described thus far indicate that the retention of the UL37 protein on ssDNA columns is dependent upon the presence of a DNA-binding competent ICP8 protein. To assess whether another

Figure 23

Analysis of n10-infected CV-1 cell proteins by ssDNA column chromatography. n10-infected CV-1 cell proteins were harvested at 24 hpi and separated on a ssDNA-agarose column by using step elutions of 150, 300, 500, and 1000 mM KCl as described. Proteins in each fraction were then separated on SDS-9% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with either UL37- (487; αUL37) or ICP8-(αICP8) specific antiserum as indicated. Bound antibody was detected using ^{125}I-labeled protein A followed by phosphorimage analysis. For detection with the 487 antiserum, individual fractions were concentrated prior to being resolved by SDS-PAGE. C, control; L, load; numbers indicate KCl concentration (millimolar).


C L 150 300 500 1000
A.
UL37
αUL37
C L 150 300 500 1000
B.
ICP8
αICP8

single-stranded DNA binding protein (SSB) could substitute for the ICP8 protein, studies utilizing the HSV-1 ICP8 homolog from varicella-zoster virus (VZV), open reading frame 29 (ORF 29; Gene 29), were conducted. The ORF 29 protein of VZV is the major VZV DNA-binding protein, which is presumed to be essential for viral DNA replication (Roberts et al., 1985; Davison and McGeoch, 1986; Kinchington et al., 1988; Gupte et al., 1991). The amino acid sequences predicted from the published DNA sequences for ORF 29 protein of VZV (Davison and McGeoch, 1986) and the gene for the HSV-1 ICP8 protein (Quinn and McGeoch, 1985) show an overall identity of 50% at the amino acid level. However, the homology ranges from greater than 90% (e.g., amino acids 678 to 714) to less than 25% homology (e.g., 100 amino acids of the proteins extending toward the carboxyl termini (Kinchington et al., 1988).

The properties of the VZV ORF 29 protein have been studied in less detail than the homologous ICP8 protein in HSV-1 but some characterization has been achieved (Roberts et al., 1985; Davidson and McGeoch, 1986; Kinchington et al., 1988; Sabella et al., 1993). Previous studies utilizing ssDNA cellulose chromatography columns demonstrated that both the VZV ORF 29 and HSV-1 ICP8 proteins possess similar single-stranded DNA-binding properties with the ORF29 protein exhibiting slightly less binding efficiency than that of the ICP8 protein (Kinchington et al., 1988). The ORF 29 gene has been expressed in a vaccinia virus recombinant (V29) and the corresponding protein (V29 protein) demonstrates DNA-binding characteristics that resemble the authentic VZV ORF 29 gene product (Sabella et al., 1993).

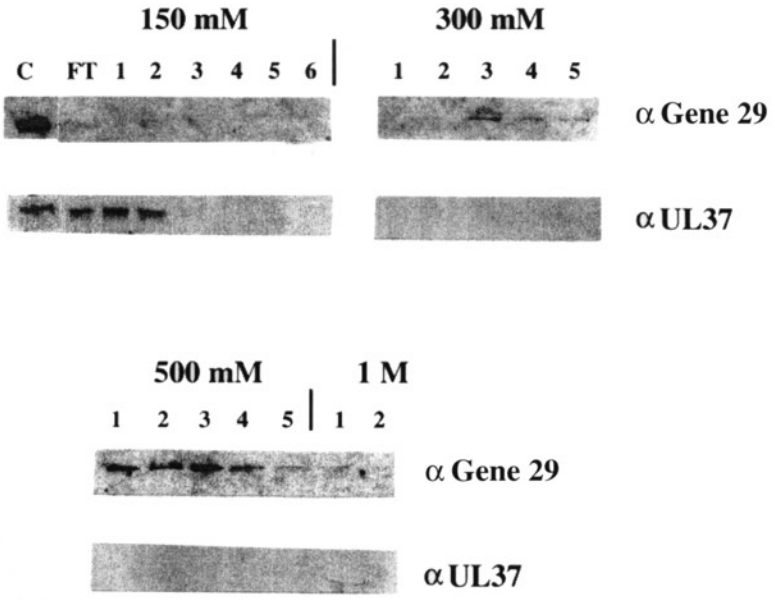
To determine whether the UL37 protein could be retained on a ssDNA column in the presence of the ICP8 equivalent SSB protein from VZV, high-salt extracts were prepared from Vero cells coinfected with both the V29 (obtained from Dr. Iain Hay, Department of Microbiology, SUNY Buffalo) and V37 vaccinia virus

recombinants and soluble proteins analyzed by ssDNA agarose chromatography. Fractions from the step-KCl elution gradient were analyzed by immunoblot for the ORF 29 (V29) and UL37 (V37) proteins. As shown in Figure 24, the majority of V29 protein eluted in fractions corresponding to 300 mM to 500 mM KCl concentration in agreement with previous reports (Roberts et al., 1985; Kinchington et al., 1988). In contrast, the V37 protein was unable to bind ssDNA under these conditions and was detected in the flow-through and initial 150 mM KCl wash fractions (as designated in Figure 24). These results suggest that the ability of the UL37 protein to bind to or be retained on the ssDNA column is dependent on a specific interaction with the HSV-1 ICP8 protein and not just an SSB- induced conformational change of the DNA structure.

e. V8-Infected Cell Proteins and malE-UL37 Fusion Proteins. To examine which domains of the UL37 protein may be involved in the UL37 and ICP8 protein interaction on ssDNA columns, ssDNA agarose chromatograpy studies were performed utilizing purified malE-UL37 fusion proteins from E. coli. This particular strategy was employed because attempts to generate HSV-1 viral recombinants with deletions in the UL37 protein have been unsuccessful thus far, and maltose binding protein (MBP) from E. coli does not exhibit an affinity for DNA on ssDNA agarose columns (data not shown). Large-scale preparations of each UL37 fusion protein were purified (as described in Material and Methods) and individually combined with high-salt extracts from V8-infected cells and loaded on separate ssDNA agarose columns for analysis. Step-KCl elution gradients were performed for each column, fractions collected, and assayed by immunoblot for the presence of the V8 protein and the respective malE-UL37 fusion protein. The two smaller UL37 fusion proteins, 1.1 and

Figure 24

Analysis of V37- and V29-coinfected cell proteins by ssDNA column chromatography. CV-1 cells were coinfected with V37 and V29 and protein extracts harvested at 48 hpi and separated on a ssDNA-agarose column by using step elutions of 150, 300, 500, and 1000 mM KCl as described. Proteins in each fraction were then separated on SDS-9% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with either UL37-(αUL37; 487) or Gene 29-(αGene 29)specific antiserum. Bound antibody was detected using ^{125}I-labeled protein A followed by phosphorimage analysis. For detection with the 487 antiserum, individual fractions were concentrated prior to being resolved by SDS-PAGE. C, control; FT, flow-through.


150 mM
300 mM
C FT 1 2 3 4 5 6
1 2 3 4 5
α Gene 29
α UL37
500 mM
1 M
1 2 3 4 5 1 2
α Gene 29
α UL37

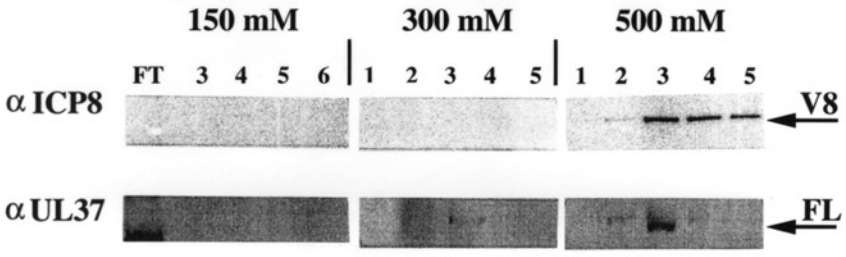
2.1, did not bind, in the presence of ICP8, to ssDNA columns (data not shown); however, the full-length malE-UL37 fusion protein (FL) did bind, co-eluting with the V8 protein in fractions corresponding to 500 mM KCl concentration (Figure 25). Some of the FL protein was detected in fractions eluted at 300 mM KCl concentration representing a population of FL protein with weaker affinity for the ICP8 protein on ssDNA. The weaker affinity observed with this population of FL protein may be due to the MBP domain causing either a steric hindrance or a change in the conformation of the UL37 protein such that the UL37 domain does not interact with the ICP8 protein as efficiently. These results suggest that the N-terminal domain of the UL37 protein may be important for interaction with the ICP8 protein on ssDNA columns. While the inability to bind may be due to conformational changes, the 1.1 and 2.1 domains are not sufficient by themselves for binding.

2. Amylose Affinity Column Chromatography

The results obtained from ssDNA agarose chromatography suggest that the presence of ssDNA may play a role in the ability of the UL37 and ICP8 proteins to interact. To examine whether the UL37 and ICP8 protein:protein interaction could be demonstrated on a different column matrix in the absence of ssDNA, amylose column chromatography was performed using full-length UL37 fusion (FL) and V8-infected cell proteins. This particular method was utilized because the FL fusion protein exhibits a strong affinity for amylose due to the maltose binding protein (MBP) moiety and in addition, the ICP8 protein did not display any intrinsic affinity for amylose when tested (data not shown). FL fusion and V8-infected cell protein extracts were prepared (as described under Materials and Methods) and either combined prior to loading or sequentially added on the amylose column. The loaded column was washed

Figure 25

Analysis of V8-infected cell proteins and the full-length malE-UL37 fusion protein (FL) by ssDNA column chromatography. Large-scale preparations of the FL fusion protein were purified (as described under Materials and Methods) and combined with high-salt extracts from V8-infected cells (prepared as described). The mixed extracts were then separated on a ssDNA-agarose column by using step elutions of 150, 300, 500, and 1000 mM KCl as described. Proteins in each fraction were separated on SDS-12% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with UL37-(αUL37; 487) or ICP8-(αICP8)specific antiserum. Bound antibody was detected using ^{125}I-labeled protein A followed by phosphorimage analysis. For detection with the 487 antiserum, individual fractions were concentrated prior to being resolved by SDS-PAGE. FT, flow-through.


150 mM
300 mM
500 mM
FT 3 4 5 6 1 2 3 4 5 1 2 3 4 5
α ICP8
V8
α UL37
FL

extensively with 150 mM KCl buffer before proteins were eluted in wash buffer supplemented with 10 mM maltose. Fractions from both the wash and elution steps were collected and analyzed by immunoblot for the presence of the FL and ICP8 proteins. As seen in Figure 26, the FL fusion protein was detected in elution fractions 3 to 5 while the V8 protein was only detected in the flow-through and wash fractions. The FL protein bands on the blot are weak due to the affinity of the UL37 antiserum (487) used. A duplicate blot probed with a higher affinity UL37 antiserum (780) detected the presence of fusion protein in greater abundance in the eluted fractions (data not shown). The results shown in Figure 26 represent fractions from a mixed extract experiment. Similar results were obtained by sequentially loading the two protein extracts (data not shown). These results suggest that the FL fusion and V8 proteins are not able to associate on this matrix (unlike the results obtained from ssDNA columns) and imply that ssDNA is required for the two proteins to form a stable interaction. It is also possible that the conformation of the FL fusion protein while bound to the amylose column is modified in such a way that the UL37 domain can no longer interact with the ICP8 protein independent of an absence of ssDNA.

3. Blue Sepharose Affinity Column Chromatography

a. V8-Infected Cell Proteins. To further evaluate the putative role of ssDNA in the UL37 and ICP8 protein interaction, other non-DNA column matrices including phosphocellulose P-11, ATP agarose, and blue sepharose CL-6B were tested for affinity for either the UL37 or ICP8 proteins using the respective vaccinia virus recombinants, V37 and V8. The initial results of column chromatography studies using these matrices demonstrated that the V8 protein has an affinity for blue sepharose, (Figure 27), but not for the other matrices, while the V37 protein did not exhibit an affinity for any of the matrices tested (data not shown). As shown in Figure 27, the

Figure 26

Analysis of the full-length malE-UL37 fusion protein (FL) and V8-infected cell proteins by amylose column chromatography. FL fusion protein and V8-infected cell protein extracts were prepared (as described under Materials and Methods) and were combined prior to loading on the column. The loaded column was washed extensively with 150 mM KCl buffer and proteins eluted in wash buffer supplemented with 10 mM maltose. Proteins in each fraction were then separated on SDS-12% polyacrylamide gles, transferred to nitrocellulose, and immunoblotted with UL37-(αUL37; 487) or ICP8-(αICP8)specific antiserum. Bound antibody was detected using ^{125}I-labeled protein A followed by phosphorimage analysis. For detection with the 487 antiserum, individual fractions were concentrated prior to being resolved by SDS-PAGE. FT, flow-through.

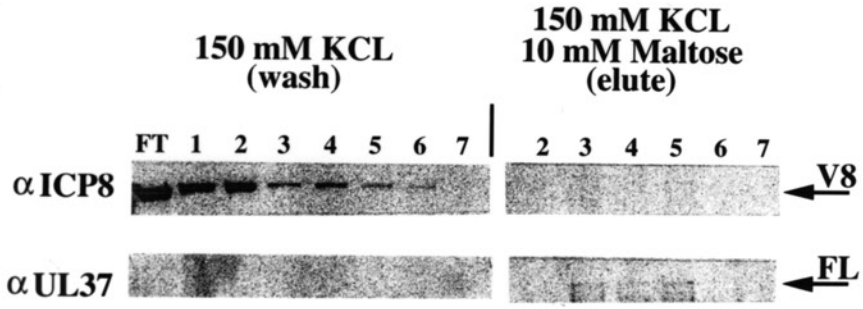
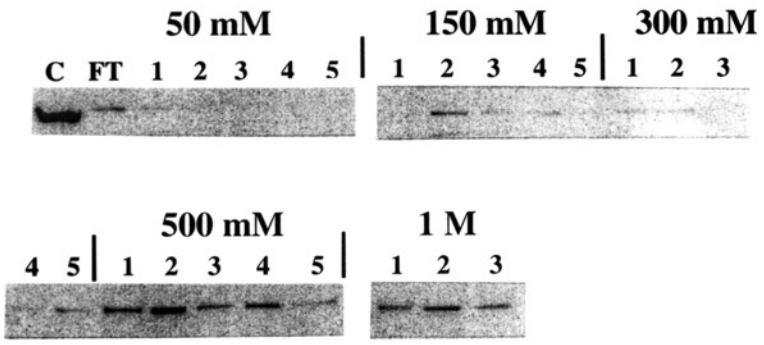

150 mM KCL
(wash)
150 mM KCL
10 mM Maltose
(elute)
FT 1 2 3 4 5 6 7
2 3 4 5 6 7
αICP8
αUL37
V8
FL

Figure 27

Analysis of V8-infected cell proteins by blue sepharose column chromatography. V8-infected cell proteins were harvested at 48 hpi and separated on a blue sepharose column by using step elutions of 50, 150, 300, 500, and 1000 mM KCl as described. Proteins in each fraction were then separated on SDS-12% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with an ICP8-specific antiserum. Bound antibody was detected using ^{125}I-labeled protein A followed by phosphorimage analysis. C, control; FT, flow-through.

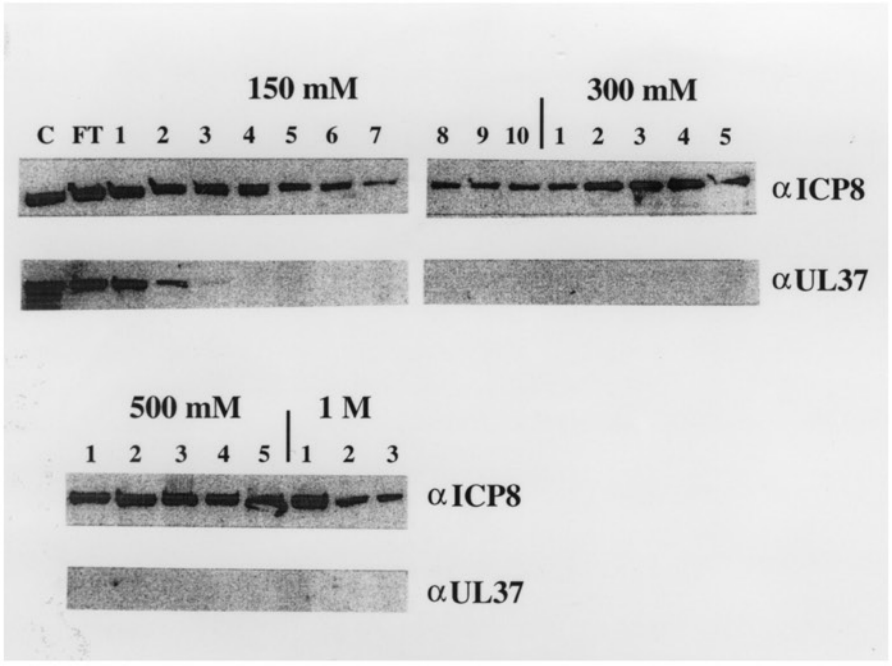

50 mM
150 mM
300 mM
C FT 1 2 3 4 5
1 2 3 4 5
1 2 3
500 mM
1 M
4 5
1 2 3 4 5
1 2 3

majority of the V8 protein was detected in the 500 mM and 1 M KCl step elution fractions indicative of an affinity for blue sepharose. Blue sepharose CL-6B contains the dye Cibacron Blue F3G-A covalently attached to the cross-linked agarose gel Sepharose CL-6B and has an affinity for a wide variety of enzymes and other proteins including albumin, lipoproteins, blood coagulation factors, and interferon. Most enzymes bind biospecifically with the dye due to its structural similarity to nucleotide cofactors (e.g., NAD+) while other proteins bind in a less specific manner by electrostatic and/or hydrophobic interactions with the aromatic anionic ligand (Wilson, 1976; Lamkin and King, 1976). Biospecifically adsorbed proteins can be eluted by low concentrations of the free cofactor while less specifically bound proteins require the use of much higher concentrations of cofactor, or salt.

b. HSV-1-Infected Cell Proteins. Based on the the affinity of the V8 protein for blue sepharose, affinity column chromatography was conducted using HSV-1-infected cell proteins to determine if the UL37 and ICP8 proteins could associate on this matrix. Immunoblot analysis of the elution profiles for both proteins is shown in Figure 28. The ICP8 protein was detected in all the fractions including flow-through and wash fractions while the UL37 protein was present only in the flow-through and initial wash fractions. One interpretation of these data is that the column was overloaded with the ICP8 protein but not the UL37 protein. Whether this is the case or not, the data from this particular experiment is still useful for the following observations. Although the unbound population of ICP8 protein was not thoroughly washed from the column before step-KCl elution began, the amount of ICP8 protein diminishes toward the end of the wash step (150 mM KCl fractions 7 to 10; as labeled in Figure 28) and increases in the step-KCl elution fractions that follow (300 mM and 500 mM; as labeled in Figure 28) indicative of a population of HSV-1 ICP8 protein

Figure 28

Analysis of HSV-1-infected cell proteins by blue sepharose column chromatography. HSV-1-infected cell proteins were harvested at 24 hpi and separated on a blue sepharose column by using step elutions of 150, 300, 500, and 1000 mM KCl as described. Proteins in each fraction were then separated on SDS-12% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with either a UL37-(αUL37) or ICP8-(αICP8)specific antiserum. Bound antibody was detected using ^{125}I-labeled protein A followed by phosphorimage analysis. C, control; FT, flow-through.


150 mM
300 mM
C FT 1 2 3 4 5 6 7
8 9 10 1 2 3 4 5
αICP8
αUL37
500 mM
1 M
1 2 3 4 5 1 2 3
αICP8
αUL37

with affinity for blue sepharose. The fact that none of the UL37 protein co-eluted with this bound population of ICP8 protein (fractions corresponding to 300 mM - 1 M KCl) may suggest that the two proteins could not form a stable association on blue sepharose, a non-DNA matrix, either due to the conformation of each protein in such an environment and/or the absence of ssDNA.

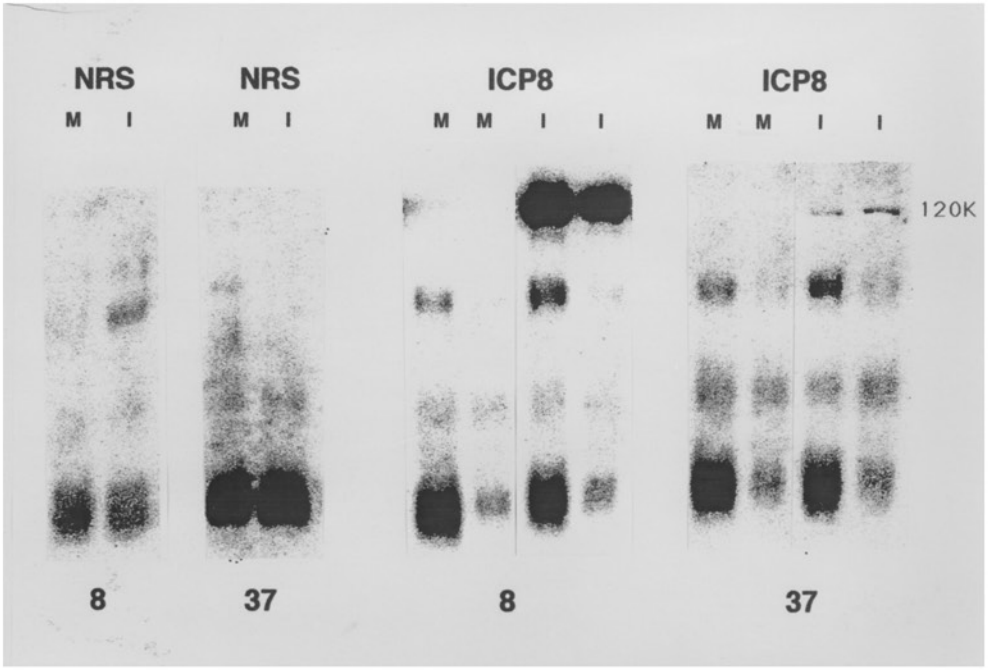
4. Immunoprecipitation Studies

a. HSV-1-Infected Cell Proteins. In addition to affinity column chromatography methods, immunoprecipitation studies were performed concurrently to investigate the association between the UL37 and ICP8 proteins. To determine whether the two proteins form an immuno-precipitable complex within the infected cell, coprecipitation experiments were performed on HSV-1 infected cell lysates using a variety of antibodies directed against either the UL37 or the ICP8 proteins. For immunoprecipitations, infected cell cytoplasmic or nuclear extracts were prepared (as described under Materials and Methods) and incubated with the appropriate volume of polyclonal or monoclonal antibody for 18 hours at 4°C. Immune complexes were then precipitated by addition of protein A-sepharose beads, pelleted and washed extensively, resuspended in sample buffer, and subjected to SDS-PAGE for subsequent analysis by immunblot.

Immunoblot analysis of HSV-1- and mock-infected cytoplasmic lysates immunoprecipitated with either a polyclonal rabbit antiserum directed against the ICP8 protein or normal rabbit serum (NRS) as a control is shown in Figure 29. The precipitating antibody is indicated at the top of each panel and the antiserum used for immunoblot below each panel. The lysate type, HSV-1-infected (I) or mock-infected (M), is designated at the top of each lane. It can be seen that both the ICP8 and UL37 proteins are coprecipitated by the ICP8-specific polyclonal antiserum and co-

Figure 29

Immunoprecipitation of HSV-1-infected cell proteins. HSV-1-(I) and mock-(M)infected cell cytoplasmic lysates (designated above each lane) were prepared as described and incubated with either a polyclonal rabbit serum directed against the ICP8 protein or normal rabbit serum (NRS) (as indicated at top of blots) for 18 hours at 4^0C. Immune complexes were then precipitated by addition of protein A-sepharose beads, pelleted and washed extensively, separated on SDS-9% polyacrylamide gels, and transferred to nitrocellulose for subsequent immunoblot analysis with either a UL37- or ICP8-specific antiserum (as designated at the bottom of each blot). Bound antibody was detected by using ^{125}I-labeled protein A followed by phosphorimage analysis. 120 K, marks the position of the co-migrating UL37 and ICP8 proteins. (Duplicate lanes represent two different volumes of ICP8 antibody used for immunoprecipitation.)

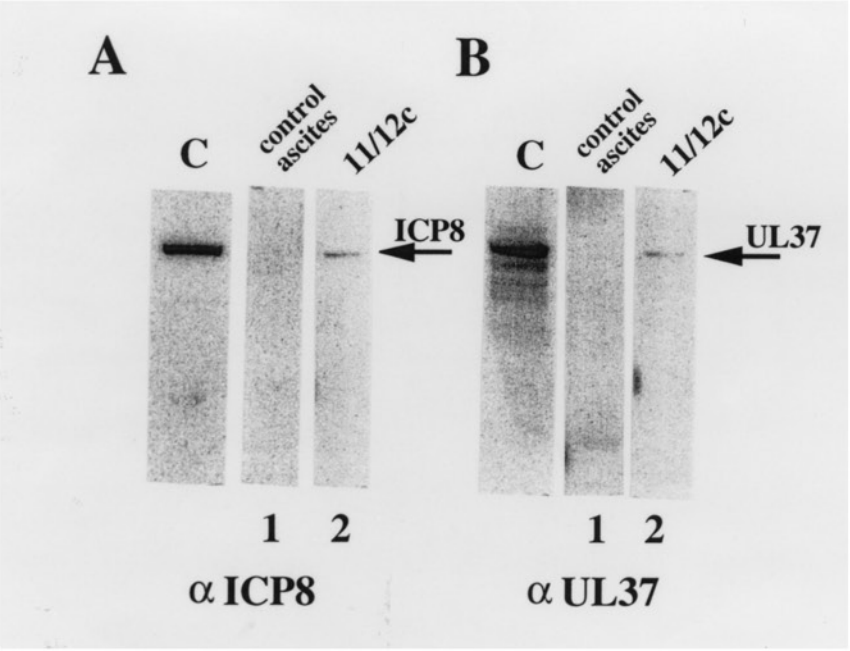

NRS
NRS
ICP8
ICP8
M I
M I
M M I I
M M I I
120K
8
37
8
37

migrate at 120 kDa on the respective blots (duplicate lanes represent two different volumes of ICP8 antibody used for immunoprecipitation). The precipitations are specific in that these 120 kDa proteins are neither present in mock-infected cells nor immunoprecipitated by the normal rabbit serum (NRS) control. The lower molecular weight bands represent spurious background proteins which are also precipitated by NRS from HSV-1- or mock-infected cells. The heavy bands at the very bottom of the blots are most likely immunoglobulin chains from the precipitating antibodies binding to the radiolabeled protein A upon immunoblotting. It should be noted that although the ICP8 protein appears to be present in much greater abundance than the coprecipitating UL37 protein based on band intensity, it is difficult to make an accurate quantitative statement about the stoichiometry of the two proteins since the ICP8 and UL37 antisera used for immunoblotting differ in affinity for the respective proteins. It should also be noted that in this experiment (data not shown) and in subsequent immunoprecipitation experiments described, attempts to coprecipitate the UL37 and ICP8 proteins using polyclonal antibodies directed towards the UL37 protein have been unsuccessful thus far (see Discussion).

To determine if the UL37 and ICP8 protein complex could be detected in the nuclei, HSV-1-infected cell nuclear extracts were prepared as described and immunoprecipitated with a monoclonal antibody (11/12c; ascitic fluid) directed against the ICP8 protein. The results are shown in Figure 30 and demonstrate that the UL37 and ICP8 proteins can be coprecipitated from the nuclei of HSV-1-infected cells (an HSV-1 protein extract and a control ascites immunoprecipitation are included as controls). These results support the hypothesis that the UL37 and ICP8 proteins form a complex within the infected cell and substantiate the association observed between the two proteins on ssDNA agarose columns.

Figure 30

Immunoprecipitation of HSV-1-infected cell proteins (nuclear extracts). HSV-1-infected cell nuclear extracts were prepared (as described under Materials and Methods) and incubated with either a monoclonal antibody directed against the ICP8 protein (11/12c) or a control ascites for 18 hours at 4^0C. Immune complexes were then precipitated by addition of protein A-sepharose beads, pelleted and washed extensively, separated on SDS-12% polyacrylamide gels, and transferred to nitrocellulose for subsequent immunoblot analysis with either an (A) ICP8-specific or (B) UL37-specific antiserum. Bound antibody was detected by using ^{125}I-labeled protein A followed by phosphorimage analysis. Arrows indicate the position of the UL37 and ICP8 proteins. C, control HSV-1 extract.

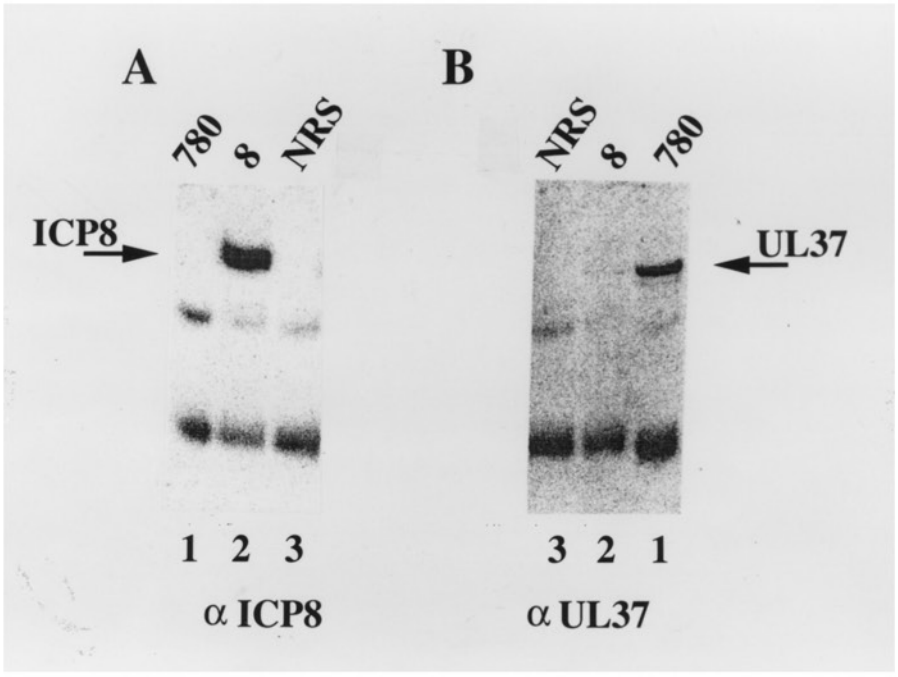

A
B
C
control ascites
11/12c
ICP8
UL37
1
2
α ICP8
α UL37

b. V37- and V8-Coinfected Cell Proteins. Results discussed earlier showed that the UL37-ICP8 protein complex could be demonstrated by ssDNA chromatography of high-salt extracts from cells infected with the V37 and V8 vaccinia virus recombinants. It was of interest to determine if this complex formation could also be demonstrated by co-immunoprecipitation experiments. To this end, cytoplasmic lysates were prepared from Vero cells coinfected with the V37 and V8 vaccinia virus recombinants (MOI = 5 for each virus) followed by immunoprecipitation with a polyclonal antibody directed against either the UL37 or the ICP8 proteins. Immunoprecipitation with NRS was used as a negative control. Immunoprecipitates were then subjected to SDS-PAGE and immunoblot analysis (Figure 31) using an ICP8-specific polyclonal antiserum (panel A) or a UL37-specific polyclonal antiserum (panel B). As can be seen, the ICP8 protein is immunoprecipitated with the ICP8 antibody (panel A, lane 2) and a faint coprecipitating band identified as the UL37 protein is seen on the corresponding blot (panel B, lane 2); arrows designate the position of the respective proteins. No coprecipitation of the UL37 and ICP8 proteins was achieved using the UL37 polyclonal antibody, 780. These results provide additional evidence that the UL37 and ICP8 proteins form a stable complex within infected cells and can associate in the absence of any other HSV-1 proteins.

c. <u>In Vitro</u> Translation. As another means to investigate complex formation between the UL37 and ICP8 proteins, <u>in vitro</u> translation of the UL37 protein was performed in the presence or absence of purified ICP8 protein. UL37 RNA templates were first generated from <u>in vitro</u> transcription reactions and then <u>in vitro</u> translated (as described under Materials and Methods) in the presence or absence of 10 μg purified ICP8 protein (see Materials and Methods). Translation products were detected on SDS-polyacrylamide gels as described or immunoprecipitated with a UL37- or ICP8-specific

Figure 31

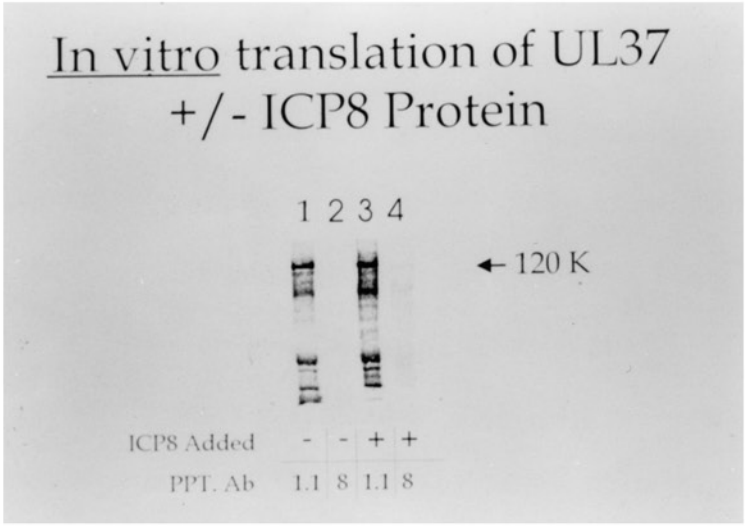
Immunoprecipitation of V37- and V8-coinfected cell proteins. Cytoplasmic lysates from V37- and V8-coinfected cells were prepared as described and incubated with a polyclonal antibody directed against either the UL37 protein (780) or the ICP8 protein (8), or normal rabbit serum (NRS) for 18 hours at 4^0C. Immune complexes were then precipitated by addition of protein A-sepharose beads, pelleted and washed extensively, separated on SDS-12% polyacrylamide gels, and transferred to nitrocellulose for immunoblot analysis with either an (A) ICP8-specific or (B) UL37-specific antiserum. Bound antibody was detected by using ^{125}I-labeled protein A followed by phosphorimage analysis. Arrows indicate the position of the UL37 and ICP8 proteins.


A
780
8
NRS
ICP8
1 2 3
α ICP8
B
NRS
8
780
UL37
3 2 1
α UL37

polyclonal antibody prior to SDS-PAGE and immunoblot analysis. Figure 32 represents the results from one such experiment showing a phosphorimage of immunoprecipitated radiolabeled in vitro translation products resolved on SDS-polyacrylamide gels and transferred to nitrocellulose for analysis by phosphorimager. The UL37 antiserum used in these experiments is 780 which is labeled 1.1 in Figure 32. Lanes 1 and 3 demonstrate that the full-length UL37 protein (120 kDa) is in vitro translated and immunoprecipitated by UL37-specific antiserum in the presence or absence of exogenous ICP8 protein. Lower molecular weight precipitated bands in these lanes most likely represent pre-mature termination or breakdown products of the UL37 protein. The appearance of darker bands in lane 3 compared to lane 1 probably reflects a more efficient translation of UL37 mRNA in the presence of a single-stranded DNA binding protein (SSB) such as ICP8. Lane 4 shows a faint radiolabeled band (120 kDa) and a smaller band at approximately 80 kDa coprecipitating with the ICP8 protein (the presence of the coincident ICP8 protein was confirmed by immunoblot analysis of a duplicate gel using ICP8-specific antiserum) (data not shown). The two faint bands in lane 4 correspond in size to the full-length translated UL37 protein and one of its byproducts seen in lanes 1 and 3; however, identification could not be confirmed by immunoblot analysis using a UL37 antiserum due to the low level of protein generated by the in vitro translation reaction (data not shown). No radiolabeled protein was precipitated non-specifically by the ICP8 polyclonal antibody (lane 2). These results suggest that the UL37 and ICP8 protein complex can be reconstituted in vitro. It should be noted that coprecipitation of the UL37 and ICP8 proteins using this system was only achieved when purified ICP8 protein was added during the UL37 in vitro translation reaction but not after (data not shown).

Figure 32

Immunoprecipitation of in vitro translated UL37 protein with exogenously added ICP8 protein. UL37 RNA templates were first generated from in vitro transcription reactions and in vitro translated (as described under Materials and Methods) in the presence (+) or absence (-) of 10 μg purified ICP8 protein (see Materials and Methods). Radiolabeled translation products were then incubated with a UL37-specific (780, labeled as 1.1) or an ICP8-specific (8) antiserum for 18 hours at 4^0C. Immune complexes were precipitated by addition of protein A-sepharose beads, pelleted and washed extensively, separated on SDS-9% polyacrylamide gels, and transferred to nitrocellulose for phosphorimager analysis (shown here) or analysis by immunoblot with either UL37 or ICP8 antiserum. 120 K, designates the position of the immunoprecipitated in vitro translated (radiolabeled) full-length UL37 protein.


In vitro translation of UL37
+/- ICP8 Protein
1 2 3 4
← 120 K
ICP8 Added - - + +
PPT. Ab 1.1 8 1.1 8

5. Renaturing Studies

Throughout all the studies performed in our laboratory investigating the nature of the UL37-ICP8 protein-protein interaction on ssDNA chromatography columns, it has been repeatedly observed that the UL37-specific antisera demonstrate varying reactivities with the population of UL37 molecules co-eluting with ICP8 (i.e., protein eluted at high-salt) (L. Shelton and F. Jenkins, unpublished data). Namely, the 487 antiserum (directed against UL37 in vitro translation products) is capable of detecting the UL37 protein in these fractions while the two malE-UL37 fusion protein antisera, 779 and 780, demonstrate little or no reactivity, respectively, against the UL37 protein in these fractions. However, the fact that all UL37-specific antisera are capable of detecting the UL37 molecules present in the flow-through and wash fractions (i.e., the population of UL37 protein that is not retained on the ssDNA column in the presence of the ICP8 protein) in these same studies led to the hypothesis that perhaps the different reactivities of the UL37-specific antisera observed with "peak fractions" is due to a conformational change in the UL37 protein induced by an interaction with the ICP8 protein on ssDNA (e.g., the formation of new intra-molecular disulphide bonds), the effect of which is not overcome even after subjection to denaturing and reduction on SDS-polyacrylamide gels. Furthermore, the observation that all UL37-specific antisera are capable of detecting the the UL37 protein in the flow-through and wash fractions, representing the unbound population of UL37 molecules, also suggested that the lack of reactivity with this portion of UL37 molecules in the peak fractions (bound molecules) was not simply due to limitations of the respective antisera.

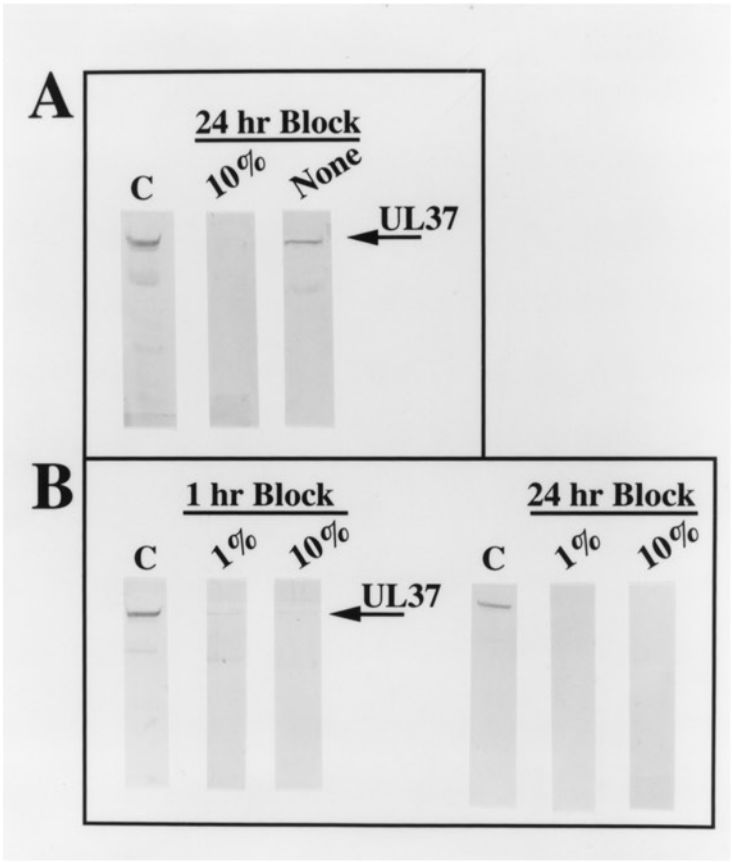
It was also reasoned that in addition to a conformational change in the UL37 protein as a result of its interaction with ICP8 on ssDNA columns, the conditions

used to non-specifically block the nitrocelluose membranes containing samples from the wash (unbound proteins) and peak (bound proteins) ssDNA fractions prior to immunoblotting contributed to a renaturing of the UL37 protein in these samples in such a way that efficient detection of the fraction of UL37 molecules co-eluting with the ICP8 protein (high-salt fractions) was precluded upon immunoblot with the 780 antisera. Incubation of membranes in a neutral buffer with a blocking agent such as nonfat dry milk or bovine serum albumin (BSA) has been shown to facilitate renaturing of some proteins even in the presence of SDS (Lacks and Springhorn, 1980; Celenza and Carlson, 1991).

Therefore, to investigate the possibility that the bound population of UL37 molecules undergo a renaturing effect which precludes detection with the 780 antiserum, a series of blocking experiments were performed using fractions eluted at high-salt (peak fractions) from ssDNA chromatography of HSV-1-infected cell extracts. It should be noted that salt concentration does not inhibit the ability of any of the UL37-specific antisera from detecting UL37 in infected cell extracts (data not shown). Duplicate samples from a high-salt eluted ssDNA chromatography fraction were subjected to SDS-PAGE and transferred to nitrocellulose as described under the Materials and Methods section. One membrane was incubated in a 10% BSA/WBII buffer blocking solution overnight; the other, WBII buffer alone. The two blots were then immunoblotted with the 780 antiserum and developed with an alkaline-phosphatase conjugated secondary antibody. The results are shown in Figure 33A. The UL37 protein is detected by the 780 antiserum in the unblocked blot but fails to be detected in the blot which received the 10% BSA block. These results suggest that the blocking conditions used for the membranes can affect the ability of the UL37-specific antiserum, 780, to detect the UL37 protein in these fractions and is most likely due to

Figure 33

Effect of blocking conditions on 780 antiserum reactivity. Fractions eluted at high-salt concentrations (peak fractions) from ssDNA chromatography of HSV-1-infected cell extracts were separated on SDS-12% polyacrylamide gels and transferred to nitrocellulose membranes. The blots were then (A) incubated in a 10% (w/v) BSA/WBII blocking solution (10% lane) or WBII solution alone (None; no blocking) for 24 hours followed by immunoblot with the 780 antiserum. To titrate the effects of blocking, identical blots were (B) incubated in a 1% or 10% (w/v) nonfat dry milk/WBII blocking solution (as designated) for either 1 hour (left panel), or 24 hours (right panel) followed by immunoblotting with the 780 antiserum. For both experiments (A and B), bound antibody was detected with alkaline phosphatase-conjugated goat anti-rabbit antiserum.


A
24 hr Block
C
10%
None
UL37
B
1 hr Block
C
1%
10%
UL37
24 hr Block
C
1%
10%

a blocking-induced renaturing of the UL37 protein which precludes its recognition. It is noteworthy that the UL37 protein contained in the flow-through and wash fractions can be detected with the 780 antiserum under the 10% blocking condition (data not shown) suggesting that the renaturing effect is unique to the bound population of UL37 molecules.

To determine if the blocking effect on the reactivity of the 780 antiserum could be titrated, similar samples were subjected to SDS-PAGE and transfer to nitrocellulose membranes as before but were incubated in varying amounts of blocking agent (1% or 10% [w/v] nonfat dry milk in WBII buffer) for either one hour or overnight. The results of this experiment is shown in Figure 33B. As can be seen, the UL37 protein was capable of being detected by the 780 antiserum under both blocking conditions when the incubation was limited to one hour (Figure 33B, left panel; faint bands indicated by arrow); however, no detection of the UL37 protein by the 780 antiserum is observed on duplicate blots that were incubated overnight with increasing amounts of the blocking agent (Figure 33B, right panel). These results demonstrate that under limiting blocking conditions, the UL37 protein is capable of being detected by the 780 antiserum most likely due to an incomplete renaturing of the UL37 protein allowing recognition by the antibody.

C. Phosphorylation Studies

The identification of a potential adenosine tri-phosphate (ATP)-binding site in the UL37 sequence (Shelton et al., 1990) led to the hypothesis that the UL37 protein either binds ATP directly or perhaps is phosphorylated. The UL37 protein did not exhibit an affinity for ATP when HSV-1 infected cell extracts were subjected to ATP agarose chromatography (as discussed in the previous section). To investigate the

possibility that the UL37 protein is phosphorylated, the following set of experiments were performed.

1. UL37 Protein Phosphorylation in Infected Cells

To determine whether the UL37 protein is phosphorylated, HSV-1-infected cells were labeled with $^{32}P_i$ from either 6 to 12 or 12 to 24 hpi and then harvested at the end of the incubation period in RIPA buffer (as described under Materials and Methods). Immunoprecipitation of the radiolabeled proteins with the 780 antiserum detected the presence of a 120 kDa phosphoprotein that was not immunoprecipitated with either the ICP8-specific or normal rabbit serum (Figure 34, right panel) (the 140 kDa coprecipitating phosphoprotein on this as well as subsequent phosphorimager blots in this section was identified as the large subunit of HSV-1 ribonucleotide reductase (ICP6) protein and is discussed in the section entitled "Stability of the UL37 Protein in HSV-1-Infected Cells". To determine if the UL37 protein was phosphorylated by an HSV-specific kinase, ^{32}P-labeled extracts from V37-infected cells were immunoprecipitated with the UL37-specific, ICP8-specific, and normal rabbit antiserum. As shown in Figure 34 (left panel), a 120 kDa phosphoprotein was immunoprecipitated with the 780 antiserum but not with the ICP8-specific or normal rabbit antiserum, suggesting that phosphorylation was the result of a cellular kinase, although it cannot be ruled out that a protein encoded by vaccinia virus is responsible for the UL37 protein phosphorylation in the V37-infected cells or perhaps that the UL37 protein can undergo autophosphorylation.

As a control to ensure that phosphorylation of the UL37 protein in the V37-infected cells was protein-specific, ^{32}P-labeled V8-infected cell protein extracts were also immunoprecipitated with the UL37-specific, ICP8-specific, and normal rabbit antiserum. The ICP8 protein has been reported to have no posttranslational modifications in HSV-

Figure 34

Identification of the UL37 protein as a phosphoprotein. ^{32}P-labeled protein extracts from V37-, V8-, and HSV-1-infected cells were immunoprecipitated with either normal rabbit serum (N), ICP8-specific antiserum (8), or UL37-specific antiserum (37). Immunoprecipitated proteins were then separated on SDS-9% polyacrylamide gels and transferred to nitrocellulose membranes. Radiolabeled proteins were detected by phosphorimage analysis.

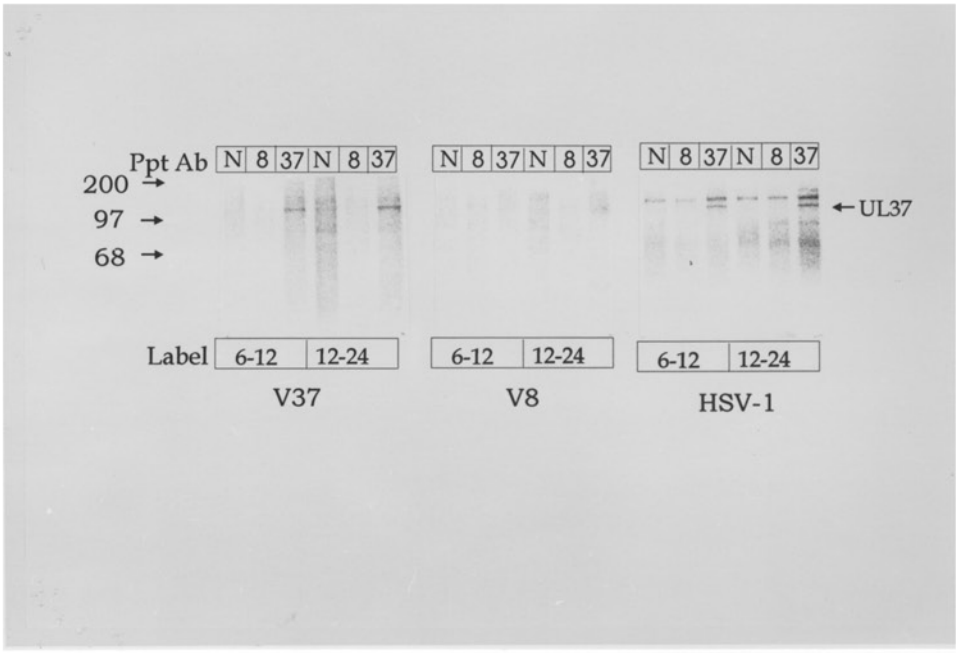

Ppt Ab
N 8 37 N 8 37
N 8 37 N 8 37
N 8 37 N 8 37
200
97
68
UL37
Label
6-12
12-24
6-12
12-24
6-12
12-24
V37
V8
HSV-1

Figure 35

Detection of the UL37 and ICP8 proteins (^{32}P-labeled HSV-1-infected cells). A duplicate of the gel shown in Figure 34 (right panel, HSV-1) was subjected to immunoblot analysis with either ICP8-specific or UL37-specific antiserum and bound antibody detected with alkaline phosphatase-conjugated goat anti-rabbit antiserum. The blot on the left was probed with ICP8-specific antiserum (α8) and the blot on the right was probed with UL37-specific antiserum (α37). Arrows indicate the position of the UL37 and ICP8 proteins.

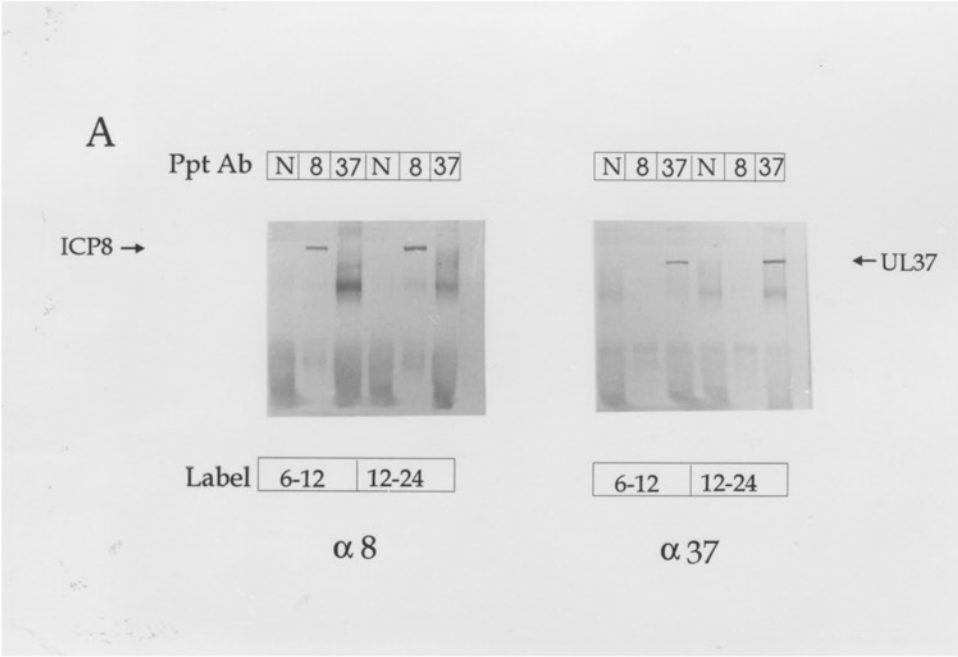

A
Ppt Ab
N 8 37 N 8 37
N 8 37 N 8 37
ICP8 →
← UL37
Label
6-12
12-24
6-12
12-24
α 8
α 37

Figure 36

Detection of the UL37 protein (^{32}P-labeled V37-infected cells). A duplicate of the gel shown in Figure 34 (left panel, V37) was subjected to immunoblot analysis with either ICP8-specific or UL37-specific antiserum and bound antibody detected with alkaline phosphatase-conjugated goat anti-rabbit antiserum. The blot on the left was probed with ICP8-specific antiserum (α8) and the blot on the right was probed with UL37-specific antiserum (α37). Arrow indicates the position of the UL37 protein; no ICP8 protein was detected (left blot).

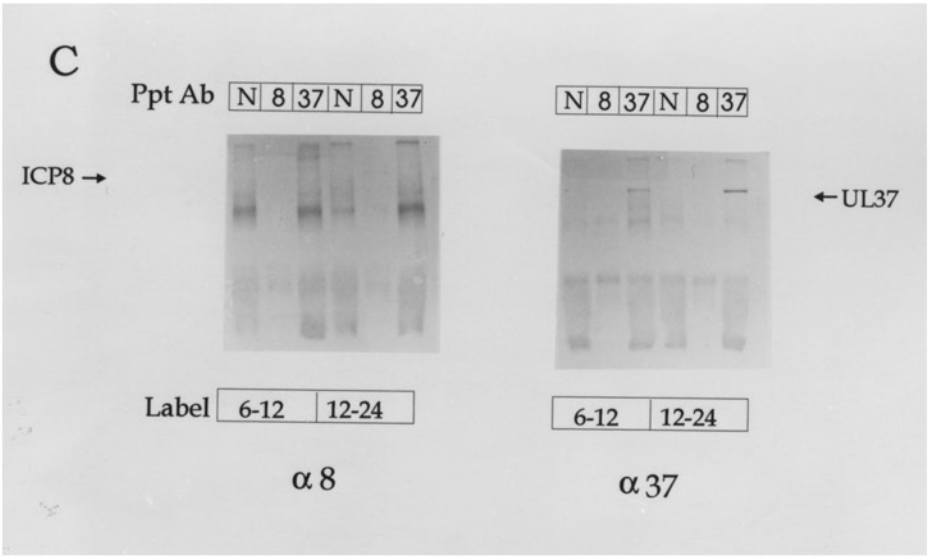
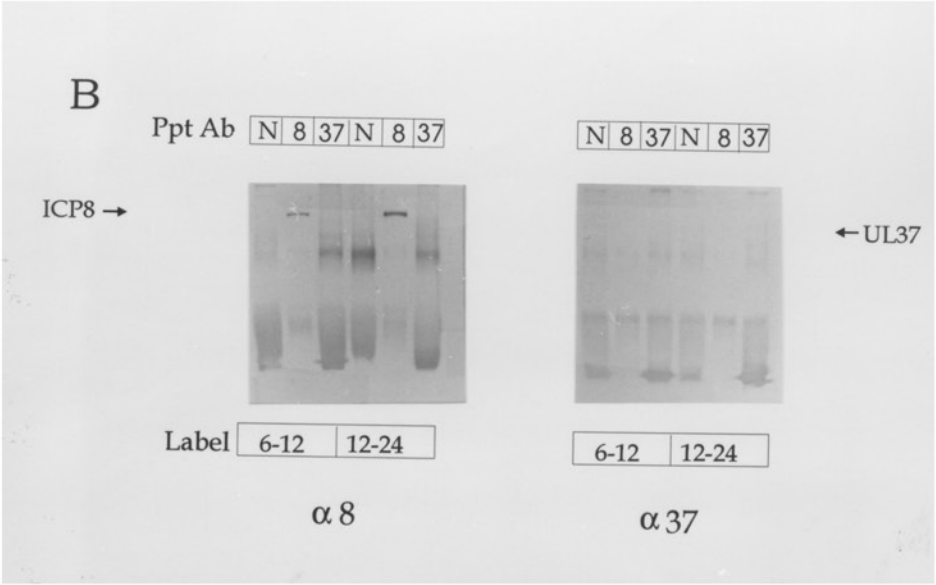

C
Ppt Ab
N 8 37 N 8 37
N 8 37 N 8 37
ICP8 →
← UL37
Label
6-12
12-24
6-12
12-24
α 8
α 37

Figure 37

Detection of the ICP8 protein (^{32}P-labeled V8-infected cells). A duplicate of the gel shown in Figure 34 (center panel, V8) was subjected to immunoblot analysis with either ICP8-specific or UL37-specific antiserum and bound antibody detected with alkaline phosphatase-conjugated goat anti-rabbit antiserum. The blot on the left was probed with ICP8-specific antiserum (α8) and the blot on the right was probed with UL37-specific antiserum (α37). Arrow indicates the position of the ICP8 protein; no UL37 protein was detected (right blot).


B
Ppt Ab
N 8 37 N 8 37
N 8 37 N 8 37
ICP8 →
←UL37
Label
6-12
12-24
6-12
12-24
α 8
α 37

1-infected cells (Knipe et al., 1982). As shown in Figure 34 (center panel), immunoprecipitations of the ^{32}P-labeled V8-infected cell extracts failed to detect any phosphoproteins.

To demonstrate that the 120 kDa phosphoprotein immunoprecipitated from HSV-1- and V37-infected cell extracts was the UL37 protein, the nitrocellulose membranes were immunoblotted with either a UL37- or ICP8-specific antiserum using a secondary goat anti-rabbit immunoglobulin antiserum conjugated to alkaline phosphatase for detection. As shown, the UL37-specific antiserum immunoprecipitated the UL37 protein from HSV-1- and V37-infected cells (Figures 35 and 36, right panels, respectively) while the ICP8-specific antiserum immunoprecipitated the ICP8 protein from HSV-1- and V8-infected cells (Figures 35 and 37, left panels, respectively). The 120 kDa phosphorylated proteins in Figure 34 (right and left panels) were coincident with the UL37 proteins identified, respectively in Figures 35 and 36 (right panels).

2. Stability of the UL37 Protein in HSV-1 Infected Cells

Before determining the kinetics of the UL37 protein phosphorylation, it was first important to assess the stability of the UL37 protein within infected cells. The UL37 protein is first detected by immunoblot analysis and immunoprecipitations in HSV-1-infected cells between 6 and 9 hpi and increases in abundance throughout the viral replication cycle (Shelton et al., 1990). To determine the stability of the UL37 protein during lytic HSV-1 replication, pulse-chase labeling with [^{35}S]methionine was performed in HSV-1-infected cells as described in Materials and Methods. HSV-1-infected or mock-infected Vero cells were pulsed with [^{35}S]methionine for 30 minutes at 6, 9, 12, 15, and 18 hpi and chased in medium containing excess cold methionine for various time periods up to 24 hpi followed by harvest in RIPA buffer. The radiolabeled infected cell proteins were then immunoprecipitated with the UL37-

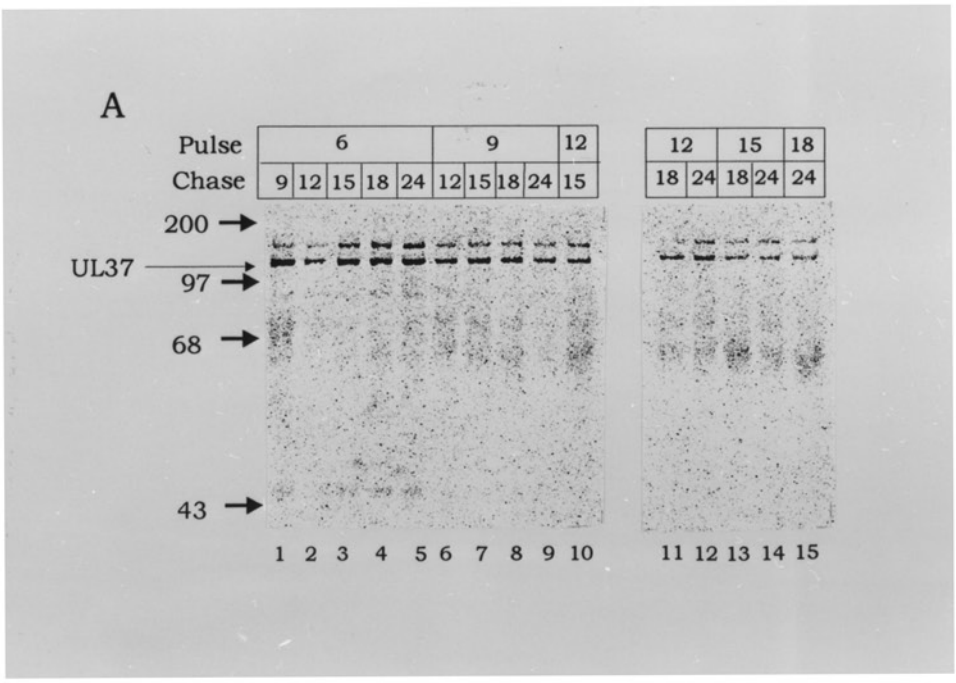
specific antiserum 780, separated by SDS-PAGE, and transferred to nitrocellulose membranes.

Phosphorimage analysis of the nitrocellulose membranes is shown in Figure 38 (A and B). Two bands of 140 and 120 kDa were immunoprecipitated with the 780 antiserum from HSV-1-infected cell extracts (Figure 38A) but not from mock-infected cell extracts (Figure 38B). Immunoblot analysis of the nitrocellulose membranes using the 780 antiserum confirmed that the ^{35}S-labeled 120 kDa band was the UL37 protein (Figure 39A) which was not detected in mock-infected cells (Figure 39B). There were no significant differences in the rate of synthesis of the UL37 protein produced at the 6, 9, and 12 hpi time points. For example, the amount of UL37 protein produced at 6, 9, and 12 hpi and harvested three hours later (9, 12, and 15 hpi, respectively) was similar (Figure 38A). Likewise, the levels of radiolabeled UL37 protein present at 15 hpi appeared to be identical for the 6-, 9-, and 12-hour pulses. There was also no noticeable reduction in the rate of synthesis of the UL37 protein produced at 15 and 18 hpi and harvested at 18 and 24 hpi. This pattern of expression is in agreement with previous data from our lab indicating that the UL37 protein belongs to the γ1 class of HSV-1 genes (Shelton et al., 1990). These results demonstrate that the UL37 protein is a stable protein in HSV-1 infected cells.

The UL37-specific 780 antiserum immunoprecipitated a 140 kDa protein from ^{35}S-labeled, HSV-1-infected cells but not from mock-infected cells (Figure 38, A and B, respectively). The 780 antiserum failed to detect the 140 kDa protein in immunblot analysis, indicating it was not the UL37 protein (Figure 39A). Similarly, a 140 kDa phosphoprotein was immunoprecipitated from HSV-1-infected cells with either UL37-specific, ICP8-specific, or normal rabbit antiserum (Figure 34, right panel). These results suggested that the 140 kDa band represented an HSV-1 phosphoprotein

Figure 38

Synthesis and stability of the UL37 protein in HSV-1-infected cells. Immunoprecipitates of (A) HSV-1-infected and (B) mock-infected cell extracts that were pulse-labeled with [^{35}S]methionine. HSV-1-infected cells were pulse-labeled and harvested at the times indicated (in hours postinfection) as described under Materials and Methods. Proteins from aliquots taken at each time point were immunoprecipitated with the UL37-specific antiserum 780, separated on SDS-9% polyacrylamide gels, and transferred to nitrocellulose membranes. ^{35}S-labeled proteins were detected by phosphorimage analysis of the membranes. Position of high molecular weight protein standards are indicated on the left (in kilodaltons)


A
Pulse
Chase
6
9
12
12
15
18
9 12 15 18 24 12 15 18 24 15
18 24 18 24 24
200
UL37
97
68
43
1 2 3 4 5 6 7 8 9 10
11 12 13 14 15

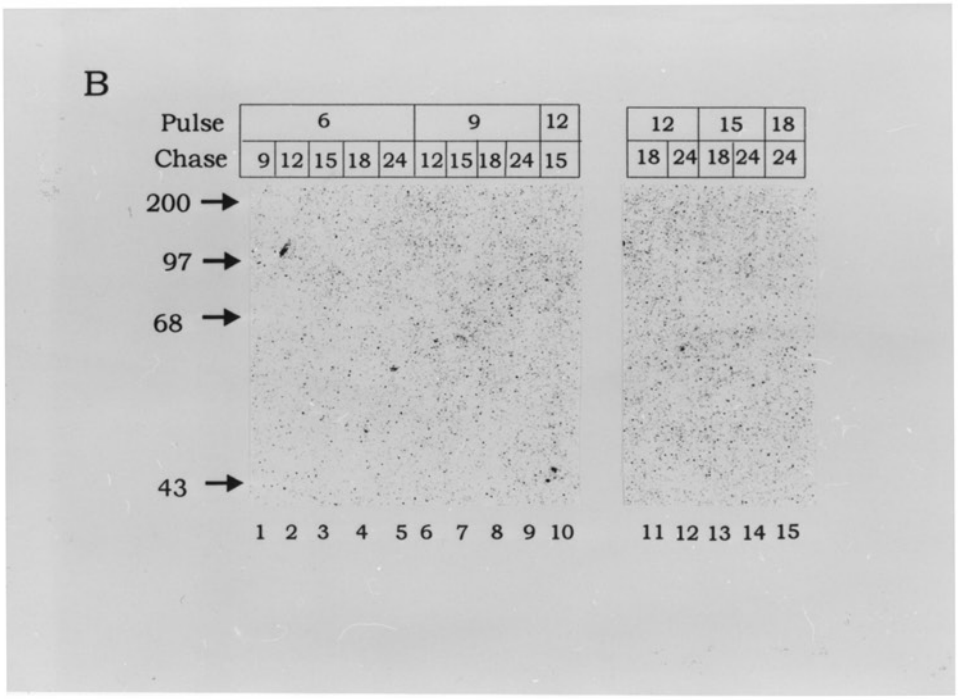
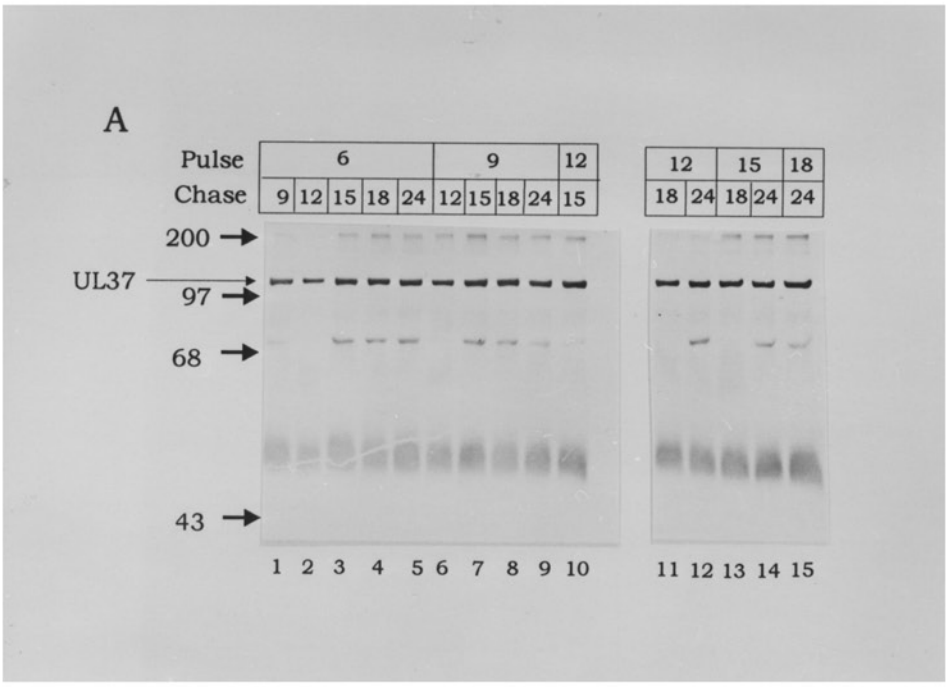
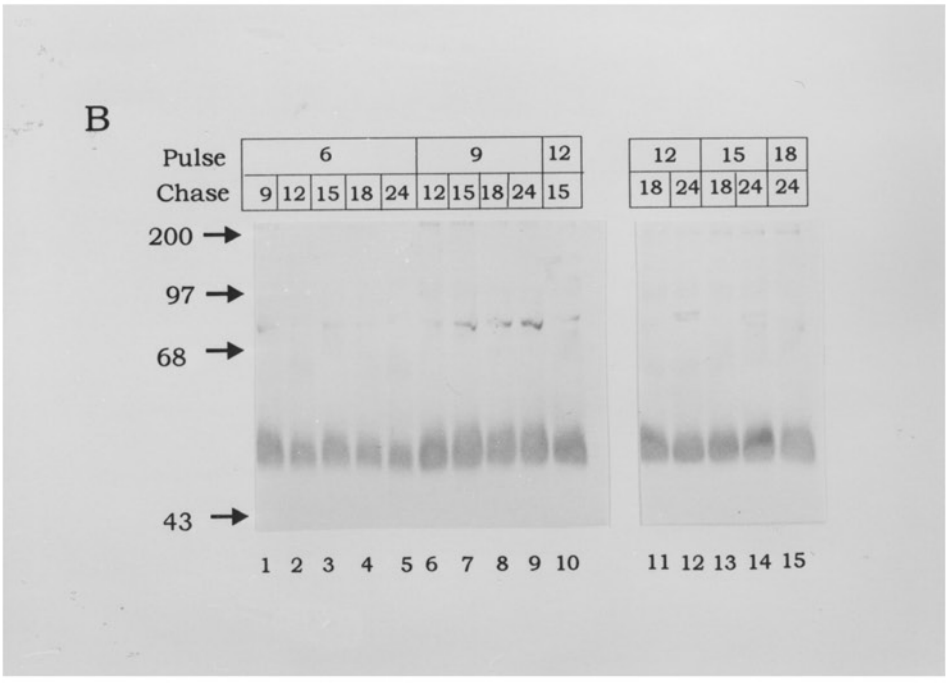

B
Pulse
Chase
6
9
12
12
15
18
9 12 15 18 24 12 15 18 24 15
18 24 18 24 24
200
97
68
43
1 2 3 4 5 6 7 8 9 10
11 12 13 14 15

Figure 39

Identification of the UL37 protein. Photograph of immunoblot analysis of (A) HSV-1-infected and (B) mock-infect cell proteins. Proteins processed as described in the legend to Figure 38 were subjected to immunoblot analysis with the 780 antiserum and bound antibody detected with alkaline phosphatase-conjugated goat anti-rabbit antiserum. Position of high molecular weight protein standards are indicated on the left (in kilodaltons).


A
Pulse
Chase
6
9
12
12
15
18
9 12 15 18 24 12 15 18 24 15
18 24 18 24 24
200
UL37
97
68
43
1 2 3 4 5 6 7 8 9 10
11 12 13 14 15


B
Pulse
Chase
6
9
12
12
15
18
9 12 15 18 24 12 15 18 24 15
18 24 18 24 24
200
97
68
43
1 2 3 4 5 6 7 8 9 10
11 12 13 14 15

that was immunoprecipitated by rabbit antiserum. It was reasoned that the most likely candidate for such a protein was the 140 kDa HSV-1 ribonucleotide reductase (ICP6) protein encoded by the UL39 gene. ICP6 is a major HSV-1 phosphoprotein and shares significant homology at the amino acid level with ribonucleotide reductases encoded in other systems (Wilcox et al., 1980; Swain and Galloway, 1986). ICP6 exhibits 38% relatedness at the amino acid level with the large subunit of ribonucleotide reductase from E. coli (Swain and Galloway, 1986). Polyclonal antiserum prepared from rabbits may also contain antibodies against various bacterial enzymes which may cross-react with homologous viral or cellular proteins. The identity of the 140 kDa band as the ICP6 protein was confirmed by comparative immunoprecipitations of ^{32}P-labeled HSV-1-infected cell extracts using either UL37-specific, ICP8-specific, and normal rabbit antiserum, and a monoclonal antibody (48S) directed against the ICP6 protein (Figure 40). As can be seen, the 140 kDa HSV-1 phosphoprotein precipitated by the various polyclonal antisera (lanes 1 to 3) comigrates with the ICP6 phosphoprotein (lane 4).

3. Stability of the UL37 Protein Phosphorylation

To determine the stability of the phosphate bound to the UL37 protein, HSV-1-infected and mock-infected Vero cells were pulse-labeled with $^{32}P_i$ for 30 minutes at 6 and 9 hpi and then either harvested immediately in RIPA buffer or chased with medium containing cold phosphate for an additional 5 hours prior to harvest. The radiolabeled proteins were then immunoprecipitated with the 780 antiserum, separated by SDS-PAGE, and transferred to nitrocellulose membranes for subsequent analysis. As shown in Figure 41A, the level of phosphorylation on the UL37 protein did not change between the one-hour pulse period and the subsequent five-hour cold chase as judged by the intensity of the label (lanes 1 to 4). The identity of the 120 kDa phosphoprotein as the UL37 protein was confirmed by immunoblot analysis with UL37-

Figure 40

Identification of the 140 kDa protein as the ICP6 protein. Vero cells were infected with HSV-1, labeled with $^{32}P_i$ from 6 to 12 hpi, and harvested at 12 hpi as described under Materials and Methods. ^{32}P-labeled protein extracts were immunoprecipitated with either normal rabbit serum (N), ICP8-specific antiserum (8), UL37-specific antiserum (37), or a monoclonal antibody directed against the ICP6 protein (48S). Immunoprecipitated proteins were separated on SDS-9% polyacrylamide gels and transferred to nitrocellulose membranes. Radiolabeled proteins were detected by phosphorimage analysis.

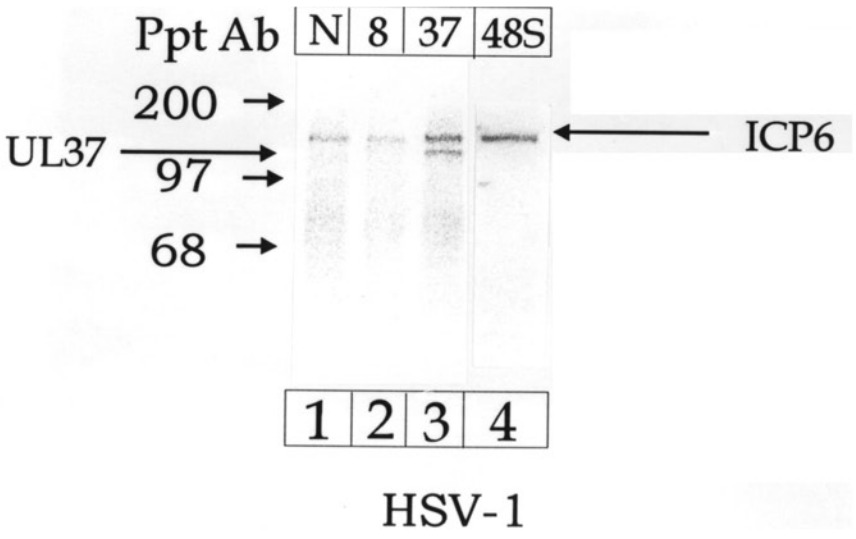
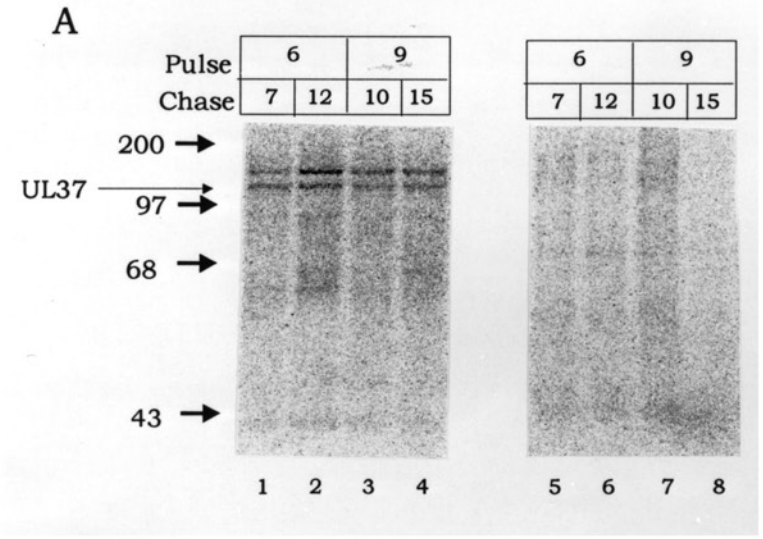
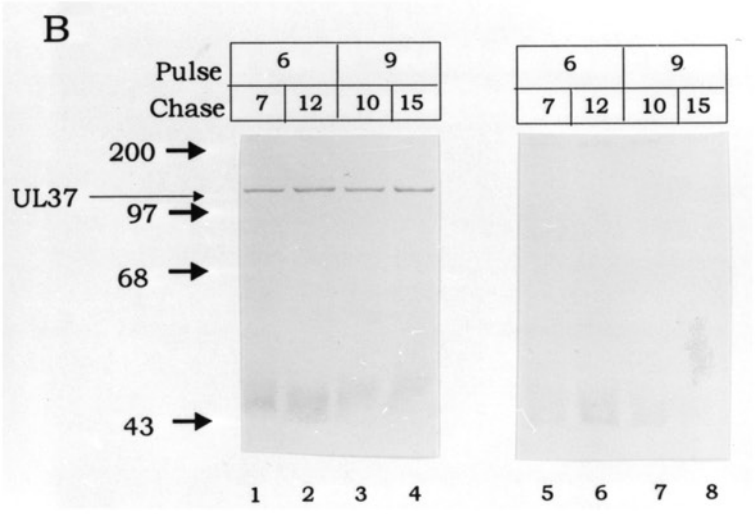

Ppt Ab
N
8
37
48S
200
UL37
97
68
ICP6
1
2
3
4
HSV-1

Figure 41

Stability of the UL37 protein phosphorylation. Immunoprecipitations of HSV-1-infected cell (lanes 1 to 4) or mock-infected cell (lanes 5 to 8) protein extracts pulse-labeled with $^{32}P_i$. HSV-1-infected Vero cells were pulse-labeled and harvested at the times indicated (in hours postinfection) as described in Materials and Methods. Proteins from aliquots taken at each time point were immunoprecipitated with the UL37-specific antiserum 780, separated on SDS-9% polyacrylamide gels, and transferred to nitrocellulose membranes. (A) ^{32}P-labeled proteins were detected by phosphorimage analysis of the nitrocellulose membranes. (B) Nitrocellulose membranes were subjected to immunoblot analysis with UL37-specific antiserum and bound antibody detected with alkaline phosphatase-conjugated goat antiserum.


A
Pulse
Chase
6
9
7
12
10
15
200
UL37
97
68
43
1
2
3
4
5
6
7
8


B
Pulse
Chase
6
9
7
12
10
15
200
UL37
97
68
43
1
2
3
4
5
6
7
8

specific antisera and alkaline phosphatase-conjugated goat antiserum (Figure 41B). Thus, the phosphate bound to the UL37 protein does not appear to cycle on and off but rather appears to be added soon after the protein is translated and remains stably associated with the UL37 protein throughout the viral replication cycle.

D. Analysis of HSV-1 Virions and L Particles

1. Demonstration of the UL37 Protein in Purified HSV-1 Virions and L Particles

Previous analysis of intact HSV-1 virus particles found that within the limits of detection, the UL37 protein did not appear to be a major component of the virion (Shelton et al., 1990). This conclusion was based on results obtained from immunoblot analysis of purified HSV-1 virions using a UL37 polyclonal antiserum (487) directed against UL37 in vitro translation products. Subsequent work in our laboratory resulted in the production of additional UL37 polyclonal antisera (see Generation of UL37-Specific Polyclonal Antiserum) that have a much stronger affinity for the UL37 protein than the 487 antiserum. Therefore, it was reasoned that re-examination of HSV-1 virions using a stronger affinity UL37-specific antiserum might demonstrate detectable amounts of the UL37 protein within virus particles.

To address this possibility, extracellular intact HSV-1 virions as well as HSV-1 L particles (light particles) were isolated and protein extracts of each subjected to SDS-PAGE for subsequent analysis as described. L particles are non-infectious virus-like particles that lack both capsids and viral DNA but do possess intact envelopes and teguments (Szilagyi and Cunningham, 1991; McLauchlan and Rixon, 1992; Rixon et al., 1992) and were included in this study for comparison purposes. Proteins were detected directly by Coomassie blue staining or by immunoblotting with antisera directed against the UL37, ICP8, and alpha trans-inducing factor (αTIF)

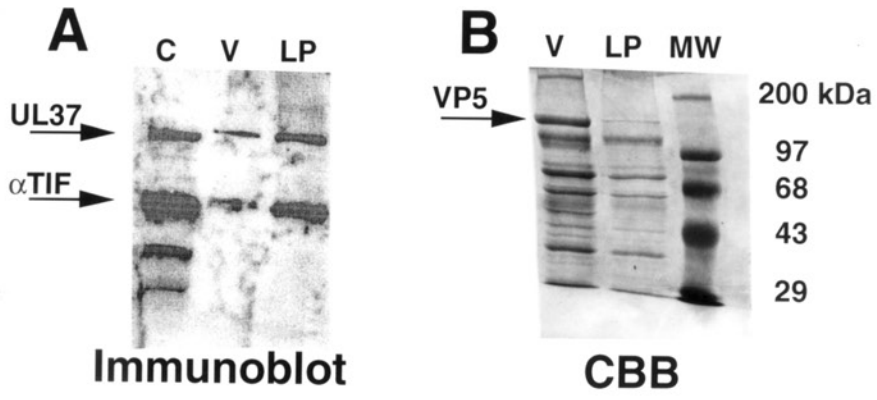
proteins (Figure 42). The αTIF and ICP8 antisera served as controls for structural and nonstructural HSV-1 proteins, respectively. Figure 42B shows the protein profiles obtained for both purified HSV-1 virions and light particles which were similar to those published by other laboratories (Spear and Roizman, 1972; Rixon et al., 1992). As shown, the most abundant polypeptide in the HSV-1 virion preparation is the 155 kDa major capsid protein (VP5); however, the L particle preparation displays only trace amounts of the major capsid protein relative to infectious virions as expected (McLauchlan and Rixon, 1992). In addition, the ICP8 protein was not detected when resolved proteins from a duplicate gel were transferred to nitrocellulose and immunoblotted with ICP8-specific antiserum (data not shown) thus verifying the purity of these preparations. Immunoblot analysis of proteins transferred to nitrocellulose from an identical gel is shown in Figure 42A. The blot was first probed with a UL37-specific antiserum (780) followed by antiserum directed towards the αTIF protein. The data from Figure 42A indicate the presence of the UL37 protein in both purified HSV-1 virions as well as L particles as compared to the HSV-1 extract control. Likewise, the αTIF protein is detected in all three protein extracts as expected. These results confirm that the UL37 protein is a component of the HSV-1 virion.

2. Localization of the UL37 Protein in HSV-1 Virions

The detection of the UL37 protein in HSV-1 L particles combined with results from previous studies which have failed to detect a 120 kDa protein in purified HSV-1 nucleocapsids (Gibson and Roizman, 1972; Heilman et al., 1979; Cohen et al., 1980) strongly suggest that the UL37 protein is associated with the envelope or tegument region of the virion. To determine the location of the UL37 protein in the virus particle, purified HSV-1 virions were first subjected to detergent treatment. The objective of this experiment was to examine the fate of the UL37 protein when the

Figure 42

Association of the UL37 protein with purified HSV-1 virions. Extracellular HSV-1 virions and light particles released from HSV-1-infected Vero cells were purified as described under Materials and Methods. Protein samples from control HSV-1 extracts (C), purified virions (V), and light particles (L) were resolved on SDS-12% polyacrylamide gels, transferred to nitrocellulose membranes, and subjected to (A) immunoblot analysis using 780 antiserum and anti-αTIF antiserum or (B) direct staining with Coomassie brilliant blue (CBB). Bound antibody was detected by using ^{125}I-labeled protein A followed by phosphorimage analysis. VP5, major capsid protein; MW, high molecular weight protein standards.


A
C
V
LP
UL37
αTIF
Immunoblot
B
V
LP
MW
VP5
200 kDa
97
68
43
29
CBB

virion envelope and associated proteins and glycoproteins were solubilized with nonionic detergents. If a protein is in (or associated with) the virion envelope, it should be solubilized by membrane-disrupting agents. Alternatively, tegument proteins would not be substantially solubilized by this treatment.

To explore this, purified HSV-1 virions were incubated with 1% NP-40, 1% NP-40 plus 0.5% deoxycholate (DOC), or no detergent as a control for 15 minutes at 37°C and then subjected to high-speed centrifugation. The resulting supernatant containing solubilized proteins and pelleted virus particles were analyzed by SDS-PAGE followed by immunoblotting. Duplicate blots were probed with either UL37-specific antiserum (Figure 43A) or antisera directed toward the HSV-1 glycoprotein B (gB) and αTIF proteins (Figure 43B). The gB and αTIF antisera served as positive controls for known HSV-1 envelope and tegument proteins, respectively. The data in Figure 43B show that most of the gB and some of the αTIF proteins were released from the purified virions into the supernatant after detergent treatment (lanes 1 and 3) but none released in the absence of detergent (lane 5) as previously reported (Yao and Courtney, 1989). In contrast, the data in Figure 43A show that none of the UL37 protein was detected in the supernatant fraction (lanes 1, 3, and 5) but remained associated with the pelleted virus particles and was not solubilized with virus envelope proteins (lanes 2, 4, and 6). These results suggest that the UL37 protein is not an envelope protein but is located internally most likely in the tegument portion of the virion.

To further explore the association of the UL37 protein with the HSV-1 tegument, experiments were performed to determine the ability of the envelope to protect the UL37 protein from protease digestion. If the UL37 protein is present within the virion and not on the surface, then protease treatment of purified virions should not

Figure 43

Effect of detergent treatment on the association of the UL37 protein with purified HSV-1 virions. Purified virion particles were either treated for 15 minutes with 1% NP40 + 0.5% DOC, or with 1% NP40 alone, or were not treated (None). The virions were separated into two fractions, soluble proteins (S) and pelleted particles (P) by centrifugation at 30,000 x g for one hour. Proteins in each fraction were resolved on SDS-12% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted using (A) UL37-specific antiserum, or (B) glycoprotein B (gB)-specific and αTIF-specific antiserum. Bound antibody was detected by using ^{125}I-labeled protein A followed by phosphorimage analysis.

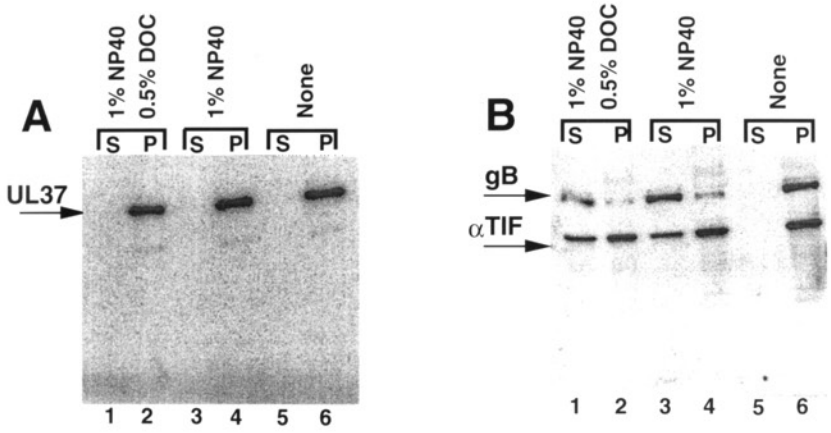
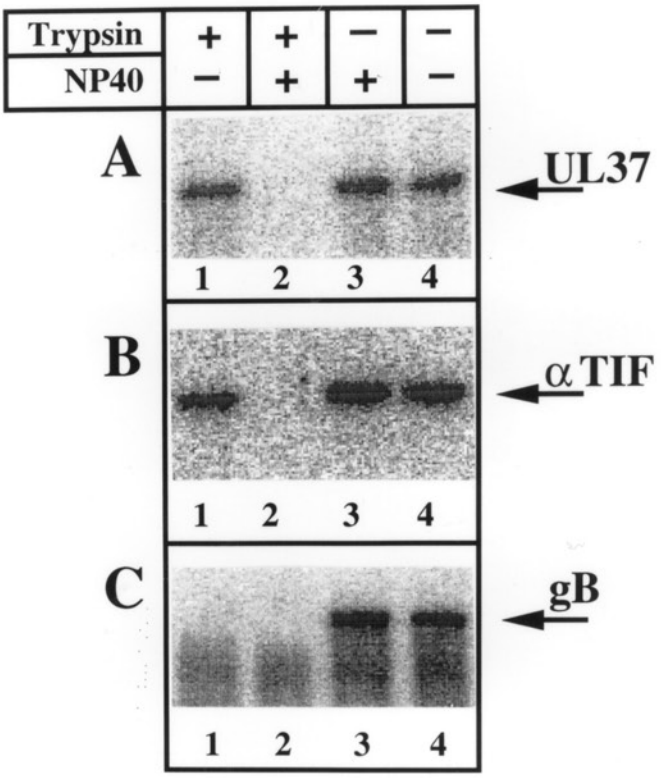

A
1% NP40
0.5% DOC
1% NP40
None
S P S P S P
UL37
1 2 3 4 5 6
B
1% NP40
0.5% DOC
1% NP40
None
S P S P S P
gB
αTIF
1 2 3 4 5 6

Figure 44

Effect of trypsin treatment on the association of the UL37 protein with purified HSV-1 virions. Purified HSV-1 virions were treated with 0.1 mg/ml trypsin in either the absence or presence of 1% NP40 for 5 minutes at 37^0C followed by the addition of TLCK, PMSF, and chicken eggwhite trypsin inhibitor. Proteins from each fraction were resolved on SDS-12% polyacrylamide gels, transferred to nitrocellulose membranes for immunoblot analysis using (A) UL37-specific antiserum, (B) αTIF-specific antiserum, and (C) glycoprotein B (gB)-specific antiserum. Bound antibody was detected by using ^{125}I-labeled protein A followed by phosphorimage analysis.


Trypsin
NP40
+ + − −
− + + −
A
UL37
1 2 3 4
B
αTIF
1 2 3 4
C
gB
1 2 3 4

affect the virion-associated UL37 protein. To test this, purified HSV-1 virions were treated with trypsin in either the presence or absence of detergent (NP-40) (as described under Materials and Methods). The proteolysis reactions were terminated by the addition of a trypsin inhibitor and samples were resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblot analysis using antisera directed against the UL37, gB, or αTIF proteins (Figure 44). The results presented in Figure 44C show that glycoprotein B (gB) was sensitive to trypsin treatment both in the absence and presence of detergent (lanes 1 and 2, respectively) as expected and previously reported (Yao and Courtney, 1989). In contrast, the data presented in Figure 44A indicate that the UL37 protein was sensitive to trypsin treatment only if the virions were also treated with detergent (lane 2). Similar results to those of the UL37 protein were also obtained for the αTIF protein, a known tegument protein which served as a control (Figure 44B). Taken together, these data demonstrate that the UL37 protein is most likely a component of the virion tegument.

IV. DISCUSSION

Previous work in our laboratory focused on the initial identification and characterization of the gene product encoded by the open reading frame (ORF) designated UL37 of HSV-1 (Shelton et al., 1990; Shelton, 1992). In these studies, it was demonstrated that the UL37 ORF encoded a 120 kDa protein belonging to the γ1 class of HSV-1 genes. In addition, the UL37 protein was found to co-elute from both ss and dsDNA columns with ICP8, the HSV-1 major ssDNA binding protein; however, results from comparative studies using a vaccinia virus recombinant, V37, and an ICP8 mutant, d21, strongly suggested that the apparent DNA binding ability exhibited by the UL37 protein from HSV-1-infected cells was due to its association with the ICP8 protein on these columns and not from an intrinsic ability to bind DNA. Furthermore, it was shown that within the limits of detection, the UL37 gene product did not appear to be a major component of the virion and was therefore classified as a nonstructural protein.

In this study, we report that the ability of the UL37 protein to bind ssDNA columns is dependent solely upon the presence of a DNA binding-competent ICP8 protein. In addition, results from other column matrices tested suggest that the UL37-ICP8 protein:protein interaction may be one that is stabilized or perhaps mediated by the presence of DNA. Furthermore, we demonstrate an ability to co-immunoprecipitate the UL37 and ICP8 proteins which provides additional evidence for a stable and perhaps functional interaction between these two proteins within infected cells. Through the development of more sensitive UL37-specific antisera, we also demonstrate that the UL37 protein is a stably phosphorylated protein within infected cells exhibiting little or no apparent turnover during the course of HSV replication. Lastly, re-

examination of purified mature HSV-1 virions by immunoblot analysis shows that the UL37 protein is present in detectable amounts and is a located in the tegument region of HSV-1 virus particles and is therefore re-classified as a structural protein.

A. Interaction Studies

Several lines of evidence presented in this study demonstrate that the UL37 and ICP8 proteins are capable of forming a stable interaction. These include comparative studies on ssDNA columns using the vaccinia virus recombinants, V37 and V8, which express the UL37 and ICP8 proteins independent of other HSV-1 genes, the HSV-1 mutant n10, as well as coprecipitation studies of HSV-1-infected, V37- and V8-coinfected cell lysates, and in vitro translated UL37 protein with exogenously added purified ICP8 protein.

The results from analyses using ssDNA chromatography along with data from previous studies from our laboratory conclusively demonstrate that the ability of the UL37 protein to bind ssDNA columns is due solely to the presence of a DNA binding-competent ICP8 protein. The UL37 protein does not possess any intrinsic ability to bind DNA (based on analysis of the V37 protein on ssDNA columns). Furthermore, the interaction between the UL37 and ICP8 proteins on ssDNA appears to be specific in that the VZV ICP8 protein equivalent, V29 (gene 29 of VZV expressed in a vaccinia virus recombinant), did not substitute for the HSV-1 ICP8 protein in similar experiments. These results suggest that the ability of the UL37 protein to be retained on the ssDNA column is dependent on a specific interaction with the HSV-1 ICP8 protein and not by just an SSB-induced conformational change of the DNA structure.

Although it is difficult from these analyses to determine at what point the

UL37 and ICP8 proteins form a complex, results from mixed or sequentially loaded extracts of V37- and V8-infected cell proteins suggest that the UL37 and ICP8 proteins can form an association outside of the infected cell in the presence of ssDNA. Although the domains of each protein responsible for the UL37-ICP8 protein interaction are unknown, the finding that the UL37 protein is retained on the ssDNA column in the presence of the n10 ICP8 mutant protein suggests that the C-terminal 36 amino acids (which includes the nuclear localization signal) of the ICP8 protein are not necessary to mediate such an interaction with the UL37 protein. In addition, the ability of the full-length malE-UL37 fusion protein (FL) and the failure of the 2.1 and 1.1 UL37 fusion proteins to bind to the ICP8 protein on the ssDNA column suggest that the N-terminal domain of the UL37 protein may be important for interaction with the ICP8 protein. Moreover, the results from UL37-specific antisera reactivity on fractions representing the population of UL37 molecules co-eluted with the ICP8 protein under high-salt conditions (peak fractions) along with the renaturation studies performed on similar fractions indicate that the bound form of the UL37 protein undergoes a conformational change induced by its retention on ssDNA, presumably due to an interaction with the ICP8 protein. Obviously, more work is required in order to elucidate the precise domains of each protein involved in this complex formation. The generation of UL37 deletion mutants either in HSV-1 or vaccinia virus will be required to adequately assess UL37 protein domains that are important for interaction with the ICP8 protein. Chromatography of infected cell extracts on ssDNA provides a system that is capable of assessing protein domains which may be involved in such an interaction.

The apparent inability of the UL37 and ICP8 proteins to form an association on non-DNA column matrices such as blue sepharose and amylose suggests that the

presence of DNA may stabilize or perhaps mediate the association between these two proteins. While we cannot rule out that the conformation of the respective proteins in the environment of each matrix tested precluded complex formation independent of an absence of DNA, precedence exists for protein:protein interactions requiring a DNA substrate for ability to interact or stimulate protein activity. This phenomenon stems from studies in E. coli where such systems have been studied in greater detail to date than their eucaryotic counterparts. One example of this is seen in the case of the E. coli SSB and DNA polymerase II proteins. SSB has a pronounced effect on the ability of some nucleic acid enzymes to bind to DNA including DNA pol II (Molineux and Gefter, 1975). E. coli DNA pol II will only bind to ssDNA in the presence of SSB; the formation of a complex betweeen SSB and pol II that is stable to sedimentation through glycerol can be observed under these conditions. DNA polymerase II, only when present in this ternary complex, degrades ssDNA in an apparent processive manner, and it is likely that the enzyme's mechanism for DNA synthesis also becomes processive through a similar stimulation by the presence of SSB. The biological role of SSB remains uncertain but is believed to be an essential component for the processes of DNA replication, recombination, repair, and gene regulation. An additional example of a protein whose DNA binding ability and activity are stimulated in the presence of a second protein bound to DNA is the SOS-inducible DNA repair protein RuvB. Work performed by Shiba et al. (1991) demonstrated that RuvB, which alone cannot bind to DNA, can bind to DNA through the interaction with RuvA. Since RuvA enhances the ATPase activity of RuvB only when it (RuvA) binds to DNA (supercoiled DNA > ssDNA > linear dsDNA), it was hypothesized that RuvA undergoes a conformational change by binding to DNA, and the DNA-bound RuvA may modify the conformation of RuvB into a form that is more active as an ATPase.

Interestingly, from sequence analysis, the UL37 protein contains a putative ATP-binding motif (Shelton et al., 1990); however, it remains to be determined whether or not the UL37 protein can actually bind or hydrolyze ATP; the UL37 protein did not display an affinity for this moiety when subjected to ATP agarose chromatography (see Results).

The ICP8 protein has been reported to interact with several other HSV-1 proteins involved in DNA replication and metabolism including the DNA polymerase, alkaline exonuclease, and origin-binding protein. These interactions have been demonstrated through a variety of methods including immunoaffinity columns, protein affinity columns, and immunoprecipitation tests (Littler et al., 1983; Vaughan et al., 1984; Thomas et al., 1992; Boehmer and Lehman, 1993). The fact that the UL37 and ICP8 proteins form an immuno-precipitable complex within infected cells as well as in an in vitro translation system strongly suggests that there is both a physical and functional interaction between the two proteins during HSV-1 replication and substantiates the detection of the complex formed between these two proteins from ssDNA chromatography studies. The inability to achieve a reciprocal coprecipitation of the UL37 and ICP8 proteins with a UL37-specific polyclonal antiserum is disappointing but not surprising since some polyclonal and monoclonal antibodies are known to disrupt protein complexes depending on the particular epitopes of the respective proteins that are bound (Thomas et al., 1992). The isolation of a panel of monoclonal antibodies against the UL37 protein would most likely alleviate such a problem. In addition, the apparent weak coprecipitation of the soluble UL37 and ICP8 proteins from cytoplasmic and nuclear lysates using the ICP8 polyclonal and monoclonal antibodies can be interpreted as evidence for a weak protein-protein interaction; however, from the results of ssDNA and non-DNA column matrices, an

apparent weak coprecipitation might imply that DNA is required in order for an efficient complex to be formed and maintained between the two proteins as discussed earlier. The apparent difference in stoichiometry of the UL37 and ICP8 proteins in cytoplasmic verses nuclear precipitations may reflect an effect caused by the method of preparation of these lysates.

B. Phosphorylation and Kinetic Studies

Pulse-labeling and pulse-chase studies demonstrated that the UL37 is a stable phosphoprotein whose synthesis can be detected initially at 6 hpi with a steady rate of synthesis of the protein maintained late into the viral replication cycle. This pattern of expression is in good agreement with previous data from our lab showing that the UL37 protein belongs to the γ1 class of HSV-1 genes and accumulates late in infection (Shelton et al., 1990). The role that phosphorylation plays in the overall function and conformation of the UL37 protein remains to be determined. By comparison of the relative intensities of major HSV-1 phosphoproteins such as ICP6, ICP0, and the UL42 protein following [^{32}P]orthophosphate labeling of HSV-1-infected cells, the phosphate content of the UL37 protein appears significantly less (data not shown) and probably explains why the UL37 protein was not detected as an HSV-1 phosphoprotein in previous studies (Wilcox et al., 1980) in the absence of UL37-specific antiserum. This has also been the case for other less abundantly phosphorylated HSV-1 proteins such as the UL36 protein (McNabb and Courtney, 1992c). While the number and locations of the phosphorylation sites within the UL37 protein are not known, there are numerous potential sites. Within the 1,123 amino acids of the UL37 protein, there are 162 serine, threonine, and tyrosine residues (71, 71, and 20 residues, respectively). Analysis of the amino acid sequence for motifs

recognized as potential sites of phosphorylation by known cellular kinases reveal two potential sites for cyclic-AMP-dependent protein kinase, 14 potential sites for casein kinase II, and 14 potential sites for protein kinase C.

Although the UL37 protein is capable of being phosphorylated by what appears to be a cellular kinase, it is interesting to speculate whether the UL37 protein might be a target of one of two known serine-threonine HSV-1 protein kinases encoded by the US3 and UL13 genes (Frame et al., 1987; Chee et al., 1989; Smith and Smith, 1989). The US3 protein kinase has been purified and has been shown to mediate phosphorylation of a virion protein encoded by the UL34 gene (Purves et al., 1991). Although less work has been reported on the UL13 gene product, Cunningham et al. (1992) identified the UL13 protein as a virion component, and correlated it with a novel protein kinase activity present in the nuclei of infected cells. Thus, the UL13 kinase may correspond to the tegument-associated protein kinase activity identified by LeMaster and Roizman (1980), which is responsible for the in vitro phosphorylation of the UL36 protein as well as other structural proteins within the virion (which may also include the UL37 protein) and possibly other proteins in HSV-1-infected cells.

The phosphorylation of the UL37 protein from V37-infected cells indicates that interaction with the ICP8 protein is not a prerequisite for phosphorylation. In addition, preliminary results have indicated that the phosphorylated form of the UL37 protein co-elutes with ICP8 protein from ssDNA agarose columns (data not shown). This suggests that phosphorylation of the UL37 protein does not inhibit the interaction of the UL37 with the ICP8 protein. The fact that the bacterially expressed full-length malE-UL37 fusion protein (FL) is capable of interacting with the ICP8 protein on ssDNA columns might suggest that phosphorylation does not play an essential role; however, the phosphorylation status of this fusion protein is not known. Further studies

are necessary to determine whether phosphorylation of the UL37 protein is required for its interaction with the ICP8 protein or perhaps the ability of the UL37 protein to localize to the nucleus (Moll et al., 1991).

C. Structural Studies

In this study, we confirm that the UL37 protein is a component of mature HSV-1 virions and is located in the tegument region. The presence of the UL37 protein in virus particles is consistent with previous data from our laboratory which demonstrate that the UL37 protein belongs to the γ1 class of HSV-1 genes and is stably synthesized late in the course of HSV-1 infection (Shelton et al., 1990; Albright and Jenkins, 1993). These data are also in good agreement with the observation that all γ proteins examined to date for presence in virus particles have been shown to be components of the virion.

Upon analyzing the protein composition of HSV-1 virions, Honess and Roizman (1973) made note of a minor virion polypeptide designated VP6A with an estimated molecular weight of 130 kDa which co-electrophoresed with the ICP8 protein. In a later report, the VP6A protein was estimated to be present at approximately 30-40 molecules per virion particle (which is considered to be low copy number in comparison to αTIF and VP5 which are both present at 800-1000 copies) (Heine et al., 1974). The characteristics of the previously unidentified VP6A protein are entirely consistent with those of the UL37 protein, and it is likely that the UL37 protein is the VP6A protein described by Honess and Roizman. The results of experiments in which virion preparations were subjected to detergent treatment suggest that the UL37 protein may be more tightly associated with the viral capsid then with the virion envelope since none of the UL37 protein was solubilized under these conditions. A similar

pattern was reported in the case of the ICP4 protein which has also been shown to be a constituent of the tegument in HSV-1 virions (Yao and Courtney, 1989). Whether this tighter association with nucleocapsids has a functional role for either protein in the virion is unknown.

The intracellular localization of the UL37 protein has been analyzed using indirect immunofluorescence and cell fractionation techniques (Schmitz et al., manuscript in preparation). The data obtained by both methods suggests that the UL37 protein is distributed throughout the nucleus and cytoplasm. Indirect immunofluorescence using the V37 vaccinia virus recombinant demonstrates that the UL37 protein can localize to the nucleus in the absence of other herpes proteins. Because viral assembly occurs in the nucleus and the UL37 protein is a structural protein, one would expect to find the UL37 protein exclusively localized in the nucleus of HSV-infected cells. In addition, the known association between the UL37 protein and ICP8, the major DNA-binding protein, in the presence of ssDNA suggests a functional role for the UL37 protein in the nucleus. The reason for the cytoplasmic localization of the UL37 protein from these studies is less clear; however, it is interesting to note the similarities between the UL37 and UL36 proteins in this regard.

The UL37 and UL36 gene products are both phosphorylated proteins located in the tegument region and are present in low copy number in the virion. Chou and Roizman (1989) demonstrated sequence-specific binding of the UL36 protein and an as yet unidentified 140 kDa viral protein with the a sequence of the HSV-1 genome. This finding implicates the UL36 protein in a potential cleavage and/or packaging role of the viral DNA during the assembly process in the nucleus, yet the UL36 protein exhibits both a nuclear and cytoplasmic distribution in infected cells (McNabb and Courtney, 1992c) similar to that observed with the UL37 protein. The authors suggest

that the large size of the UL36 protein (270 kDa) may preclude the efficient transport of the protein into the nucleus; however, this is not likely the case with the UL37 protein since the ICP8 protein which is of comparable size (120 kDa) is readily transported to the nucleus (Knipe and Spang, 1982; Quinlan et al., 1984). Additional studies using both immunofluorescence and cell fractionation will be necessary in order to ascertain the kinetics of the UL37 protein transport into the nucleus and to determine if the UL37 protein preferentially localizes in regions of the nucleus similar to that observed with the ICP8 protein (co-localization). We cannot rule out the possibility that the UL37 protein may also play some functional role in the cytoplasm of HSV-infected cells.

D. Potential Functions

The genome of HSV-1 encodes at least 77 genes (Baines et al., 1994) of which thirty or more of these proteins have been reported to be structural components of the virion (Spear and Roizman, 1972; Heine et al., 1974; Cassai et al., 1975; Powell and Watson, 1975). Approximately one-half of the structural polypeptides which compose the HSV-1 virions are probably located in the tegument; however, the function(s) of many of these proteins within HSV-infected cells and virions remains unknown. The studies described in this dissertation have provided a further characterization of the UL37 protein and its interaction with the ICP8 protein; however, the current challenge is to understand the function of the UL37 gene product in the replication cycle of HSV-1. The construction of an HSV mutant containing a null allele of the UL37 gene would provide a valuable tool in experiments designed to address this question. Many attempts at constructing such a mutant have been made but with no success thus far in our laboratory, implying that the UL37 protein may

represent an essential gene product. Although ascribing a putative function to the UL37 protein is difficult in the absence of a viral mutant, a potential function(s) of the UL37 protein can possibly be deduced from what is already known about other proteins located in the tegument region as well as the various roles performed by the ICP8 protein in the replication cycle.

Various functions have been assigned to proteins within the tegument, including the trans-activation of immediate-early gene transcription (Batterson and Roizman, 1983), the inhibition of host protein synthesis (Fenwick and Walker, 1978; Read and Frenkel, 1983), and a protein kinase activity (LeMaster and Roizman, 1980). In addition, although the function of the UL36 protein is not known, reports have suggested that this gene product (which is also located in the tegument in low copy number) may be involved in the cleavage and/or packaging of DNA (Chou and Roizman, 1989) as discussed earlier. In addition, studies by Batterson et al. (1983) have demonstrated that the mutation responsible for the temperature-sensitive phenotype of an HSV-1 mutant (HSV-1 HFEM tsB7) maps within the UL36 gene, implying that the UL36 protein is an essential gene product. At the nonpermissive temperature, tsB7 was found to be defective in several viral functions including release of the viral DNA from the nucleocapsids during the initial stages of infection, viral DNA synthesis, and late gene expression (Batterson et al., 1983). The UL36 polypeptide may be involved in the release of the viral genome from the nucleocapsid (uncoating) to initiate an HSV infection, and subsequently function in the cleavage and/or packaging of the viral genome into progeny virus during the latter stages of the replicative cycle. From this example, it is conceivable that the UL36 protein and perhaps other proteins in the tegument such as the UL37 gene product may play a seemingly dual role perfoming functions at two critical stages (i.e., early and late) in the replication cycle of HSV-

1.

In addition to their functions immediately following infection, tegument proteins (particularly those in high copy number such as the αTIF and the virion host shut-off [vhs] protein are believed to perform a structural role in the virion perhaps by stabilizing the virus particle (Weinheimer et al., 1992). Such a function is analagous to matrix proteins (M proteins) commonly found in enveloped RNA viruses which lie beneath the lipid bilayer and presumably bridge between the viral envelope and the nucleocapsid core. Studies on the M protein of vesicular stomatitis virus (VSV) demonstrate an ability of the M protein to associate with plasma membranes in vivo in addition to an ability of membrane-bound M protein to interact with nucleocapsid cores in vitro supporting a model of VSV assembly in which membrane-bound M protein could nucleate sites for viral assembly at the cell surface (Chong and Rose, 1993; Chong and Rose, 1994). Given the observation that the UL37 protein may be more tightly associated with nucleocapsids, it is interesting to speculate whether the UL37 protein might be involved in an analagous assembly process of HSV-1 virions in the nucleus. It is unknown at this time whether or not the UL37 protein can associate with membranes or glycoproteins tails which presumably would be necessary in such a role. The UL37 protein lacks a distinct stretch of hydrophobic residues indicative of a membrane-associating region, and modification by fatty acid has not been demonstrated; however, the same characteristics are true of the M protein of VSV suggesting that other regions of such proteins can be involved in membrane interaction (Chong and Rose, 1994).

Other possible functions for the UL37 protein may be deduced from its interaction with the ICP8 protein, an essential multifunctional protein in the HSV replication cycle. The ICP8 protein (as outlined in the Introduction) is involved in

several important processes in the viral replication cycle including viral DNA replication (and perhaps recombination) and HSV gene regulation. The requirement for ICP8 in HSV DNA replication early in infection has been firmly established by genetic studies using both temperature-sensitive (ts) and deletion mutants in the ICP8 gene. It is unlikely that the UL37 protein plays a role in such early stages of viral DNA replication since maximal production of the UL37 protein does not occur until after the onset of viral DNA replication. For the same reason, it is unlikely that the UL37 protein is involved in HSV-1 recombination since time course analysis of HSV-1 recombination and DNA replication indicate that these two processes parallel each other, and direct quantitation of the products of recombination by Southern analysis demonstrate that recombination occurs during or subsequent to the last round of DNA replication (Dutch et al., 1992). However, the complexing of the UL37 and ICP8 proteins may possibly result in a modification of ICP8 protein function late in viral replication which may be necessary for the ICP8 protein's role in gene regulation or perhaps other processes in the viral replication cycle. Since levels of the ICP8 protein reach peak levels early during an infection, but remain high through late times in the replication cycle, it is reasonable to think that the ICP8 protein may a different role late in infection.

The ICP8 protein has been implicated in the regulation of viral gene expression since it is able to down-regulate the expression of viral genes from parental genomes (Gao and Knipe, 1993) and stimulate late-gene expression from progeny templates (Gao and Knipe, 1991). Roles in both DNA replication and gene regulation is a common theme amongst other ssDNA binding proteins including the adenovirus DNA binding protein (E2A) (Chang and Schenk, 1990), T4 bacteriophage gene 32 protein (gp32) (Gauss et al., 1987; Herendeen et al., 1989; Herendeen et al., 1990).,

and E. coli SSB (Haynes and Rothman-Denes, 1985). Specifically, the ICP8 protein has been implicated in the negative regulation of ICP4, the major immediate-early regulatory protein of HSV-1 (Godowski and Knipe, 1983; Gao and Knipe, 1989; Gao and Knipe, 1991). Mutations in the ICP8 protein have also been shown to result in increased transcription of several early and late HSV genes (Godowski and Knipe, 1985; Godowski and Knipe, 1986). Gao and Knipe (1991) recently described a trans-dominant mutant of the ICP8 protein that inhibited the expression of several late proteins during HSV replication. These results have led to the hypothesis that at late times in infection, the ICP8 protein binds either to small ssDNA regions, keeping promoter regions open for transcription, or to specific structures or sequences in HSV-1 late-gene promoters. In order for the ICP8 protein to recognize these late-gene promoters, it may require direct interactions with other viral and/or cellular proteins. From the results of earlier studies in our laboratory (Shelton et al., 1990) as well as the work constituting this dissertation, we have postulated that the UL37 protein is a reasonable candidate for such a viral protein.

Another interesting speculation about what function the UL37-ICP8 protein complex may have in the HSV-1 replication cycle comes from studies on host range temperature-sensitive (ts) mutants in the adenovirus DNA binding protein (DBP). Nicolas et al. (1983) reported the phenotype of one such mutant, r(ts107)202, which failed to produce infectious virus or virus particles when grown at the nonpermissive temperature in 293 cells. This mutant also synthesized its viral DNA at a lower rate and extent than wild-type virus, but produced a perfectly normal set of late adenovirus proteins. However, in spite of the fact that r(ts107)202 produced all the detectable late viral proteins normally, it failed to assemble virus particles at 39^0. Thus, it was suggested that the DBP of adenovirus might play a role in assembly of virions, either

directly or indirectly (via an altered DNA structure) and was postulated to interact with a cellular or perhaps viral function in this process. Recently, the large T antigen of simian virus 40 (SV40), which is a multifunctional protein necessary for the initiation and maintenance of viral DNA replication, autoregulation of early gene expression, and stimulation of late gene expression, has been implicated in SV40 virion assembly through similar studies using host range mutants (Spence and Pipas, 1994). The HSV-1 alkaline exonuclease, in addition to its function in DNA metabolism and putative role in cleavage and/or packaging of viral DNA, has recently been shown to be required for efficient egress of capsids from the nucleus (Shao et al., 1993). From these examples, it is clear that precedence exists for non-structural proteins such as DNA binding proteins involved in DNA replication and/or gene regulation (and perhaps auxiliary viral or cellular proteins) to perform functions at a much later stage in the replication cycle such as virion assembly or maturation. Whether the ICP8 protein plays such a role in late events has yet to be determined. No conditional-lethal or deletion mutants have been described thus far to implicate the ICP8 protein in virion assembly; however, such a role has been difficult to assess since the ICP8 protein is essential for viral DNA replication and mutants in this protein are usually blocked at this stage in the replication cycle.

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