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ANALYSIS OF THE ESSENTIAL IN VIVO ROLE OF HERPES SIMPLEX VIRUS TYPE I DNA POLYMERASE ACCESSORY PROTEIN, UL42, AND IN VITRO CHARACTERIZATION OF ITS PROTEIN-PROTEIN INTERACTION WITH THE HERPES-ENCODED ORIGIN-BINDING PROTEIN, UL9

DISSERTATION

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

By
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The Ohio State University
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ABSTRACT

Replication of the DNA genome of herpes simplex virus type 1 (HSV-1) requires seven viral-encoded proteins: the trimeric helicase-primase complex (UL5, UL8, UL52), the major single-stranded DNA binding protein (ICP8), the DNA-dependent DNA polymerase (pol), a pol accessory factor (UL42), and an origin-binding protein (UL9). UL42 and pol have been shown to physically and functionally interact. To investigate whether an essential in vivo function of UL42 is to provide pol accessory function, I examined whether the same regions of UL42 which affect its ability to bind to and stimulate pol activity in vitro are also important for its ability to complement the replication of CgalA42, a UL42 null virus. The strong correlation between the abilities of UL42 proteins to bind to and stimulate pol activity in vitro and to complement replication of CgalA42 indicate that a predominant in vivo role of UL42 is as a pol accessory factor, although additional functions for UL42 cannot be ruled out.

Recombinant viruses harboring insertion mutations at amino acids 140 or 206 were generated since these mutations resulted in low-level complementation. Although these UL42 mutations were not lethal, they did reduce the efficiency with which infectious progeny virus was produced. These results confirm that UL42 functions predominantly as a pol accessory factor.
An additional protein-protein interaction was demonstrated between UL42 and
UL9 using affinity chromatography with glutathione-S-transferase fusion proteins. The
regions of UL42 important for interaction with UL9 most likely reside in multiple,
noncontiguous domains of UL42. Residues of UL9 important for interaction with UL42
reside within the N-terminal portion of the protein and are distinct from those required for
interaction of UL9 with either UL8 or ICP8.

These data validate the use of in vitro assays and simple in vivo complementation
assays as initial screens to delineate the domains of UL42 required for pol accessory
function. The recombinant viruses will be important to elucidate the mechanism by
which UL42 increases pol processivity within cells. Moreover, the interaction between
UL42 and UL9 may suggest an additional role for UL42 in DNA replication.
Dedicated to my supportive and loving husband and parents
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Virions of the Herpesviridae family include a core containing a linear double-stranded DNA genome, an icosahedral capsid (T = 16), an amorphous tegument surrounding the capsid, and a viral-glycoprotein-spiked envelope (Roizman, 1990). This family of viruses has been classified into three subfamilies. The alphaherpesvirinae subfamily includes herpes simplex virus type 1 and 2 (HSV-1 and HSV-2), and varicella-zoster virus (VZV). This subgroup is characterized by its variable host range, relatively short reproductive cycle, efficient destruction of infected cells, and ability to establish latent infections primarily, but not exclusively, in sensory ganglia (Roizman, 1990). Epidemiologically, this subfamily is associated with fever blisters (HSV-1), severe genital infection (HSV-2), chicken pox and shingles (VZV; reviewed in Boehmer and Lehman, 1997).

The remaining two subgroups, betaherpesvirinae and gammaherpesvirinae, include human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV), respectively. These, too, are significant human pathogens and are associated with retinitis in immunocompromised people (HCMV; reviewed in Fields et al., 1996) and infectious
mononucleosis, Burkitt’s lymphoma, and nasopharyngeal carcinoma (EBV; reviewed in Boehmer and Lehman, 1997).

Because human herpes viruses are considerable pathogens, much time and effort has been spent on understanding the replication and latency of these viruses in an attempt to identify targets for antiviral agents. One of the most intensely studied members of this family, and the focus of my research, is HSV-1.

**HSV-1 genome structure and model of genome replication.** The sequence of the entire 152-kbp HSV-1 genome is known (McGeoch et al., 1988) and encodes at least 75 open reading frames (Boehmer and Lehman, 1997). The linear genome consists of two covalently-linked unique regions termed U_L (unique long) and U_S (unique short). Each unique region is flanked by inverted repeats designated ab and b'a' for the U_L segment and a'c' and ca for the U_S segment (reviewed in Roizman and Sears, 1996). Within every population of virus, there is an equimolar mixture of four isomeric forms of the DNA which differ in their relative orientations of the L and S regions. The HSV-1 genome contains three functional origins of replication: one copy in each of the two c sequences that flank the U_S segment and one copy in the U_L segment located between the UL29 and UL30 genes (Stow, 1982; Weller et al., 1985).

Early postinfection, in the absence of protein synthesis, viral DNA accumulates in the nucleus and adopts an “endless” configuration, consistent with circularization (Jacob and Roizman, 1977; Becker et al., 1978; Jacob et al., 1979; Jongeneel and Bachenheimer, 1981; Davison and Wilkie, 1983; Poffenberger and Roizman, 1985; Garber et al., 1993; Severini et al., 1994). It is thought that in this form, the parental DNA serves as a
template for origin-dependent theta replication, although little evidence exists to support this hypothesis.

Several groups have shown that during late stages of genome replication, the theta mechanism is replaced by a rolling-circle mechanism (Jacob et al., 1989; Roizman, 1979; Kaerner et al., 1981; Vlazny and Frunkel, 1981). Jacob and colleagues (1979) demonstrated replicating HSV-1 DNA consisted of rapidly sedimenting bodies of tangled DNA. In addition, DNA labeled during a pulse is of an extremely high molecular weight, but is chased into unit length molecules (Jacob and Roizman, 1977). Likewise, analysis of newly replicated DNA by restriction enzyme digestion suggests that the DNA does not possess detectable termini (Jacob et al., 1979; Jongeneel and Bachenheimer, 1981). Taken together, these data suggest that DNA replication results in the formation of large head-to-tail concatamers consisting of tandem repeats of the viral genome. Supporting evidence of a rolling-circle mechanism has been provided by electron microscopy (Shlomai et al., 1976), pulsed-field gel electrophoresis (Severini et al., 1994), and two dimensional gel electrophoresis (Severini, et al., 1996). In addition, Stow (1982) demonstrated that large head-to-tail concatemers are generated from HSV-1 ori-containing plasmids when cells were coinfected with HSV-1.

**Seven viral gene products essential for origin-dependent DNA replication.**

Seven viral encoded proteins are sufficient to promote origin-specific DNA synthesis in transient replication assays (Wu et al., 1988; Stow, 1992) and are essential for viral DNA synthesis in infected cells (reviewed in Challberg, 1991; and Weller, 1991). Viral temperature-sensitive (ts) mutants were first used to identify seven complementation groups that directly affected HSV-1 DNA replication (reviewed in Challberg and Kelly,
1989). In addition, a transient DNA replication assay was developed in which cloned segments of HSV DNA were tested for their ability to support the replication of a cotransfected ori-containing plasmid (Challberg, 1986). Taken together, these studies demonstrated that seven viral-encoded proteins are required for origin-specific DNA replication: UL5, UL8, UL52, UL29, UL9, UL30, and UL42 (Wu et al., 1988 and McGeoch et al., 1988). In addition, the necessity of these seven genes has been demonstrated by targeted null mutations in the UL5 (Zhu and Weller, 1988; Zhu and Weller, 1992a; and Zhu and Weller, 1992b), UL8 (Carmichael and Weller, 1989), UL52 (Goldstein and Weller, 1988), UL29 (Conley et al., 1981; Powell et al., 1981; and Purifoy and Powell, 1981), UL9 (Carmichael et al., 1988; Malik et al., 1992), UL30 (Chartrand et al., 1980; Coen et al., 1982, Honess, et al., and Coen et al, 1984), and UL42 (Johnson et al., 1991) genes. These viral null mutants all display a DNA negative phenotype.

Interestingly, Stow (1992) demonstrated baculovirus recombinants expressing the seven essential replication proteins were able to support the replication of origin-containing plasmids in insect cells. These findings indicate that any host function required for origin-dependent viral replication in mammalian cells is also present in insect cells, and therefore, well conserved.

a. UL5, UL8, and UL52: DNA Helicase-Primase. The DNA helicase-primase holoenzyme consists of a trimeric complex of UL5, UL8, and UL52 in a 1:1:1 stoichiometry (Crute, 1989; Dodson et al., 1989; Crute and Lehman, 1991). First identified as a virus-induced DNA helicase (Crute et al, 1988), the DNA helicase-primase complex possesses both primase and DNA-dependent ATPase activities (Crute et al,
Crute and colleagues (1988) demonstrated that the complex was able to unwind short oligodeoxyribonucleotides annealed to single-stranded M13 DNA in a 5' to 3' direction by translocation along the lagging strand. After purification of the complex, it was found that the helicase consists of three protein subunits encoded by the UL5, UL8, and UL52 genes (Crute et al., 1989). In addition, Crute and colleagues (1989) demonstrated the trimeric helicase also possessed primase activity.

Six conserved ATP-binding and DNA helicase motifs, as defined by Gorbalenya et al. (1989), were identified using amino acid sequence analysis of the UL5 protein (Zhu and Weller, 1992). Analysis of mutant proteins with alterations in any of the conserved motifs further confirmed UL5, alone, possesses both ATPase and helicase activities (Zhu and Weller, 1992).

Site-directed mutagenesis of UL52 at a proposed divalent metal-binding motif conserved among DNA polymerases and primases, conclusively demonstrated that the UL52 protein is responsible for the primase activity of the trimeric complex (Klinedinst and Challberg, 1994). The necessity of this region of UL52 was later confirmed by Dracheva et al. (1995) who demonstrated mutagenesis of this region abolished origin-specific DNA replication in vivo. A subassembly of UL5 and UL52 retains DNA-dependent ATPase, helicase, and primase activities and is therefore considered the core enzyme (Calder and Stow, 1990; Dodson and Lehman, 1991).

While it has been shown that UL5 and UL52 are sufficient for helicase-primase enzymatic activities, UL8 is thought to play a role in stabilizing the interaction between subunits (Sherman et al., 1992). Tanguy and colleagues (1996) demonstrated that UL8 was required for optimal DNA helicase, DNA-dependent nucleoside triphosphatase, and
primase activity in the presence of ICP8. In addition, UL8 may serve a tethering function for the helicase/primase activities as it has been shown to interact with the origin-binding protein, UL9 (McLean et al., 1994) and is necessary for efficient nuclear localization of the helicase-primase complex (Calder et al., 1992).

b. UL29: Major single-stranded DNA-binding protein. UL29, or infected cell polypeptide 8 (ICP8), was one of the first HSV-1 DNA replication proteins to be identified and characterized. It binds cooperatively to DNA (Ruyechan, 1983) and preferentially to single-stranded DNA (Lee and Knipe, 1985; Powell and Purifoy, 1976), much like the single-stranded DNA binding protein of E. coli (Schneider and Wetmur, 1982; Meyer and Laine, 1990). UL29 possesses a helix-destabilizing activity as a result of its ability to unwind short regions of duplex DNA in an ATP- and direction-independent manner (Powell et al., 1981; Boehmer and Lehman, 1993). This action is extremely rapid and cooperative, and requires saturating concentrations of UL29 (Boehmer and Lehman, 1993).

UL29 has been shown to interact with several other proteins including viral DNA polymerase, DNA helicase-primase (via the UL8 subunit; Hamatake et al., 1997), and UL9 proteins. UL29 acts by modifying the enzymatic activities of these proteins. The role of some of these interactions will be considered below (see Protein-Protein Interactions).

c. UL9: Origin-binding protein. Using oriS as bait, Elias and colleagues (1986) identified an HSV-1 induced factor that bound specifically to sequences within oriS. Elias and Lehman (1988) showed the protein specifically bound to two inverted
repeats that flank the A + T region within ori. By amino acid sequence analysis, Gorbalenya and colleagues (1989) identified conserved ATP-binding and DNA helicase motifs. Using single amino acid changes and insertion mutations (Martinez et al., 1992), UL9 null virus (Malik et al., 1992), and dominant negative assays (Stow et al., 1993), these motifs have been shown to be essential for viral replication.

Whether bound to its target sequence or free in solution, UL9 exists as a homodimer (Makhov et al., 1996a; Makhov et al., 1996b; Bruckner et al., 1991; Fierer et al., 1992; Stabell and Olivo, 1993; and Fierer and Challberg, 1995). A leucine-zipper motif located in the amino-terminal portion of the protein is thought to mediate dimerization (Elias et al., 1992 and Hazuda et al., 1992). The carboxy-terminal portion of the protein possesses sequence-specific DNA binding activity (Deb and Deb, 1991; Weir et al., 1989; Arbuckle and Stow, 1993; and Martin et al., 1993) and when expressed by itself, exists as a monomer (Elias et al., 1992). Not surprisingly, when only the DNA-binding domain of UL9 is expressed, it exhibits a dominant-negative effect on viral replication probably due to its nonproductive occupation of the origin (Stow, 1992; Stow et al, 1993; and Perry et al., 1993).

In addition to specifically binding the origin, UL9 possesses both DNA-stimulated nucleoside triphosphatase and DNA helicase activities (Bruckner et al., 1991; Fierer and Challberg, 1992; Dodson and Lehman, 1993; and Boehmer et al., 1993). Boehmer and colleagues (1993), when studying the kinetics of DNA unwinding by UL9, first observed that unwinding appeared to be a stoichiometric rather than a catalytic event. The existence of a lag in helicase activity at early times in the unwinding reaction suggests an inactive UL9 enzyme that assembled into an active multimer.
Because of its ability to recognize elements within the origin of replication and to act as a DNA helicase, UL9 is likely to be a DNA replication initiator protein whose role is to make the DNA at the origin accessible to the replication machinery. Indeed, some of the properties of UL9 strongly resemble those of the large T antigen of Simian Virus 40 (SV40) and polyoma virus (Borowiec, 1996). Consistent with its role as an initiator protein, UL9 induces structural changes in the DNA at the origin. Elias and colleagues (1990) demonstrated that binding of UL9 protein to ori₅ induces hypersensitivity to DNase I in the A + T region which separates the strong UL9-binding regions. Stabell and Olivo (1993) showed that this hypersensitivity to DNase I digestion requires protein-protein interactions between the UL9 protein dimers bound to the flanking regions within the origin. Based on these and other observations, it was proposed that protein-protein interactions between ori₅-bound UL9 protein dimers lead to looping, distortion, and destabilization of the A + T region.

Confirming and extending the role of UL9 as a replication initiator, Makhov and colleagues (1996) used electron microscopy to confirm the UL9 protein bound to ori₅ as a pair of dimers that bent the DNA. In addition, when ATP was added, stem-loop structures were expelled from the base of the UL9 protein complex. These observations lead to a model of origin-specific unwinding. In short, the protein-protein interactions between UL9 dimers bound within the origin lead to bending, looping, and distortion of the A + T region. Because these interactions would prevent ATP-dependent translocation of the UL9 dimers, the DNA must move through the protein complex and be released as single-stranded DNA. Since UL9 and ICP8 have been shown to physically and functionally interact, it is speculated that this interaction, in the presence of ori sequences,
would stimulate the helicase activity of UL9 while also stabilizing the released single-stranded DNA.

d. UL30 and UL42: DNA polymerase holoenzyme. Keir and colleagues (1966) were first to show induction of a DNA polymerase (pol) following infection with HSV-1. Since that time, HSV polymerase has been studied both as a model for eukaryotic DNA replication and target for antiviral therapies. Several groups demonstrated that the catalytic core of the pol is a large (130 – 140 Kd) polypeptide encoded by the UL30 gene (Quinn and McGeoch, 1985; Jofre et al., 1977; Purifoy et al., 1977; Gibbs et al., 1985; and Hall et al., 1986; Parris et al., 1988). Using amino acid and DNA sequence comparisons, the 1235 amino acid protein, UL30, has been shown to exhibit significant similarity to other viral and cellular DNA polymerases including human DNA polymerase α-primase, Saccharomyces cerevisiae DNA polymerase δ, E. coli DNA polymerase I, and bacteriophage T4 DNA polymerase (Digard and Coen, 1990; Blanco et al., 1991; and Hall et al., 1995).

Once purified from virus-infected cells, the HSV-1 DNA pol was shown to be stably associated with another protein of lower molecular weight (Gallo et al., 1988; and Crute and Lehman, 1989). Using hybrid-arrested translation experiments and anti-peptide antibodies, Parris and colleagues (1988) were able to identify the 65-kDa protein as the product of the UL42 gene. It is now clear that the stable heterodimer comprised of a 1:1 mix of pol:UL42 makes up the pol holoenzyme (Crute and Lehman, 1989; Gottlieb et al., 1990).

The HSV-1 catalytic subunit, pol, possesses deoxynucleotide polymerization and 3’-5’-exonuclease activities that are intrinsic to the UL30 polypeptide (Knopf, 1979;
Ostrander et al., 1980; O'Donnell et al., 1987; Weissbach et al., 1973; Derse and Cheng, 1981; Abbotts et al., 1987; Marcy et al., 1990; Weisshart et al., 1994; and Hall et al., 1996). Pol retains catalytic activity in the absence of the UL42 polypeptide when assayed on simple primer-templates such as activated DNA. Catalytically active pol has been expressed by in vitro transcription/translation (Dorsky and Crumpacker, 1988; Haffey et al., 1988; and Crute and Lehman, 1989), in yeast (Haffey et al., 1988), and in insect cells using a recombinant baculovirus (Marcy et al., 1990; Hernandez et al., 1991). The exonuclease activity is active on a variety of DNA substrates including gapped DNA, DNA hairpin structures, and single-stranded oligodeoxyribonucleotides (O'Donnell et al., 1987 and Derse and Cheng, 1981).

The pol accessory protein encoded by the UL42 gene contains 488 amino acid residues (McGeoch et al., 1988) and is an abundant double-stranded-DNA (dsDNA)-binding protein with no apparent sequence specificity (Bayliss et al., 1975; Powell and Purifoy, 1976; Marsden et al., 1987; Gallo et al., 1988). UL42 physically associates with HSV pol by copurification from infected cells (Gallo et al., 1988; Crute and Lehman, 1989; Gottlieb et al., 1990), forms heterodimers with pol in heterologous expression systems (Digard et al., 1990; Hernandez and Lehman, 1990; Gottlieb et al., 1990), and colocalizes with pol in nuclei of infected cells (Goodrich et al., 1990). This physical interaction is not without function as UL42 protein stimulates pol activity in high salt and increases the processivity (Gallo et al., 1989; Hernandez and Lehman, 1990; and Gottlieb et al., 1990).

UL42 is similar in function to DNA polymerase processivity factors such as the β subunit of E. coli DNA polymerase III (Stukenberg et al., 1991 and Kong et al., 1992),
the eukaryotic proliferating cell nuclear antigen (PCNA; reviewed in Hubscher et al.,
1996), and bacteriophage T4 gene 45 protein (reviewed in Kuriyan and O'Donnell, 1993)
which serve to increase the processivity of their cognate polymerases. The latter proteins
form a torus encircling the DNA and stimulate the processivity of their respective
polymerases by direct protein-protein interactions. Only UL42 has been shown to bind
DNA. Moreover, the β subunit, PCNA, and gene 45 protein all require specific sets of
proteins that mediate ATP-dependent loading of the accessory proteins onto the DNA
(reviewed in Hubscher et al., 1996). By contrast, UL42 does not appear to require other
proteins to load onto the DNA template (Marsden et al., 1987; Gottlieb and Challberg,
1994). All of the aforementioned processivity factors prevent dissociation of the pol
from the primer-template, thereby increasing processivity of polymerization. However,
because of its ability to bind DNA directly as opposed to simply encircling it, UL42 is
thought to tether the HSV-1 polymerase to the DNA (Chow and Coen, 1995). Indeed,
Chow and Coen (1995) demonstrated that stimulation of DNA pol required the DNA-
binding activity of UL42, providing further support of the tethering model.

The closest functional similarity exists between UL42 and the T7 replicative pol
(gene 5 protein) processivity factor thioredoxin. The host E. coli protein, thioredoxin,
forms a 1:1 complex with T7 pol to increase its processivity. Although thioredoxin
possesses no inherent DNA binding activity (Huber et al., 1986), it has been shown to
contact the DNA template upstream of the pol when complexed to it (Bedford et al.,
1997) and to decrease the K_d of the pol for template 80-fold compared to that without
thioredoxin (Huber et al., 1987). This increased affinity of the pol for template results in
a 1000-fold increase in processivity, allowing the pol to incorporate thousands of nucleotides in one association event (Tabor et al., 1987)

Using gel shift and filter binding analysis, Gottlieb and Challberg (1994) showed that the affinity of the pol enzyme for the model primer/template was increased 10-fold by the presence of UL42. Footprinting experiments demonstrated that the pol subunit bound to the primer template at a position which was approximately centered on the 3' terminus of the priming strand in such a way as to make close contacts with both the single-stranded template ahead of the growing chain and the double-stranded product DNA (Gottlieb and Challberg, 1994). Upon addition of UL42, no effect was seen on the interaction with single-stranded template, but the contacts of the enzyme with double-stranded DNA behind the 3' end of the primer increased. These results suggest that UL42 acts as a clamp that reduces the dissociation of the polymerase from the DNA (Gottlieb and Challberg, 1994).

**Protein-protein interactions.** A recurring theme throughout DNA replication is the importance of specific protein-protein interactions. They are important in the formation of a functional replisome at an origin of replication. As indicated above, many important protein-protein interactions have been detected among HSV-1 DNA replication proteins. Although the seven HSV-1 DNA replication proteins are involved in several protein-protein interactions, only those relevant to my study will be discussed below. Additional protein-protein interactions are reviewed in Boehmer and Lehman (1997).

As previously indicated, UL9 self-interacts to form homodimers (Bruckner et al., 1991; Elias et al., 1992; Fierer and Challberg, 1992). These homodimers then act cooperatively to bind the origin and alter its conformation (Elias et al., 1990; Fierer and
Challberg, 1992). Once UL9 has bound and contorted the origin, it most likely plays an important role in recruiting the other replication proteins. In fact, UL9 has been shown to physically associate with several HSV-1 proteins.

UL9 both physically and functionally interacts with the HSV-1 single-stranded DNA-binding protein (ICP8). Several groups have reported that ICP8 can stimulate the DNA-dependent ATPase and the DNA helicase activities of UL9 protein in a species-specific manner \textit{in vitro} (Fierer and Challberg, 1992; Dodson and Lehman, 1993; and Boehmer \textit{et al.}, 1993). Using indirect immunofluorescence, Boehmer and colleagues (1994) showed that these two proteins colocalize during HSV-1 replication \textit{in vivo}. The carboxyl-terminal domain of UL9 appears to be required for interaction with ICP8 (Boehmer and Lehman, 1993), as deletion of the 27 C-terminal residues of UL9 greatly reduces both DNA binding and stimulation of DNA unwinding by ICP8 (Boehmer \textit{et al.}, 1994).

Once the origin has been unwound, UL9 most likely recruits the HSV-1 helicase-primase. Using baculovirus recombinants expressing UL9 and UL8, a subunit of the helicase-primase complex, Stow \textit{et al.} (1994) were able to coimmunoprecipitate UL9 and UL8 using a monoclonal antibody to UL8. Interestingly, in contrast to ICP8, the amino-terminal two-thirds of UL9 was required for interaction with UL8 (McLean \textit{et al.}, 1994). In addition, while the UL8 component of the helicase-primase is not required for enzymatic helicase/primase activities by a subassembly of the UL5 and UL52 proteins, only the holoenzyme (UL5/8/52) is stimulated by ICP8 (Hamatake \textit{et al.}, 1997). This interaction presumably serves to recruit a helicase/primase to the functional origin of
replication, although Tenney and colleagues (1995) showed that the minimal ori, does not act as a template for the primase activity of the HSV-1 helicase-primase.

That a different primase may be used for initiation of DNA replication is suggested by the fact that UL9 interacts with DNA polymerase α (Lee et al., 1995). A physical interaction between UL9 and DNA polymerase α was displayed using coimmunoprecipitation of recombinant baculovirus proteins with antibodies to either UL9 or pol α and a modified ELISA technique (Lee et al., 1995).

Finally, Monahan et al. (1998) and this report, showed that UL42 specifically interacts with UL9. Monahan et al. (1998), using coimmunoprecipitation and glutathione-S-transferase (GST) fusion protein column chromatography, demonstrated a specific interaction between UL42 and UL9 that was independent of protein-DNA interactions. Monahan et al. (1998) and my study defined a subdomain of UL9 required for interaction with UL42. This subdomain is distinct from regions required for interaction with UL8 and ICP8. Thus, UL9 interacts with at least one partner of each of the viral protein complexes required for processive DNA synthesis, suggesting its importance in the assembly of the DNA replication machinery at functional origins of replication in the infected cell.

Another important protein-protein interaction involving HSV-1 DNA replication proteins is that of UL42 and UL30. This interaction is quite strong (1 X 10^8 M^-1; Hamatake et al., 1993) and has been shown to be essential for viral replication (Stow, 1993; Digard et al., 1993; Digard et al., 1993; and Reddig et al., 1994). UL42 has been shown to stimulate pol activity four- to sevenfold on activated calf thymus DNA (Gallo et
al., 1989) and to increase the processivity of pol on primed single-stranded DNA templates (Gottlieb et al., 1990; Hernandez and Lehman, 1990).

Using deletion mutations, Digard and Coen (1990) mapped the site of interaction between UL30 and UL42 to the C-terminal 227 residues of the UL30 protein. Deletion of this region of UL30 had no effect on the pol activity of UL30 but completely abolished interaction with and stimulation by UL42. Subsequent investigations to localize essential regions of pol important for interaction with and stimulation by UL42 have found the C-terminal 27 residues of pol are required for this physical and functional interaction (Stow et al., 1993; Digard et al., 1993a; Tenney et al., 1993; Marsden et al., 1994; Digard et al., 1995). Deletion of the C-terminal 27 residues from pol abolishes its ability to interact with or be stimulated by UL42 without affecting pol’s enzymatic abilities.

The site of interaction in the UL42 protein has been more difficult to elucidate. Several groups have supported the presence of multiple regions of UL42 important for both physical and functional interaction with the polymerase. Using deletion and insertion mutations, several groups have found the N-terminal 340 residues of UL42 sufficient to bind duplex DNA, interact with UL30 protein, and support viral replication (Digard et al., 1993a; Digard et al., 1993b; Monahan et al., 1993; Tenney et al., 1993; Reddig et al., 1994), however the specific regions within the N-terminal 340 residues remain unclear. In addition, Hamatake et al. (1993) identified two protease-resistant peptides derived from the N-terminal regions of UL42 that are required for physical and functional interactions with UL30. However, that the region within the N-terminal 340 residues of UL42 required for interaction with pol is not a linear domain is supported by findings of Owsianka and colleagues (1993) in which overlapping peptides spanning the
entire UL42 protein were unable to disrupt the functional interaction between the UL30 and UL42 proteins. Indeed, that the C-terminal 150 residues of UL42 are dispensable in vivo as well as in vitro, is supported by viral mutant n338, which lacks residues 339-488, but displays no apparent differences to the wild-type virus with regard to nuclear localization of UL42, DNA replication, and growth in Vero cells (Gao et al., 1993).

Another DNA binding protein has been shown to stimulate UL30 activity — ICP8, the single-stranded DNA binding protein (Ruyechan and Weir, 1984; Gottlieb et al., 1990). ICP8 has been shown to stimulate pol activity 2-fold on an oligonucleotide-primed single-stranded DNA template (Ruechan and Weir, 1984). However, it is interesting that stimulation of pol by ICP8 on this template appears non-specific since other single-stranded binding proteins also increase pol activity. Although ICP8 stimulates pol 2-fold, UL42 is predicted to be the pol accessory factor as it stimulates pol 4- to 7-fold over pol activity alone. It is probable that ICP8 stimulates pol, or the pol:UL42 heterodimer, by coating the single-stranded DNA, reducing the frequency of nonproductive binding events, thereby increasing the efficiency with which pol is assembled at the primer termini. The ability of a single-stranded binding protein to stimulate the replicative DNA polymerase appears to be a common phenomenon, as replication factor A (RF-A) stimulates DNA polymerase α activity on either primed M13 DNA or oligo dT-primed poly-dA, resulting in short products (reviewed in Wang, 1991).

Because the 140-kDa subunit possesses substantial polymerase activity when expressed alone (Dorsky and Crumpacker, 1988; Gallo et al., 1989; Gottlieb et al., 1990; Hernandez and Lehman, 1990; Marcy et al., 1990; Haffey et al., 1988), the question arose whether the essential in vivo activity provided by the accessory protein correlates
with known activities of these proteins \textit{in vitro}. It is intriguing that UL42 protein exists in excess over pol with which it forms heterodimers in wild-type virus-infected cells (Gallo \textit{et al.}, 1988; Crute and Lehman, 1989; Gottlieb \textit{et al.}, 1990). It is possible that all or part of its essential function \textit{in vivo} may reside in activities other than or in addition to those mediated through pol activity. To investigate whether or not there was a correlation between the \textit{in vitro} activities ascribed to UL42 and its apparent activity in the virus-infected cell, I have taken advantage of the availability of a null mutant of HSV-1 deleted for the UL42 gene. The UL42 null mutant fails to synthesize viral DNA in nonpermissive cells (Johnson \textit{et al.}, 1991).

In my study, a transient complementation assay was used to determine the ability of various mutant UL42 proteins to complement the replication of the UL42 null mutant in nonpermissive host cells. This assay provided a means to determine the correlation between the domains of UL42 required to stimulate pol activity \textit{in vitro} and those necessary for virus replication \textit{in vivo}. Where only partial complementation was observed, recombinant viruses were created to further study the role of UL42 in a true viral background. The hypothesis that drove my research was that the major essential \textit{in vivo} function of UL42 is to act as a polymerase accessory factor.

In addition to confirming the major \textit{in vivo} role of UL42 as a processivity factor, my research has demonstrated, for the first time, a specific protein-protein interaction between UL42 and UL9. My results suggest an additional role for UL42 in virus replication.
CHAPTER 2

MATERIALS AND METHODS

Cells and viruses.

African green monkey kidney (Vero) and baby hamster kidney (BHK) cells were cultivated in Dulbecco modified Eagle minimum essential medium (Flow Laboratories, Inc., McClean, VA) supplemented with 5–7.5% fetal bovine serum (Intergen, Purchase, NY), 100 U of penicillin/ml, 100 μg streptomycin/ml, and 0.075% NaHCO₃ for closed vessels or 0.225% NaHCO₃ for open vessels. V9 cells, which contain the HSV-1 UL42 gene and have been described previously (Johnson et al., 1991), were propagated as above except that G418 (geneticin; GIBCO Laboratories, Grand Island, NY) was added at alternate passages to a concentration of 200–400 μg (active)/ml. The Sf9 insect cell line used to propagate baculovirus was kindly provided by Fred Hink (Ohio State University, Columbus, OH). Sf9 cells were propagated in TNM-FH insect medium (Sigma Chemical Company, St. Louis, MO), 100 U of penicillin/ml, 100 μg of streptomycin sulfate/ml, and 10% fetal bovine serum (TNM-FH complete media; Intergen) at 27°C.

The HSV-1 parental (wild-type) strains used were KOS (Smith, 1964) and wtCgal+ (Johnson et al., 1991). The deletion mutant CgalΔ42 was constructed from
wtCgal+ (Johnson et al., 1991). The deletion mutant UL42/lacZ was a kind gift of Min Gao (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ) and has been described previously (Gao et al., 1993). Virus stocks were prepared by low multiplicity passage under permissive conditions and tested for retention of the defect by plaque assay under permissive and nonpermissive conditions.

Recombinant baculovirus (Autographica californica) encoding the UL9 deletion mutant Δ4 was a kind gift of Nigel Stow (MRC Virology Unit, Glasgow, Scotland). Stock of recombinant baculovirus was prepared by low multiplicity passage.

Plasmids and cloning.

Plasmids were maintained in Escherichia coli (E. coli) host strains JM101, JM109, or DH5α. Large scale preparation of plasmid DNA was performed using Qiagen column chromatography (Qiagen Inc., Santa Clara, CA) according to the instructions of the manufacturer. Recombinant plasmids were constructed by standard methods (Sambrook et al., 1989) and will be described in detail below. All restriction enzymes were purchased from either GIBCO BRL (Gaithersburg, MD) or New England Biolabs (Beverly, MA).

A series of plasmids containing the wild-type and mutant forms of HSV-1 UL42 were constructed in the phagemid vector pTZ 19U (United States Biochemical, Cleveland OH). The UL42 mutants were derived from the wild-type construct pLBN 19A, which encodes full-length UL42 (Gallo et al., 1989). C-terminal deletions of UL42 were produced from the wild-type plasmid via insertion of BamHI linkers (8-, 10-, and 12-
mer; New England Biolabs, Beverly, Mass) to introduce frameshift (fs) mutations. N459 and N206 encode polypeptides containing wild-type UL42 amino acids from the amino terminus (N terminus) to the residue indicated because of frameshifts created at the NruI or HpaI restriction sites, respectively. Plasmids i206 and i140 contain in-frame insertion mutations at the HpaI and NdeI sites, respectively. A BamHI linkers were introduced at the indicated sites, inserting amino acid residues RGSA and PDFV, respectively. Deletion mutations were made within the UL42 gene by removing internal restriction fragments. The d129 – 163 mutation was created by digestion of pLBN 19A with NdeI and AatII, removal of the single-stranded overhangs with S1 nuclease, and religation. The deletion mutation d37 – 282 was created by removal of the internal 738bp PstI fragment which resulted in an in-frame mutation. To create the d202-337 deletion mutation, the BamHI-to-EcoRI fragment from the poly linker of pLBN 19A was removed to delete the SstI site in the vector (upstream of the UL42 gene). Then the 389bp SstI fragment within the UL42 gene was removed. Since removal of the SstI fragment placed the C-terminal region out of frame, a BamHI linker was introduced at the unique SstI site to create the in-frame d202 – 337 mutation.

Nested deletions within the UL42 open reading frame were created at unique restriction sites by digestion with exonuclease III (United States Biochemical, Cleveland, Ohio), removal of single-stranded ends with mung bean nuclease, and ligation with T4 DNA ligase. Deletion d140 was generated from the NdeI site of pLBN 19A. The d137 – 142 mutant was obtained by cleavage of the wild-type plasmid with NdeI, digestion with exonuclease III (0.5 units/µg DNA) for 10 min, and treatment with mung bean nuclease.
(0.37 units/μg DNA). The resulting linear DNA was religated, yielding a sequence encoding a UL42 protein deleted for amino acid residues 137 – 142 and containing an insertion of an alanine residue at position 137. The d274 – 288 mutant was obtained by exonuclease III digestion from the unique PstI site of a cloned 398bp SstI fragment corresponding to sequences encoding UL42 amino acids 202 – 337. Following religation, the mutated SstI fragment was used to replace the wild-type SstI fragment in the parental plasmid pLBN 19A. Mutations d241 – 261, d256 – 282, d282 – 283 were created by digestion of the UL42 SstI fragment with exonuclease III for various periods of time, removal of the 5' overhang with T4 DNA polymerase, and blunt-end ligation of a 12-mer BamHI linker. The resulting product was cloned into a pLBN 19A derivative lacking the SstI fragment, producing plasmids which encode UL42 proteins deleted for the indicated amino acid residues.

The point mutations R134D and T142A were constructed using an overlap extension polymerase chain reaction (PCR) method (Ho et al., 1989). Briefly, two pairs of primers were used to direct the synthesis of two DNA fragments with complementary ends possessing the desired mutation, followed by annealing of the two fragments and further PCR amplification prior to cloning. All mutations were confirmed by restriction endonuclease mapping and by DNA sequencing in the region of the mutation.

Plasmids pGEM3-UL9, encoding the wild-type UL9 protein, and UL9 d534-851 (9NT), encoding the N-terminal 533 amino acid residues, were kindly provided by Sumitra Deb (University of Texas Health Sciences Center, San Antonio, TX) and have
been previously described (Deb and Deb, 1991). Mutations in the UL9 gene were cloned into plasmid pGEM3-UL9 by domain swapping as described below.

**a. Cloning of PCR products.** PCR amplification of UL9 and UL9 mutants was performed as described. Products of PCR were electrophoresed through 1% low melting point (LMP) agarose at 90 volts for 90 min. The bands corresponding to the UL9 wild-type or mutant sequences were excised from the gel and purified with Glassmilk (Bio101 Inc., Vista, CA) according to the instructions of the manufacturer. Ten nanograms of each purified DNA was mixed with 50 ng PCR™ II Vector (TA vector; Invitrogen), 1 μl 10X ligation buffer (Invitrogen, San Diego, CA), and 1 μl T4 DNA ligase (10 U/ml, Gibco BRL, Grand Island, NY) in a total volume of 10 μl. Ligations were incubated 18 – 24 hr at 16°C. One microliter of this ligation mixture was used to transform *E. coli* strain DH5α competent cells (GIBCO BRL) as described by the manufacturer. Transformed cells were cultured on Luria broth (LB) agar plates containing 100 μg/ml ampicillin. Immediately before use, the agar plates were spread with 50 μl of a 30 mg/ml stock of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; Gibco BRL) in N-N-dimethyl formamide. Plates were incubated for 18 hr at 37°C and white colonies selected for screening. To verify that the correctly-sized insert had been cloned, DNA was isolated from 3 ml LB containing 100 mg/ml ampicillin using the Wizard™ miniprep DNA purification system (Promega Biotec, Madison WI) according to the instructions of the manufacturer. DNA was digested with EcoRI and electrophoresed through 1% agarose gel to separate the insert from vector sequences.
b. **Cloning of GST/UL9.** Full length UL9 was amplified from KOS infected cell DNA using primers UL9-up and UL9-down (Table 1) as described under "PCR amplification of UL9 DNA". PCR products were purified and sub-cloned into the TA vector as described above. The UL9 gene was subsequently sub-cloned into pGEX-2T (Pharmacia Bitech, Piscataway, NJ) by in-frame ligation of the EcoRI fragment containing the full-length UL9 gene. Upon induction of the lacZ promoter with isopropyl-B-D-thiogalactopyranoside (IPTG; GIBCO BRL), a fusion protein (GST/UL9) is produced in which the GST moiety is fused to the N-terminus of the full-length UL9 protein.

c. **Construction of UL9 mutations.** A restriction digest map of the UL9 gene is shown in Figure 1. Plasmid 9CT, encoding UL9 residues 1-10 linked to residues 534-851, was produced by removal of the 1536-bp BstXI fragment from the N-terminal portion of the UL9 ORF in plasmid pGEM3-UL9, followed by in-frame ligation to the C-terminal portion at the unique BamHI site in the ORF. Plasmid pGEM3-UL9.591 was created by PCR amplification of the UL9 gene from plasmid pGEM3-UL9 with primers UL9-1320 and UL9-2813 (Table 1). Primer UL9-2813 encoded a stop codon at amino acids 592. PCR products were purified and cloned into the TA vector as described above. The TthIII/EcoRI fragment of TA-UL9.591 was sub-cloned by in-frame ligation into TthIII/EcoRI-cleaved pGEM3-UL9, resulting in pGEM3-UL9.591.

UL9 mutation UL9Δ4.591 was generated from baculovirus recombinants. DNA from UL9 recombinant baculovirus was recovered as described under “Virion DNA purification” (see below). For UL9Δ4.591, a region of the recombinant baculovirus DNA
containing the UL9Δ4 deletion was amplified from purified viral DNA using primers UL9-up and UL9-2813 (Table 1; see below). The 1239 bp PCR product was cloned into the TA vector downstream of the T7 RNA polymerase promoter. No further sub-cloning was necessary for expression of UL9Δ4.591. Control in vitro transcription/translation (IVTT) reactions were carried out as described below to verify the predicted protein product.

d. Cloning of UL42 and UL42 mutations into pcDNA3.1+. Wild type UL42 and all UL42 mutations were PCR amplified as described below using PCR65K-8 and PCR65K-9 (Table 1) from the corresponding mutant gene within the pTZ 19U/UL42 plasmid series. PCR products were purified and cloned into the TA vector. Wild type and mutant UL42 gene inserts were removed from the vector by cleavage with EcoRI, and sub-cloned into EcoRI-cleaved, calf-intestinal-alkaline-phosphatase-(CIP)-treated pcDNA3.1+ (Invitrogen). Orientation of the fragment within the host vector was confirmed by cleavage of the pcDNA-UL42 (or UL42 mutation) plasmid with HindIII and XhoI.

PCR Amplification of UL42, lacZ, and UL9.

a. PCR amplification of UL42 and lacZ for detection of recombinant viruses.
Total infected cell DNA was generated as described below and used as a template for PCR amplification of portions of the UL42 and/or lacZ genes. The optimized PCR reaction contained the following: 1X PCR reaction buffer (10mM Tris-HCl, pH 8.4; 50 mM KCl; GIBCO BRL), 1.5 mM MgCl₂, 250 μM of each of four dNTP’s (dATP, dCTP,
dGTP, TTP; Perkin Elmer, Norwalk, CT), 2.5 U Taq DNA polymerase (GIBCO BRL), 200 pmoles of each primer, 5% v/v dimethyl sulfoxide (DMSO), and 0.005-100 ng of DNA. The PCR mixtures were overlaid with 100 µl of mineral oil (Sigma Chemical, St. Louis, MO) in 0.5 ml Gene-Amp™ reaction tubes (Perkin Elmer) and subjected to cycling conditions in a Perkin Elmer Cetus DNA Thermal Cycler (model N8010100). Thermal cycling conditions consisted of 30 cycles of a one-minute denaturation step at 95°C, a 30 second annealing step at 65°C, and a one-minute extension step at 72°C. For amplification of a portion of the UL42 gene, primers UL42-1 and UL42-2 (Table 1) were used. When amplifying the UL42/lacZ junction, primers UL42-1 and lacZ-1 (Table 1) were used.

b. PCR amplification of UL42 for sub-cloning into pcDNA3.1+ and for detection of recombinant virus with insertion mutations. For amplification of the UL42 gene in preparation of cloning into pcDNA3.1+, the pTZ 19U/UL42 plasmid series containing the mutated genes was used for template. Total infected cell DNA was generated and was used as a template for PCR-amplification of the UL42 gene from recombinant virus containing insertion mutations. The optimized PCR conditions were as described above except that they contained 200 pmoles of PCR65K-8 and PCR65K-9 (Table 1). Cycling conditions were as described above except the annealing step was performed at 55°C instead of 65°C.

c. PCR amplification of UL9 and UL9Δ4. For amplification of the wild type UL9 gene, total KOS infected cell DNA was generated as described under "Total infected cell DNA purification" (see below). This DNA was then used as a template for PCR
amplification of the UL9 gene. The optimized PCR reaction contained the following: 1X PCR reaction buffer (10mM Tris-HCl, pH 8.4; 50 mM KCl; GIBCO BRL), 1.0 mM MgCl₂, 250 μM of each of four dNTP's (dATP, dCTP, dGTP, dTTP; Perkin Elmer), 2.5 U Taq DNA polymerase (GIBCO BRL), 200 pmoles of each primer (UL9-up and UL9-down for full-length UL9; UL9-up and UL9-2813 for the deletion mutant; Table 1), 5% v/v dimethyl sulfoxide (DMSO), and 100 ng of DNA. For amplification of the UL9Δ4, recombinant baculovirus DNA was recovered as described under “Purification of Virion DNA from recombinant baculovirus” and was used as template DNA for amplification of the UL9 deletion mutation. The reaction conditions were the same as for wild-type UL9 except 1.5 mM MgCl₂ was used instead of 1.0 mM. Thermal cycling conditions consisted of 30 cycles of a one-minute denaturation step at 95°C, a 30 second annealing step at 64°C, and a 3.5-min extension step at 72°C. The last cycle included a final 10-min extension step at 72°C.

Viral DNA purification.

a. Total infected cell DNA purification. Monolayers of Vero or V9 cells on 60 mm plates were inoculated with virus at approximately 5 PFU/cell. Upon reaching 4+ CPE, cells were harvested by scraping the monolayer into the media. Cells were pelleted in a table-top centrifuge. The supernatant was removed and the cell pellet was resuspended in 200 μl of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄; pH7.4). Total infected cell DNA was purified from these cells using the Easy-DNA™ Kit (Invitrogen) according to the instructions of the manufacturer. The resulting DNA was used as template for PCR.
b. Purification of recombinant baculovirus virion DNA. Sf9 cells were seeded into a 75 cc flask to 70% confluency in 15 ml of TNM-FH complete media. Cells were infected with virus at a multiplicity of infection (moi) of 10 PFU/cell and incubated at 27°C for six days. The cells were harvested and pelleted by centrifugation in a TJ-6 table-top centrifuge (Beckman). The virus was recovered from the supernatant by centrifugation for 1 hr at 12,300 x g using the JA-14 rotor in the Beckman J2-21 centrifuge. Viral pellets were resuspended in 2 ml of TNE (100mM Tris-HCl, pH 8.0; 10mM EDTA, 1M NaCl) and SDS was added to a final concentration of 1 %. After gentle mixing, sodium perchlorate was added to a final concentration of 1.25 M. A single extraction with an equal volume of phenol was followed by three extractions with equal volume of chloroform: isoamyl alcohol (24:1). The resulting aqueous layer was dialized for 4 hr at room temperature against several changes of water. The resulting DNA was used as template for PCR.

Purification of UL42/lacZ virion DNA. Virions were purified from combined cytoplasmic and extracellular fractions as follows. V9 cells were seeded to 70% confluency onto 100-mm tissue culture plates. When the cells were completely confluent (approximately 24 hr later), they were inoculated at a moi of 0.1 (PFU/cell). When cells exhibited full cytopathic effect, they were harvested by scraping into the media and pelleted by low-speed table-top centrifugation. The supernatant, which contained extracellular virus, was collected and placed on ice. The cell pellet was resuspended in RSB (10 mM Tris-HCl, 10 mM NaCl, 3 mM MgCl₂; pH 7.4). After swelling on ice for 10 min, the cells were disrupted with 12 strokes in a Dounce homoginzer (Wheaton) with
a size B pestle. Nuclei were pelleted by low-speed centrifugation and the supernatant containing the cytoplasmic fraction was combined with the supernatant containing the extracellular medium. The combined virus was pelleted by centrifugation for 1 hr at 12,400 x g using the JA-14 rotor in the Beckman J2-21 centrifuge to pellet the virus. Virions were suspended in 10 ml of TNE and further purified by sucrose density gradient centrifugation using a 20-60% (w/w) gradient. The gradient was centrifuged at 10,000 x g for 1 hr in the SW27 rotor of the Beckman L5-75 Ultracentrifuge. The virus band was visualized and collected by fractionation of the gradient. The virus was diluted in TNE and concentrated by centrifugation at 4000 x g for 15 min in the SW27 rotor or the Beckman Ultracentrifuge. The viral pellet was resuspended in a total volume of 9 ml of TNE, and SDS was added to 1%. DNA was prepared as described above for recombinant viruses. DNA was dialyzed against 0.1 x SSC, concentrated with solid polyethylene glycol 6000, and dialyzed against three changes of 0.1 x SSC. DNA was stored in aliquots at -80°C.

Creation of recombinant viruses.

Recombinant viruses were obtained by cotransfection of permissive cells with UL42lacZ virion DNA and plasmids containing mutated UL42 genes. Briefly, V9 cells were seeded in 60-mm culture dishes to approximately 80% confluency. After 24 hr, the cells were washed twice in medium lacking serum. The transfection mixture was produced by mixing 5 μg of UL42/lacZ virion DNA with 5 μg of either wild-type UL42 plasmid (pLBN 19A) or the respective plasmid harboring the desired UL42 mutation in a final volume of 100 μl serum-free medium. The DNA solution was then mixed with a
solution containing 15 μl Lipofectase™ (formerly Transfectase™; GIBCO BRL) and 85 μl serum-free medium and was incubated at room temperature for 10 min. The mixture was brought to a final volume of 1 ml with serum-free medium and added to each washed cell monolayer. Cells were incubated in a humidified CO₂ (5%) incubator at 37°C for 1 hr after which an additional 1 ml of serum-free medium was added. Two hours later, the mixture was removed and the cells were washed once with serum-free medium. Medium containing 5% serum was added to the monolayers and the cells were incubated in a humidified CO₂ (5%) incubator at 34°C. When cells exhibited 50% cytopathic effect (approximately 2 – 3 days post-transfection), they were harvested in media, disrupted by sonication, and stored at -80°C.

Progeny virus was titrated in permissive (V9) and nonpermissive (Vero) cells by plaque assay. Resulting plaques were stained with neutral red solution and X-gal (6 mg/ml plate). The following day, clear plaques were amplified in Vero cells seeded in culture tubes and plaque purified 2 additional times. From these plaque-purified isolates, viral stocks were produced as described above.

Complementation analysis.

BHK cells in 60-mm culture dishes which were approximately 80% confluent were transfected 24 hr after seeding. Cells were transfected with Lipofectase using 2 μg of plasmid DNA for every plate of cells, as described above. At 29 hr post-transfection, cells were infected with CgalΔ42 (2.5 PFU/cell). After virus adsorption for 1 hr at 37°C, 5 ml of medium containing 5% fetal bovine serum was added and the cells were
incubated for an additional hour at 37°C. The fluids were removed by aspiration and residual virions were removed with an acid-glycine-saline wash (Cai et al., 1988) followed by two washes in TBS (137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 5.6 mM dextrose, 24.8 mM Trizma base, 0.5 mM MgCl₂, and 0.9 mM CaCl₂). Medium containing 5% serum was added and the cells were incubated at 34°C until 18 hr p.i. The cells and medium were harvested together and disrupted by sonication.

The yield of progeny was determined by plaque assay in V9 and Vero cells. The complementation index (CI) was calculated as the yield of virus (as titered in V9 cells) from cells transfected with the test plasmid, divided by the yield from cells transfected with the wild-type UL42 plasmid (pLBN 19A) times 100. Complementation was scored as (++) if the CI was >25, (±) if the CI was between 1 and 25, and (-) if it was <1. The percentage of recombinants in the samples was determined by the ratio of plaques observed by titration on Vero cells compared to that observed by titration on V9 cells. We observed <0.240% recombinants/test plasmid

**Preparation of proteins by *in vitro* translation.**

Proteins were expressed by coupled IVTT in rabbit reticulocyte lysates (RRL) using the TnT™ Kit (Promega) according to the instructions of the manufacturer. Plasmids TA-UL9 and TA-UL9Δ4.591 were transcribed using T7 RNA polymerase, while plasmids encoding the wild-type, 9NT, 9CT, and UL9.591 were transcribed using SP6 RNA polymerase.
Preparation of GST fusion proteins.

The plasmid encoding the GST fusion with UL42 amino acid residues 20-456 (GST/42) was created by cloning the MluI fragment within the UL42 ORF of HSV-1 strain KOS, flanked with BamHI linkers (New England Biolabs), into the BamHI site of plasmid pGEX-2T, which encodes GST (Pharmacia). Plasmids encoding GST or GST fusion proteins were propagated in JM 109 or DH5α cells at 37°C in LB containing 50 μg ampicillin/ml. Protein expression was induced following addition of IPTG (0.1 mM) for 3-12 hr, depending on the optimum time of expression for each of the fusion proteins. Extracts were prepared by suspension of the bacterial cell pellets in TED buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg/ml each of aprotinin, leupeptin, and pepstatin and 10 μg/ml β-lactalbumin, followed by the addition of lysozyme to 20 μg/ml and Triton X-100 to 0.1%. The lysates were sonicated to reduce viscosity and centrifuged at 10,000 g for 15 min at 4°C. GST or GST fusion proteins were collected in the supernatant. The amount of native GST or fusion protein in each lysate was determined spectrophotometrically using the transferase-mediated reaction between reduced glutathione and 1-chloro-2,4-dinitrobenzene (CDNB) according to the directions of the manufacturer (Pharmacia).

Affinity chromatography.

Glutathione-agarose beads (Sigma) were charged with various concentrations of GST or fusion proteins overnight at 4°C in TNB buffer (TED containing 0.1 M NaCl and
50 mg/ml bovine serum albumin). The beads were subjected to low-speed centrifugation and washed four times with 10 vol of the same buffer. In vitro-expressed proteins were mixed with beads charged with GST or GST/42 for 3 hr at 4°C. The suspension was loaded into disposable pipette tips plugged with glass wool and washed with 4 column vol of TNB. The GST and GST/42, together with any proteins which bound to them, were eluted from the columns by the addition of 5 mM glutathione. The first three elutions collected were lyophilized, suspended in dissociation buffer, analyzed by SDS-PAGE, and fluorographed as described previously (Monahan et al., 1993). Radioactivity in selected bands was quantified using a PhosphorImager (Molecular Dymanics, Sunnyvale, CA) and analyzed using ImageQuant software.

**Antibodies and Immunoblotting.**

The UL9-specific antiserum, RH7, provided by Robert Hamatake and Daniel Tenney (Brystol-Myers Squibb, Wallingford, CT), was prepared in rabbits using a glutathione S-transferase (GST) fusion with residues 531 to 851 of the UL9 protein. The UL42-specific antipeptide serum, 834, was prepared in rabbits against residues 360 to 377 of the UL42 protein linked to a promiscuous T-cell epitope of measles virus as previously described (Monahan et al., 1993).

For immunoblotting, samples were subjected to SDS-PAGE analysis followed by electrophoretic transfer to nitrocellulose for 6 hr at 450 mAmps as previously described (Towbin et al., 1979). Nitrocellulose filters were blocked in TBS containing 3% gelatin and 0.02% sodium azide, washed with TBS, and probed with the appropriate antibody which had been diluted in TBS containing 1% gelatin. After washing, the blot was
probed with 3 µCi [$^{125}$I] *Staphylococcus aureus* protein A (specific activity 60 mCi/mg; ICN Biochemicals, Inc., Irvine, CA) in TBS containing 1% gelatin. The blot was then exposed to Kodak X-OMAT film at -80°C.

**Indirect Immunofluorescence**

Vero cells were seeded to 60% confluency on 4-well chamber slides. Cells were transfected with 2 µg of plasmid as indicated above. 24 hr post-transfection, cells were fixed with 3.7% formaldehyde at room temperature. Cells were washed with PBS and permeabilized with acetone for 2 min. UL42-directed antipeptide antibody, 834, was adsorbed to the cells in a humidified chamber at 37°C for 1 hr followed by incubation with FITC-conjugated goat anti-rabbit IgG antibody. Immunofluorescence was detected using the Zeiss Photomicroscope III.

**Direct yield of progeny assay**

Equivalent numbers of Vero cells were seeded into 60-mm culture dishes, inoculated with KOS, UL42lacZ, i140/UL42, or i206/UL42 at various multiplicity of infection (MOI) of 0.001, 0.01, 0.1, 1, and 10 PFU/cell, and incubated at 37°C. At 18 h postadsorption (p.a.), cells were scraped into the medium and disrupted by sonication and total virus yield was determined by plaque assay in Vero cells at 34°C.

**One-step growth curve**

Equivalent numbers of Vero cells were seeded into 35-mm culture dishes, inoculated with KOS, UL42lacZ, i140/UL42, or i206/UL42 at a multiplicity of infection
(MOI) of 0.5 PFU/cell, and incubated at 37°C. At various times postinfection, cells were scraped into the medium and disrupted by sonication and total virus yield was determined by plaque assay in Vero cells at 34°C.

**Quantitative immunoblot**

Vero and V9 cells in 100-mm culture dishes were mock infected or infected as described above at an MOI of 0.5 PFU/cell, harvested 18 h postinfection, and washed with Tris-buffered saline (TBS). Cells were disrupted by sonication in 400 μl of extraction buffer containing 200 mM Tris (pH 7.5), 2 mM ATP, 2 mM MgCl₂, 3 mM dithiothreitol, and 20% glycerol; this was followed by low-speed centrifugation to remove debris. Serial two-fold dilutions of each extract were made in TBS and applied to nitrocellulose by using a vacuum manifold system. The filter was blocked then probed with UL42 antipeptide antibody, 834, and [¹²⁵I]-labeled protein A as previously described (Gallo *et al.*, 1988). The protein content of each sample was determined by the method of Bradford (Bradford, 1976), using bovine serum albumin as a standard.
<table>
<thead>
<tr>
<th>PCR Primer*</th>
<th>Sequence</th>
<th>Gene</th>
<th>Position</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL9-up</td>
<td>CGAAGAATTTCCCCCGGTCATGCCTTTCGT</td>
<td>UL9</td>
<td>268</td>
<td>→</td>
</tr>
<tr>
<td>UL9-down</td>
<td>GCATGAATTCTACAGC GC GCCACAGAGTC</td>
<td>UL9</td>
<td>3368</td>
<td>←</td>
</tr>
<tr>
<td>UL9-1320</td>
<td>GGCTGCGGAAGCGGAATCCACT</td>
<td>UL9</td>
<td>582</td>
<td>←</td>
</tr>
<tr>
<td>UL9-2813</td>
<td>TTAGGCCATGGGAAACGC GGAGACA</td>
<td>UL9</td>
<td>2076</td>
<td>←</td>
</tr>
<tr>
<td>PCR65K-8</td>
<td>CCGGGATGGTGCGAGAGT</td>
<td>UL42</td>
<td>1510</td>
<td>←</td>
</tr>
<tr>
<td>PCR65K-9</td>
<td>CCAGCCCGCGTTGGGATGAC</td>
<td>UL42</td>
<td>-15</td>
<td>→</td>
</tr>
<tr>
<td>UL42-1</td>
<td>AACGCGACC CCGGGCGGACT</td>
<td>UL42</td>
<td>567</td>
<td>→</td>
</tr>
<tr>
<td>UL42-2</td>
<td>CGGGGGT CGTGAGGAAGAAGA ACT</td>
<td>UL42</td>
<td>885</td>
<td>←</td>
</tr>
<tr>
<td>LACZ-1</td>
<td>CTCAACAGCAGCACCACCATCACC</td>
<td>LacZ</td>
<td>1175</td>
<td>←</td>
</tr>
</tbody>
</table>

*aName of primer used for polymerase chain reaction (PCR) amplification

bSequence of primer used for PCR amplification in 5' to 3' direction

cGene to which the primer is complementary

dPosition of primer in DNA sequence. For UL9, 0 bp is as indicated in Figure 1. For UL42, 0 bp is located at the transcription start site. For LacZ, 0 bp is at the transcription start site for the UL42/LacZ fusion gene of UL42lacZ virus.

eOrientation of the primer with respect to UL9 or UL42 coding sequences.

**Table 1. Names and sequences of primers used for PCR**
Figure 1. **Restriction digest map of the UL9 gene.** The top line indicates the size in kbp of the region studied. The second line displays the positions of relevant restriction enzyme cleavage sites used for cleavage within the ORF and for cloning (B, BstY I). The large arrow below depicts the sequences corresponding to the full-length UL9 ORF (2553 bp), encoding the WT protein of 851 amino acids.
CHAPTER 3

ABILITY OF UL42 TO PROVIDE REPLICATION FUNCTION IN TRANS

The functions and activities specified by UL42 during viral infection have not been completely established. However, some of the in vitro properties of UL42 are known to include (i) the ability to bind DNA (Marsden et al., 1987; Gallo et al., 1988), (ii) the ability to form a complex with HSV DNA polymerase (pol; Crute and Lehman, 1989; Gallo et al., 1988; Digard and Coen, 1990), and (iii) the ability to stimulate HSV pol activity (Gallo et al., 1989; Gottlieb et al., 1990; Hernandez and Lehman, 1990; Tenney et al., 1993). In vitro studies utilizing mutant UL42 proteins synthesized in reticulocyte lysates demonstrated that all known biochemical activities of UL42 are contained within the first 315 residues of the protein (Tenney et al., 1993). Gao and colleagues (1993) constructed a UL42 mutant virus, n338, which encodes the first 338 residues of the protein. Phenotypic analysis of n338 revealed no apparent differences to the wild-type virus with regard to nuclear localization of UL42, DNA replication, and growth in Vero cells. These results demonstrated that the first 338 residues of UL42 retain all of the activities necessary for viral DNA replication and growth in lytic infection of permissive tissue culture cells (Gao et al., 1993). To further identify regions of UL42 required in vivo, I employed an in vivo complementation assay in which the
ability of UL42 mutant proteins to complement the replication of CgalΔ42, a UL42 null virus, in nonpermissive cells was examined (Fig. 2). In this assay, plasmids containing well-characterized lesions in the UL42 gene were transfected by liposome-mediated transfection into BHK cells. BHK cells are more efficiently transfected than Vero cells, but are equally nonpermissive for productive replication of CgalΔ42 (Johnson et al., 1991). Twenty-nine hours after transfection, cells were infected with CgalΔ42 at a multiplicity of infection (MOI) sufficient to ensure the delivery of 1 infectious virus particle to 100% of the cells (2.5 PFU/cell). The yield of progeny virus present in the combined intracellular and extracellular fractions following a single cycle of replication (18 hr) was determined by plaque assay in V9 cells, which provide UL42 protein in trans (Johnson et al., 1991). For yields to reflect true complementation rather than replication of viral recombinants, the yield of progeny was also determined on nonpermissive Vero cells.

In initial experiments, only a 10-fold higher virus yield was observed when cells were transfected with wild-type UL42 plasmid compared to that in cells transfected with control phagemid vector (Table 2; compare average titer on V9 of WT and pTZ 19U, respectively). The high background observed in this experiment likely reflected residual viral particles which remained on the outside of the cells following several washes of the cell monolayer with media and/or a low transfection efficiency. As a result, the complementation analysis was not sufficiently sensitive.

To reduce the background, residual viral particles were removed from the cell monolayers using an acid-glycine-saline wash (Cai et al., 1988) followed by two washes of the cell monolayer with TBS. Using this method, the average yield of progeny
observed in the presence of control phagemid vector pTZ 19U was reduced by at least 10-fold (compare average titer on V9 cells of pTZ 19U in Table 2 and Table 3). By contrast, the presence or absence of the acid-glycine-saline wash had very little effect on the yield of progeny produced upon transfection of the BHK cells with plasmid encoding wild-type UL42 protein (compare average titer in V9 cells of WT in Table 2 and Table 3). Thus, the acid-glycine-saline wash resulted in at least a 100-fold difference in yields of progeny obtained with wild-type UL42 encoding plasmid compared to that with control vector (Table 3; compare average titer on V9 of WT and pTZ19U), and increased sensitivity of the complementation assay.

The ability of plasmids containing well-characterized lesions in the UL42 protein were examined for their ability to complement the replication of CgalΔ42 in nonpermissive cells. The abilities of mutant UL42 plasmids to complement CgalΔ42 fell into three groups – those which complemented at a level indistinguishable from the wild-type plasmid (+), those which produced less than 25% of the yield produced by the wild-type plasmid (±), and those which yielded no significant virus above that detected in cells transfected with the control phagemid vector or in cells which were not transfected (–) (Table 4). A diagram showing the various deletion, insertion, and point mutations of the UL42 protein tested in this system is shown in Figure 3.

The UL42 mutant plasmids i459, d140, R134D, T142A, and d270, all effectively complemented CgalΔ42 virus replication in BHK cells (Table 4). UL42 mutant plasmids d37 – 282, d129 – 163, d137 – 142, N206, d202 – 337, d241 – 261, d274 – 288 and d256 – 282 all failed to complement the null virus (Table 4). Interestingly, only low level complementation (9 – 23%) was observed by UL42 mutant plasmids i140, i206, and
d282 – 283. Inconsistency in the ability of N459 to complement the replication of CgalΔ42 was observed, and these low levels were consistently observed in numerous experiments. Taken together, these data indicate the importance of residues previously defined as Region I (129 – 163) and Region II (202 – 337) in the function of UL42 in the infected cell (Monahan et al., 1993). A summary of the complementation results is shown in Figure 3.

It was conceivable that the low, but reproducible, number of progeny virions observed when plasmids i140, i206, and d282 – 283 were transfected was, in fact, due to recombination of the superinfecting virus with the endogenous UL42 sequences in the V9 cells to yield wild-type virus. Although a few recombinants capable of replicating in nonpermissive Vero cells were observed, such recombinants represented less than 0.240% of the total progeny (data not shown; Reddig et al., 1994). Therefore, the presence of recombinant wild-type virus was unlikely to account for the level of the complementation observed.

It was also possible that negative or low-level complementation of the replication of the UL42 null mutant by some of the plasmid constructs was due to lack of or inefficient availability of the mutant protein in nuclei. Therefore, localization of mutant proteins was determined by indirect immunofluorescence using the UL42 antipeptide antibody 834. This antibody has been previously described in detail elsewhere (Monahan et al., 1993). As previously described (Goodrich et al., 1989), the wild-type UL42 protein expressed by the wild-type plasmid, pLBN 19A, localized exclusively to nuclei of Vero cells transfected with the plasmid, even in the absence of viral infection (Figure 3). In fact, it was observed that all of the plasmids expressed a UL42 protein that localized
exclusively to nuclei, indicating that they each contained a functional nuclear localization signal. Thus poor or negative complementation was not due to lack of availability of the UL42 protein in nuclei.

In order to investigate whether poor complementation might reflect poor expression of the mutant UL42 proteins, the wild type and mutated genes were cloned downstream of the strong constitutive human cytomegalovirus (HCMV) immediate-early promoter. Nevertheless, the i140 and i206 proteins expressed from the HCMV promoter complemented CgalΔ42 to the same extent as those plasmids containing the endogenous UL42 viral promoter (Table 5). A Western blot was performed to compare expression levels driven by the authentic UL42 promoter and the HCMV promoter under the conditions of the in vivo complementation assay (Figure 4). Although such analysis is only semi-quantitative, comparable levels of protein appeared to be expressed regardless of the promoter used. These results indicate that under the conditions used in the in vivo complementation assay, equivalent expression of UL42 by either promoter was obtained. However, it is likely the level of UL42 present in transfected cells is significantly less than that present in cells infected with wild-type virus.

In this study, the in vivo complementation assay was used to determine the ability of various UL42 deletion, insertion, and point mutants to complement the growth of UL42 null virus, CgalΔ42. The plasmids tested by in vivo complementation contained well-characterized lesions in the UL42 protein. These mutations previously were tested in vitro for their ability to bind dsDNA and pol, and to stimulate HSV pol activity. Briefly, affinity chromatography with glutathione-S-transferase (GST) fusion proteins of wild type and mutant UL42 was used to measure the degree of interaction of the fusion
proteins with double-stranded DNA and pol. As a measure of UL42 mutant proteins to provide pol accessory function, the ability of the fusion proteins to stimulate the activity of pol in high salt buffer using activated calf thymus DNA as template was tested. In addition, the abilities of the mutant UL42 proteins to amplify ori-containing plasmids in vivo when the other DNA replication proteins were supplied by superinfection with the UL42 null mutant CgalΔ42 was observed. Although performed in vivo, the latter experiment served only to test the ability of mutant UL42 proteins to support ori-dependent DNA replication, not productive viral replication as in the in vivo complementation assay. A summary of the in vitro and in vivo activities of each of the mutant UL42 proteins is found in Figure 30 (Chapter 6).

Generally, concordance was observed between the in vitro ability of UL42 to bind pol and its ability to complement the null virus replication. UL42 mutants d129 – 163, d137 – 142, d202 – 337, d256 – 282, and d274 – 282 failed both in their ability to bind pol in vitro and to complement the null virus. In addition, those UL42 mutants capable of binding pol in vitro to any extent, d140, R134D, T142A, i206, N459, and i459 were able to, at least partially, complement replication of the null virus. Taken together, these data suggest a strong correlation between the ability of UL42 to bind pol and its ability to function in vivo.

Discordance was observed between the in vitro and in vivo abilities of UL42 mutant d241 – 261. In vitro, this mutant displayed wild-type abilities to bind ds DNA, bind pol, and stimulate pol. However, mutant d241 – 261 was completely unable to complement growth of the null virus and allowed only a small amount of ori-dependent DNA replication in vivo compared to wild-type UL42. Digard et al (1993) found UL42
deletion mutant d242 – 250 retained wild-type ability to bind ds DNA and pol, and stimulate pol activity, although its ability to function in vivo was not tested. In addition, Hamatake et al (1993) demonstrated that trypsin digestion of UL42 in the presence of DNA generates two protease-resistant fragments: one comprised of amino acids 1 to 245 and one starting at residue 255. A complex of the two protease-resistant fragments was able to bind pol as well as stimulate its activity. Similar to our in vitro data for d241 – 261, these data suggest that regions of UL42 around residues 241 to 261 are dispensable for physical and functional interaction with pol in vitro. These data suggest that three regions within UL42 are required for activity in vitro: residues 137 – 142, 202 – 241, and 261 - 288. In contrast, the ability of UL42 to function in vivo appears to require sequences between residues 241 and 261 as deletion of these residues abrogates its ability to complement viral growth of the null mutant. It is possible that these sequences are involved in protein-protein interactions, such as to UL9 or other viral or host proteins, or in functions of UL42 as yet undetected.

Another mutant displaying discordance between the in vitro and in vivo activities was i206, an insertion mutant containing residues RGSA at codon 206. This mutant exhibited wild-type ability to bind pol, bound ds DNA but with an altered elution profile, and was able to stimulate pol, although not to wild-type levels. Despite these abilities, mutant i206 was only able to partially complement growth of the null virus (< 15%). Interestingly, Chow and Coen (1995) also described creation of an insertion mutant I206, in which the four amino acid sequence CIDA was inserted at codon 206. This mutant protein displayed a reduced affinity for ds DNA and a greatly reduced ability to stimulate pol, not unlike our i206 mutant. In addition, similar to our i206 mutant, their I206 mutant
retained wild-type ability to bind pol. However, i206 was unable to support viral replication in a transient-complementation assay despite proper nuclear localization. This is similar to our i206 mutant that very weakly complemented the null mutant. Using the Robson-Garnier secondary-structure prediction algorithm (Garnier et al., 1978), these authors proposed that residues 206 to 209 lie in a flexible region of the UL42 protein and depending upon the amino acid residues inserted, the flexibility of the region was effected. The reduced or complete lack of flexibility could then manifest in the ability of UL42 to function \textit{in vitro} and \textit{in vivo}. Perhaps the residual flexibility of our i206 protein, in the presence of other viral proteins in an infected cell, is capable of assuming a conformation which allows limited ability to function as a pol processivity factor.

Discordance between the \textit{in vitro} and \textit{in vivo} abilities was also observed for UL42 insertion mutant, i140, and deletion mutant d282 - 283. Interestingly, these mutant proteins lacked the ability to bind ds DNA and pol, and lacked the ability to stimulate pol \textit{in vitro}. However, i140 and d282 - 283 demonstrated limited ability to complement growth of the null mutant (~11\% and 23\%, respectively). Perhaps, \textit{in vivo}, these mutants assume a conformation in the presence of other viral and cellular proteins that they are unable to assume \textit{in vitro}. It is also possible that the amount of UL42 present \textit{in vivo} is able to overcome a threshold level which enables the mutant protein to interact with pol and provide at least partial ability to stimulate pol.

For the most part, the ability of the mutant proteins to support ori-dependent replication \textit{in vivo}, reflected their ability to support growth of the null virus in the \textit{in vivo} complementation assay. Interestingly, though, each of the low complementing plasmids (i140, i206, and d282 - 283) was found to support ori-dependent DNA replication at
close to wild-type levels. These data suggest that the i140, i206, and d282 - 283 mutant proteins function adequately in vivo to replicate an ori-containing plasmid. However, this assay does not measure the quality of the DNA replicated. It is possible that the DNA produced in the ori-dependent replication assay contains a higher number of mutations or gaps in the DNA, neither of which would be detected by Southern blot analysis. Problems with the quality of DNA produced would, therefore, go undetected in the aforementioned assay but would have been manifest in the in vivo complementation assay as the reduced ability to complement replication of the null mutant.

Overall, there is a general concordance between the in vitro and in vivo data suggesting the major essential role of UL42 in the infected cell is that of processivity factor. The ability to bind pol in vitro appears to correlate strongly with the ability of the protein to function in vivo. Discordance was noted only for those which yielded weak or no complementation of CgalΔ42. However, where discordance was observed, differences in conformation of the mutant UL42 proteins in vitro compared to in vivo, differences in the amounts of mutant proteins in vitro compared to in vivo, possible interactions with other viral or host proteins present in vivo but not in vitro, or functions of the UL42 protein other than stimulation of pol activity, cannot be ruled out. Structural information would be of great assistance in understanding how these mutations affect the protein's local architecture and would shed more light on the different phenotypes of the mutants. In addition, recombinant viruses containing these mutations would provide insight into the role of these proteins under natural conditions of virus infection.
Table 2. Complementation of CgalΔ42 in nonpermissive cells (media wash)
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Average titer (PFU/ml)</th>
<th>Complementation index(^d) (n)(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8.00 x 10(^1)</td>
<td>0.3 (2)</td>
</tr>
<tr>
<td>pTZ 19U</td>
<td>2.51 x 10(^2)</td>
<td>0.9 (4)</td>
</tr>
<tr>
<td>pLBN 19A (WT)</td>
<td>2.76 x 10(^3)</td>
<td>100.0 (4)</td>
</tr>
</tbody>
</table>

\(^a\)Cells were washed once with the acid-glycine-saline wash following the 1 h virus adsorption period.

\(^b\)BHK cells were transfected with 2 \(\mu\)g of the indicated plasmid and infected with CgalΔ42 (2.5 PFU/cell) 29 hr later. Cells were washed once with acid-glycine-saline wash. Cells were harvested 18 hr p.i.

\(^c\)Determined by plaque assay in V9 cell monolayers. Titers represent the average of the replicate samples indicated.

\(^d\)\[
\frac{\text{Titer in V9 for test plasmid}}{\text{Titer in V9 for WT plasmid}} \times 100
\]

\(^e\)Sample size

Table 3. Complementation of CgalΔ42 in nonpermissive cells (acid-glycine-saline wash)\(^a\)
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Complementation index(^b) ± s.d.(^c) (n)(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLBN19A</td>
<td>100.0 ± 31.2 (14)</td>
</tr>
<tr>
<td>d37 – 282</td>
<td>0.2 ± 0.1 (6)</td>
</tr>
<tr>
<td>d129 – 163</td>
<td>0.2 ± 0.1 (6)</td>
</tr>
<tr>
<td>d137 – 142</td>
<td>0.4 ± 0.1 (6)</td>
</tr>
<tr>
<td>d140</td>
<td>145.0 ± 25.9 (6)</td>
</tr>
<tr>
<td>i140</td>
<td>11.2 ± 7.7 (12)</td>
</tr>
<tr>
<td>R134D</td>
<td>66.0 ± 12.3 (4)</td>
</tr>
<tr>
<td>T142A</td>
<td>86.0 ± 27.6 (4)</td>
</tr>
<tr>
<td>d202 – 337</td>
<td>0.1 ± 0.1 (12)</td>
</tr>
<tr>
<td>i206</td>
<td>13.0 ± 3.9 (11)</td>
</tr>
<tr>
<td>N206</td>
<td>0.4 ± 0.2 (6)</td>
</tr>
<tr>
<td>d241 – 261</td>
<td>&lt;0.001 (4)</td>
</tr>
<tr>
<td>d256 – 282</td>
<td>&lt;0.001 (4)</td>
</tr>
<tr>
<td>d270</td>
<td>101.5 ± 12.0 (2)</td>
</tr>
<tr>
<td>d274 – 288</td>
<td>0.2 ± 0.1 (6)</td>
</tr>
<tr>
<td>d282 – 283</td>
<td>23.0 ± 5.0 (5)</td>
</tr>
<tr>
<td>N459</td>
<td>37.3 ± 26.9 (8)</td>
</tr>
<tr>
<td>i459</td>
<td>161.6 ± 27.7 (6)</td>
</tr>
<tr>
<td>pTZ19U</td>
<td>0.1 ± 0.3 (14)</td>
</tr>
<tr>
<td>None</td>
<td>0.1 ± 0.1 (9)</td>
</tr>
</tbody>
</table>

\(^a\)BHK cells were transfected with 2 \(\mu\)g of the indicated plasmid and infected with Cgal\(\Delta42\) (2.5 PFU/cell) 29 hr later. Cells were harvested 18 hr p.i.

\(^b\)Virus yields were determined by plaque assay in permissive V9 cells. The complementation index (CI) was calculated as follows:

\[
CI = \frac{\sum Titer \ with \ test \ plasmid_{exp,1} \cdot \frac{Avg. \ titer \ with \ WT_{exp,1}}{n_{exp,1} + n_{exp,2} + \ldots} + \sum Titer \ with \ test \ plasmid_{exp,2} \cdot \frac{Avg. \ titer \ with \ WT_{exp,2}}{n_{exp,1} + n_{exp,2} + \ldots}}{n_{exp,1} + n_{exp,2} + \ldots} \times 100
\]

\(^c\)Standard deviation

\(^d\)Sample size

Table 4. Summary of complementation of Cgal\(\Delta42\) replication in nonpermissive cells
Plasmid$^a$ | Promoter$^b$ | Average titer$^c$ | Complementation index$^d$
--- | --- | --- | ---
PTZ19U | T7 | <10 | <1.0
pLBN19A (wt) | Endog. | 4.55 x 10^4 | 100.0
i140 | Endog. | 5.60 x 10^3 | 12.3
i206 | Endog. | 1.15 x 10^4 | 25.3
pcDNA 3.1+ | HCMV | <10 | <1.0
pcDNA-i140 | HCMV | 5.60 x 10^3 | 12.3
pcDNA-i206 | HCMV | 1.21 x 10^4 | 25.4

$^a$ BHK cells were transfected with 2 μg of the indicated plasmid and infected with CgalΔ42 (2.5 PFU/cell) 29 hr later. Cells were washed once with acid-glycine-saline wash. Cells were harvested 18 hr p.i.

$^b$ Endog. indicates endogenous UL42 promoter. HCMV indicates HCMV immediate early promoter.

$^c$ Determined by plaque assay in V9 cell monolayers. Two replicate samples were conducted for each test sample.Titers represent the average of the replicate samples.

$^d$ \[
\left(\frac{\text{Titer in V9 for test plasmid}}{\text{Titer in V9 for WT plasmid}}\right) \times 100
\]

Table 5. Complementation of CgalΔ42 replication in nonpermissive cells (endogenous vs HCMV promoter)
Figure 2. **Flow diagram of in vivo complementation assay.** BHK cells were transfected with plasmids encoding wild type or mutant UL42. After cells were allowed to recover, the cells were infected with CgalΔ42 at 2.5 PFU/cell. Progeny virus was harvested 18 hr post infection and assayed on permissive (V9) and nonpermissive (Vero) cells.
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**Figure 3. Summary of complementation and protein localization of mutant UL42 polypeptides.** Dashed lines represent deletions within the open reading frame (ORF) of UL42. Arrowheads below the ORF mark deletion of single amino acid residues at the indicated position. Arrowheads above the ORF denote in-frame insertion, at the indicated positions. “X” on the ORF indicate locations of point mutations. (+) indicates complementation at a level indistinguishable from wild-type plasmid. (±) indicates those which produced less than 25% of the yield produced by the wild-type plasmid. (-) indicates those which yielded no significant progeny virus. N, nuclear. ND, not done.
Figure 4. Western blot analysis of UL42 expression in transfected cells. BHK cells were transfected with 10 µg of the indicated plasmid. 29 h post-transfection, cells were infected with CgalΔ42 (10 PFU/cell). Cells were harvested 18 h p.i. Protein in the cellular extracts was separated by SDS-PAGE and electroblotted onto nitrocellulose. The filter was then probed with antipeptide antibody to UL42 (834, 1: 400 dilution). Immune complex on the filter was detected by [125I]-protein A. The arrow indicates the position of bands corresponding to UL42 polypeptide.
CREATION, DETECTION, AND ANALYSIS OF i140/UL42 AND i206/UL42 RECOMBINANT VIRUSES

Most of the functional analysis of the UL42 protein has been done in vitro using reticulocyte lysates or baculovirus expressed proteins to measure their abilities to bind DNA, bind pol, and stimulate pol activity in vitro. To assess the abilities of the protein to function in a cellular environment, complementation assays have been performed. Although a relatively effective way to study the in vivo role of UL42, the complementation assay is limited by low transfection efficiencies. The most powerful way to evaluate the true in vivo role of UL42 in the infected cell, and thus its ability to support productive viral replication, is through the use of recombinant viruses.

To date, few recombinant viruses harboring mutations in the UL42 gene have been generated. Gao et al (1993) created the UL42lacZ virus that encoded a fusion protein, containing the N-terminal 206 amino acid residues of UL42 fused to β-galactosidase, expressed under the control of the UL42 promoter. This mutant demonstrated that the first 206 residues of UL42 were insufficient for viral growth since viral replication occurred only in the permissive cell line, a30, which provided UL42 in trans, and not in parental Vero cells.
In view of previous in vitro studies which indicated that all known biochemical activities of UL42 were contained within the first 315 residues of the protein (Tenney et al., 1993), Gao and colleagues (1993) created mutant virus m338. This mutant virus encoded the first 338 residues of UL42. Analysis of the phenotype of this mutant virus revealed no apparent differences, compared to the wild-type virus, in nuclear localization of UL42, DNA replication, or replication abilities in Vero cells. The results indicate the first 338 residues of UL42 retain all of the activities necessary for viral DNA replication and production of infectious progeny virus.

To further investigate the effect of the various deletion, insertion, and point mutations on viral DNA replication and growth, I attempted to create recombinant viruses containing each of the mutations which I had tested by in vivo complementation. To generate these viral mutants, the UL42lacZ virus (a kind gift of Min Gao, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey) was used as a recipient in marker transfer experiments to introduce specifically mutated UL42 alleles into the viral genome.

Virion DNA was purified from cells infected with UL42lacZ virus. The infectious DNA (representing ~15 to 20 PFU) and plasmid containing the desired UL42 mutation were co-introduced into V9 cells by liposomal-mediated transfection. V9 cells provide UL42 in trans and are permissive for replication of UL42 viral mutants. In the cell, homologous recombination can occur between the plasmid and the viral DNA via the overlapping segments of the UL42 gene in each (Fig. 5). The progeny virus, consisting of parental and recombinant HSV-1, were plaqued on V9 cells and putative recombinants were identified by the lack of blue stain with X-gal.
To aid in identifying parental and recombinant genomes, a polymerase-chain-reaction (PCR)-based detection system was developed. To detect the presence of \textit{lacZ} gene sequences, primers were selected such that the amplified product spanned the UL42/\textit{lacZ} junction in the parental UL42\textit{lacZ} virus (Fig. 6). UL42-1 (Primer 1) was located in the UL42 sequences upstream of the inserted \textit{lacZ} gene while LacZ-1 (Primer 2) was located within the \textit{lacZ} gene itself (Fig. 6). Successful amplification of this junction was expected to produce a product of 629 bp, the presence of which indicated that no recombination had occurred in the region and no further study of such viruses was necessary.

The absence of \textit{lacZ} sequences, however, was suggestive of a successful recombination event. To confirm that recombination had taken place, a second set of primers were used. These primers amplified a region within the UL42 gene upstream (primer 1: UL42-1) and downstream (primer 3; UL42-2) of the recombination event (Fig. 6). Amplification of this region of DNA was predicted to produce a product of 319 bp. The absence of \textit{lacZ} sequences and the presence of UL42 sequences suggested a true recombinant virus had been created.

Before this approach was used to detect recombinant viruses, amplification of the UL42 and \textit{lacZ} sequences was first attempted on known DNA samples. Vero and V9 cell monolayers were infected with HSV-1 strain KOS and UL42\textit{lacZ} virus, respectively, and total infected cell DNA was generated. This DNA was then used as template for amplification of the UL42 and \textit{lacZ} sequences using the appropriate primers (as indicated in Figure 6). Figure 7 shows the successful amplification of the UL42 319 bp product.
from KOS infected cell DNA (Figure 7A) and of the lacZ 629 bp product from the UL42-lacZ fusion in UL42lacZ infected cell DNA (Figure 7B).

This PCR approach was used to analyze infected cell DNA from potential viral recombinants. However, when DNA from V9 infected cells was used, we found the PCR procedure was sensitive enough to detect UL42 sequences in the uninfected V9 cells (Figure 8, lane 4). To eliminate background amplification of the UL42 sequences resulting from the UL42 transgene in the V9 cellular DNA, serial 10-fold dilutions of template DNA were used for amplification. However, this did not eliminate the background amplification of UL42 sequences present in V9 cells (Fig. 8). In addition, all potential recombinants positive for UL42 amplification, were also positive for lacZ amplification (data not shown).

A third selection method of viral growth in Vero cells was employed which was expected to sacrifice production of recombinants with lethal mutations in UL42. Since Vero cells were nonpermissive for growth of the parental UL42lacZ virus, only true recombinants would be detected, and these plaques could be screened without interference from endogenous UL42 sequences. Plaques were detected on Vero cells following infection with virus harvested from cells transfected with i140 or i206 plasmid and UL42lacZ. Upon staining with X-gal, clear and light blue plaques were detected in both samples. These plaques were purified twice and infected cell DNA was tested by the PCR approach indicated above. Amplification of the UL42 and lacZ sequences was detected using primers UL42-1, UL42-2, and LacZ-1. Absence of lacZ sequences was detected with the i140 progeny indicating that plaques contained pure recombinant virus (Fig. 9A and B, lanes 7). However, a plaque from the i206 recombination revealed
amplification products for both intact UL42 and UL42/lacZ fusion genes (Fig. 9A and B, lanes 9). These results indicated that further plaque purification of i206/UL42 was necessary. After two additional plaque purification steps in Vero cells, pure recombinant i206/UL42 virus was obtained (Fig. 10C, lanes 4 and 6).

It was possible that recombination between UL42 sequences present in the UL42/lacZ virus and the endogenous UL42 sequences in the stable transfectant V9 cells could have occurred. Such an event would generate wild-type virus rather than one containing the indicated UL42 mutation. To confirm the presence of the UL42 gene, the entire UL42 gene was amplified from the mutant i140 and i206 infected cell DNA using PCR65K-8 and PCR65K-9 (Fig. 10A). Amplification of the full-length UL42 gene would produce a product of 1525 bp. The UL42 gene was successfully amplified from KOS, i140/UL42, and i206/UL42 infected cell DNA indicating the presence of a full-length UL42 gene (Fig. 10B, lanes 4, 7 and 8, respectively). We confirmed the capability of this procedure to amplify the entire UL42/lacZ gene fusion from UL42/lacZ infected cell DNA (Fig. 10B, lane 5). Therefore, these results confirm that a full-length UL42 gene was present in each virus and that there was no contamination with UL42/lacZ parental virus.

Because the insertion mutations in i140 and i206 were generated with in-frame Bam HI linkers, the presence of a Bam HI site in i140 and i206 would confirm transfer of the mutation to the virus. Although Bam HI failed to cleave the KOS product, both the i140 and i206 products were successfully cleaved resulting in fragments of the expected sizes (Figure 11A; Figure 11B, lanes 3 and 6). These results demonstrate that i140 and
i206 were true viral recombinants with the structure in the UL42 gene shown with the mutated plasmid.

It was hypothesized that since both the i140/UL42 and i206/UL42 viruses were isolated from Vero cells, in which a virus with a lethal UL42 mutation could not grow, their phenotypes would resemble that of the wild-type virus. However, a multiplicity dependent defect could possibly be observed. Multiplicity dependent defects have been observed for viruses with lesions in “non-essential” genes such as ICP0 (Stow and Stow, 1986; Sacks and Schaffer, 1987; Everett, 1989; Cai and Schaffer, 1991), ICP22 (Sears et al., 1985), and ICP47 (Longnecker and Roizman, 1986). To determine whether the viruses might display a non-lethal replication deficiency, we measured the yield of progeny as a function of multiplicity of infection (MOI). Vero cell monolayers were infected with KOS, UL42/lacZ, i140/UL42, or i206/UL42 virus multiplicities of infection ranging from 0.001 to 10. The infected cells were harvested 18 hr post-infection, the time required for a single cycle of replication by the wild-type virus. Virus yields were determined by plaque assay in Vero cells. A day of assay control was used to determine the true plaque forming units added to each plate the day of the infection for each virus at each dilution.

Interestingly, the i140/UL42 and i206/UL42 viruses produced less virus at each multiplicity than the wild-type KOS virus (Fig. 12). However, the slopes for each virus in the logarithmic range are nearly parallel suggesting that the yield per infected cell (or the burst size) is independent of the multiplicity of infection. However, the reduced yield of virus at each multiplicity most likely indicates that the UL42 mutations reduced the efficiencies of infectious viral production in some manner. Table 6 shows the average
burst size for wild-type and recombinant virus at 18 h p.i. In the logarithmic range, KOS infection of Vero cells produced approximately 70 progeny virus per infected cell while only 5 and 1 infectious viral particles were produced by i140/UL42 and i206/UL42 virus infected cells, respectively. These data suggest that although a multiplicity effect is not observed, the mutant viruses display reduced burst size compared to wild-type virus.

One possible reason for reduced burst size at 18 h was an extended replication cycle of the recombinants compared to that of wild-type virus. To address this possibility, a one-step growth curve was performed. Vero cells were infected with virus at a relatively low multiplicity (0.5 PFU/cell) and harvested various times post-infection. Virus yields at each time point were determined by plaque assay on Vero cells. Figure 13 demonstrates that both KOS and i140/UL42 display similar replication rates in Vero cells. However, the growth rate of i206/UL42 was slower than that of both KOS and i140/UL42. These data indicate that an extended growth cycle was most likely not responsible for the decreased yield of progeny observed with i140/UL42 although the severe drop in virus at 8 h p.i., a time of rapid DNA synthesis, may indicate a slower onset of DNA synthesis and the possible need to achieve a critical level of some viral product for better viral production. However, the slow rate of production of i206/UL42 progeny may have been partially responsible for the low burst size at 18 h p.i.

Another possible explanation for reduced burst size was a lower level of UL42 expression in the infected cells. If the mutant UL42 proteins were not expressed at an adequate level, the progeny produced from viral infection might be affected. To investigate this possibility, a quantitative immunoblot was used to measure UL42 expression (Fig. 14). Vero cells were infected at a multiplicity of 0.5 PFU/cell. Infected
cells were harvested 18 h p.i. Serial two-fold dilutions of extracts from infected cells were applied to nitrocellulose filters and subsequently probed with UL42 antipeptide antibody 834. The results demonstrate that approximately 8-fold more UL42 protein was expressed in Vero cells infected with i140/UL42 virus compared to that expressed in KOS infected cells (Fig. 14). However, expression of i206 protein in Vero cells was greatly reduced (approximately 8-fold) compared to that observed in either KOS or i140/UL42 infected cells suggesting the reduced expression or instability of the i206 protein may contribute to the reduced progeny observed upon i206/UL42 infection.

It is interesting that more expression of i140 protein than wild-type protein is observed at the same multiplicity. Since multiplicity is based upon infectious virus and not total virus particle count, it is possible that i140/UL42 infection produced an unusually low number of infectious units to total particle count. If this is the case, infection of cells at the same multiplicity would require more total particles when infected with i140/UL42 than with wild-type virus. The increased particles present in an i140/UL2 infection could be sufficient to overwhelm the replication, transcription, and translation machinery of the virus-infected cell, reminiscent of a high multiplicity infection, and therefore, result in a lower yield of progeny. This phenomenon is demonstrated in Table 6. When most likely only one infectious genome is present per infected cell (multiplicities ranging from 0.001 to 1), production of viral progeny has not exceeded the abilities of the viral and cellular machinery and therefore, a similar number of infectious progeny are produced by each infectious center, regardless of the input multiplicity. However, at a multiplicity of 10 PFU/cell, when most likely several infectious genomes are present in an infected cell, virus replication has exceeded the
capabilities of the viral and cellular machinery and they are unable to support viral replication above a maximum threshold level resulting in a reduced burst size per infectious center (Table 6; compare average burst size in the linear range to that observed at a multiplicity of 10).

In this study, I have isolated two recombinant viruses, i140/UL42 and i206/UL42. As previously mentioned, by the methods used, UL42 mutant i140 was incapable of binding ds DNA or pol, or stimulating pol \textit{in vitro}. UL42 mutant i206, however, displayed wild type ability to bind pol, bound ds DNA although with an altered elution profile, and stimulated pol, although not to wild-type levels. However, both mutations, i140 and i206, displayed partial ability to complement growth of the null virus (~11% and 13%, respectively). Despite only partial ability of each mutant to complement growth of the null virus, each recombinant virus was isolated from Vero cells suggesting conformation of UL42 in the infected cell was maintained to support viral replication in a cell line nonpermissive for null viral growth.

It was hypothesized that since both the i140/UL42 and i206/UL42 viruses were isolated from Vero cells, the viruses might display a non-lethal replication deficiency such as that observed with the non-essential genes ICP0, ICP22, and ICP47 (Stow and Stow, 1986; Sacks and Schaffer, 1987; Everett, 1989; Cai and Schaffer, 1991; Sears \textit{et al.}, 1985; Longnecker and Roizman, 1986). However, a direct yield of progeny assay demonstrated that although no multiplicity-dependent effects were observed, both i140/UL42 and i206/UL42 viruses were deficient in the burst size produced at each multiplicity when compared to wild-type levels (4 PFU/cell, 1 PFU/cell, and 70 PFU/cell, respectively).
One possible reason for the reduced burst size observed between wild type and the mutant viruses was a delay in the production of progeny. A one-step growth curve revealed similar growth patterns of wild type and i140/UL42 suggesting an extended growth cycle was not responsible for the difference observed in the yield of progeny. Interestingly, though, initial viral progeny production for wild type virus began at 6 h p.i. while in Vero cells infected with i140/UL42, viral progeny production began at 8 h p.i. This may suggest a slower onset of DNA synthesis in i140/UL42 infected cells compared to wild-type infected cells. Perhaps a critical level of a viral function must be achieved in order to attain better DNA and viral production. A slightly elongated growth cycle was observed for i206/UL42. The elongated rise period suggests a slight delay in the production of progeny genomes important for the production of encapsidated viral progeny, possibly due to low UL42 production in the infected cell and/or to the inability of i206 mutant protein to stimulate pol efficiently. Regardless, an extended growth cycle was not responsible for the reduced burst size observed for either mutant.

It was possible that the reduced burst observed in either case was due to low levels of UL42 in the infected cells. A quantitative immunoblot revealed the level of UL42 present in i140/UL42 infected Vero cells was 4-fold greater than that present in wild-type infected cells. How could more i140 mutant UL42 protein than wild-type protein be expressed when cells were infected at the same multiplicity? It is important to remember that multiplicity of infection is based upon infectious units, not total particle count. For all animal viruses, the number of virus particles in any given preparation exceeds the number of infectious units. It is possible that the phenotype observed for i140/UL42 reflects an unusually high number of total viral particles compared to
infectious particles. Therefore, to achieve the same multiplicity of infection between the 140/UL42 and the wild-type viruses, many more 140/UL42 total particles would be necessary. With the increased total particle count necessary to achieve 140/UL42 infection at a certain multiplicity, compared to the total particle count necessary for wild-type virus, the expression of viral proteins could be enhanced, therefore exceeding the ability of the viral and cellular replication machinery to support viral replication. This could result in an increased level of all viral expression, including UL42 (such as that observed), compared to wild-type virus, at the same multiplicity of infection and at the same time, a reduced burst size. It is possible that this high level of mutant UL42 protein present in the infected Vero cells reflects a threshold amount of UL42 required to permit limited physical and functional interaction between the mutant UL42 and pol. Perhaps no physical or functional interaction was detected *in vitro* because this threshold level was not achieved. Since it is possible that the phenotype of the 140/UL42 virus reflects an unusually high total particle count compared to infectious units, it will be necessary to determine the total particle count compared to infectious particles present in 140/UL42 infected cells.

At least 4-fold less UL42 protein was observed in the 206/UL42 infected Vero cells compared to wild-type levels. This reduced expression could reflect lower UL42 mRNA levels, shorter half-life of the 206/UL42 mRNA, or shorter half-life of the UL42 protein itself. The reduced level of mutant 206 UL42 protein in Vero infected cells may be responsible for the delayed growth cycle and reduced progeny production. It is interesting to note Johnson *et al* (1991) observed wild-type levels of viral DNA synthesis and infectious virions in V9 cells infected with the null virus, CgalΔ42, in the presence of
less than 1% the level of UL42 produced in a wild-type infected cell. These data suggest only small quantities of wild-type UL42 are required for efficient HSV-1 replication. However, the UL42 present in i206/UL42 infected Vero cells is not wild type and may require a higher threshold amount of mutant UL42 protein to efficiently functionally interact with pol. In addition, in vitro studies demonstrated that the i206 mutant protein displays a reduced affinity for DNA as suggested by its altered elution profile. This, too, may manifest itself as a reduced yield of progeny in vivo as processivity of the pol on the DNA template may be affected. Regardless, these studies indicate that both mutant UL42 proteins are being efficiently expressed in the infected cell.

The reduced burst size observed for the recombinant viruses could be due to a problem with the quantity of DNA produced resulting from a reduced yield of total viral DNA due to inefficient stimulation of pol or from an increased threshold level of mutant UL42 protein. Alternatively, the reduced burst size could reflect a problem with the quality of the DNA produced due to a high mutation frequency and low pol fidelity, or from gapped progeny genomes resulting from inefficient pol processivity. It is also possible UL42 plays an additional role in the infected cell in addition to that of processivity factor. To further understand the phenotypes of the i140/UL42 and i206/UL42 viruses, it will be necessary to examine possible deficiencies in total DNA synthesis or synthesis of full length viral DNA, resulting from the mutations introduced into the UL42 protein. This can be done by in vivo labeling of newly synthesized DNA with \(^{3}H\)thymidine and Southern blot analysis of DNase I resistant viral DNA, respectively. The yield problem could also be a reflection of the inability of the mutant proteins to stimulate pol to wild-type levels. This can be resolved by determining the pol
activity present in extracts of cells infected with i140/UL42 and i206/UL42 viruses as described in Reddig et al (1994).
Vero cells were infected at 1, 0.1, 0.01, and 0.001 PFU/cell. Cells were harvested 18 h p.i. Virus yields were determined by plaque assay in Vero cells.

Average burst size \((\text{in the logarithmic range; MOI of 0.001 - 1})\) was calculated as follows:

\[
\frac{\sum_{\text{MOI} = 1} \frac{\text{PFU produced}_{\text{MOI} = 1}}{\text{PFU input}_{\text{MOI} = 1}} + \frac{\text{PFU produced}_{\text{MOI} = 0.1}}{\text{PFU input}_{\text{MOI} = 0.1}} + \ldots}{4}
\]

Standard deviation

Burst size in the non-logarithmic range (multiplicity of 10) was calculated as follows:

\[
\frac{\text{PFU produced}_{\text{MOI} = 10}}{\text{PFU input}_{\text{MOI} = 10}}
\]

Table 6. Burst size of UL42 recombinant viruses at 18 h p.i.
Figure 5. **Creation of recombinant viruses.** Open bars represent the UL42-encoding sequences within the viruses and plasmid. The hatched bar denotes the frame of the *lacZ* gene, encoding β-galatosidase. Crossed lines indicate regions where homologous recombination could take place.
PCR detection of UL42 R' viruses

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Figure 6. Polymerase chain reaction (PCR) detection of recombinant viruses. Arrows indicate locations and directions of the selected primers. Open bars indicate the UL42 sequences. The hatched bar denotes the lacZ ORF. Sizes of predicted amplified products are indicated. 1, UL42-1 primer; 2, LacZ-1 primer; 3, UL42-2 primer.
Figure 7. PCR amplification of UL42 and lacZ sequences. Vero or V9 cells were infected with KOS and UL42/lacZ, respectively. Cells were harvested 24 h p.i. and infected cell DNA was generated. Amplification of products was performed as described under Materials and Methods and analyzed by 1.5 % agarose gel electrophoresis. Arrows indicate the correct products of this amplification. (A) UL42 amplification; (B) LacZ amplification.
Figure 8. PCR amplification of UL42 sequences from V9 cells or potential recombinant viruses. V9 cells were infected with virus and harvested 24 h p.i. Total infected cell DNA was generated. Products were amplified as described under Materials and Methods and analyzed by 1.5% agarose gel electrophoresis. Control V9 cell DNA or infected cell DNA was serially diluted 10-fold and used as template for amplification. Arrow indicates amplification product of the correct size. Lane 1, marker; lane 2, no DNA control; lane 3, pLBN 19A control; lanes 4–6 V9 DNA; lanes 7–9, recombinant 1.1; lanes 10–12, recombinant 1.2; lanes 13–15, recombinant 1.3; lanes 16–18, recombinant 1.4; lanes 19–20, recombinant 1.5.
Figure 9. **PCR detection of UL42 and lacZ sequences from i140/UL42 and i206/UL42 infected cells.** Vero cells were infected with i140/UL42 (A) or i206/UL42 (B) virus. Infected cells were harvested 24 h p.i. and total infected cell DNA was generated. Products were amplified as described under Materials and Methods and analyzed by 1.5% agarose gel electrophoresis. Arrows indicate amplification products of the correct size. (C). i206/UL42 virus was plaque purified a total of 4 times in Vero cells. PCR amplification was performed as described above. Arrows indicate amplification products of the correct size.
Figure 10. **PCR amplification of full length UL42.** (A) Location and direction of primers required for amplification of full length UL42 are indicated by arrows. The open box depicts the open reading frame (ORF) of UL42. Lines indicate sequences flanking the UL42 ORF. (B) Full length UL42 was amplified as described under Materials and Methods from i140/UL42 and i206/UL42 total infected cell DNA. Products were analyzed by 1 % agarose gel electrophoresis. Amplified products are indicated by arrows.
Figure 11. Cleavage of full length UL42 products by Bam HI. (A) Map showing locations of i140 and i206 insertion mutations within the UL42 gene. Cleavage products and their predicted molecular weights are shown. (B) Full length UL42 was amplified as described under Materials and Methods from KOS, i140/UL42 and i206/UL42 total infected cell DNA. PCR products were purified and cleaved with Bam HI. Products were analyzed by 1% agarose gel electrophoresis. Large arrow indicates uncleaved wild-type UL42 gene, dots indicate products resulting from Bam HI cleavage.
Figure 12. Effect of input multiplicity on virus yields. Vero cells were infected with KOS, UL42lacZ, i140/UL42, and i206/UL42 at various multiplicities of infection. Cells were harvested 18 h p.i. and yields of virus were determined by plaque assay on Vero cells. Points have been adjusted for true multiplicity using a day of assay control. Points indicate the average of two replicate samples and error bars represent the range.
Figure 13. Replication rates of KOS, i140/UL42, and i206/UL42 in Vero cells. Cells were infected at an MOI of 0.5 PFU per cell, and the total virus yield (PFU/ml) from each sample at the times indicated was determined by plaque assay in Vero cells. (●) KOS virus; (■) i140/UL42; (▲) i206/UL2.
Figure 14. Quantitative immunoblotting of UL42 production in recombinant viruses. Vero cells were infected with KOS, i140/UL42, i206/UL42, and CgalΔ42 or mock infected as indicated and harvested at 18 h p.i. Dilutions of extracts as indicated were applied to nitrocellulose, and the blot was probed with UL42 antipeptide antibody 834. The most concentrated spot represents one-fourth of each sample. Protein concentrations (milligrams per milliter) of undiluted samples were as follows: (A) KOS, 0.15; i140/UL42, 0.07; i206/UL42, 0.11; CgalΔ42, 0.11; and mock, 0.17.
CHAPTER 5

SPECIFIC PROTEIN-PROTEIN INTERACTION BETWEEN UL42 AND UL9

Using \textit{in vitro} and \textit{in vivo} assays, it has been determined that the main essential role of UL42 in the infected cell is that of a pol accessory protein. However, additional roles of UL42 in the infected cell cannot be ruled out. It is intriguing that UL9 has been shown to physically interact with the helicase/primase complex (McLean \textit{et al.}, 1994) and ICP8 (Boehmer and Lehman, 1993; Boehmer \textit{et al.}, 1994), suggesting a role of UL9 as an origin-directed replisome-assembly mediator.

The HSV-1 UL9 protein binds to functional origins of replication (Elias \textit{et al.}, 1986; Elias and Lehman, 1988; Olivo \textit{et al.}, 1988). In addition, UL9 also functions as a helicase on partially double-stranded templates unwinding in the 3' to 5' direction (Bruckner \textit{et al.}, 1991; Boehmer \textit{et al.}, 1993; Fierer and Challberg, 1992). The UL9 protein is organized into at least two separate functional domains (Fig. 15). The N-terminal 534 amino acid residues contain essential motifs characteristic of helicases (Gorbalenya \textit{et al.}, 1989; Martinez \textit{et al.}, 1992). In addition, this region is required for dimerization of UL9 (Bruckner \textit{et al.}, 1991; Fierer and Challberg, 1992), cooperative binding to the origins of replication (Hazuda \textit{et al.}, 1992; Elias \textit{et al.}, 1992), and specific protein-protein interaction with UL8 (McLean \textit{et al.}, 1994). The C-terminal
317 amino acid residues are required for sequence-specific DNA binding (Weir et al., 1989; Deb and Deb, 1991) and specific protein-protein interaction with ICP8 (Boehmer and Lehman, 1993; Boehmer et al., 1994).

Since it binds the origin, UL9 is likely to form a focal point around which the other proteins required for DNA replication can assemble for initiation of viral DNA synthesis within cells. Although interactions of UL9 with a component of the helicase-primase complex (McLean et al., 1994) as well as with the single-stranded DNA-binding protein ICP8 (Boehmer and Lehman, 1993; Boehmer et al., 1994) have been reported, no interaction has yet been demonstrated between UL9 and a member of the heterodimeric DNA polymerase complex, the remaining viral-encoded component required to form the viral replisome.

The ability of antibodies directed to either UL42 or UL9 protein to coimmunoprecipitate both proteins suggested a direct protein-protein interaction between UL42 and UL9 (Monahan et al., 1998). As an independent means of demonstrating the physical association between UL42 and UL9, affinity chromatography using GST fusion proteins was performed. In order to ascertain whether the UL42 portion of the fusion protein displayed the wild-type conformation of UL42, GST/UL42 was tested for its ability to stimulate the activity of the HSV-1 pol catalytic subunit in high salt using activated calf thymus DNA as template.

GST/UL42 was partially purified by glutathione agarose affinity chromatography as described under Materials and Methods. The pol catalytic subunit was prepared by purification from Sf9 cells infected with a recombinant baculovirus expressing pol. Figure 16 shows that the basal pol activity was increasingly stimulated by addition of
increasing amounts of GST/UL42. Although not shown for this experiment, GST alone failed to stimulate pol activity above the basal level. These results demonstrate that amino acid residues 20 – 456 of the UL42 protein exist in a conformation capable of providing pol accessory function even when covalently linked to the heterologous GST protein moiety, indicating the validity of using GST/UL42 for determining interactions with UL9 by affinity chromatography.

A flow chart demonstrating the steps involved in GST fusion protein affinity chromatography is shown in Figure 17. Briefly, extracts of the GST fusion protein were made from IPTG-induced bacterial cultures. GST fusion protein in the extract was bound to glutathione agarose followed by removal of unbound protein with several buffer washes. In vitro transcribed and translated (IVTT) protein was then incubated with the affinity matrix. After removal of any unbound protein by washing, the fusion protein, along with any bound protein, was eluted with 5 mM glutathione. Products of these elutions were analyzed by SDS-PAGE and signal was quantified using PhosphorImager analysis.

The wild-type UL9 protein was expressed by coupled in vitro transcription/translation of plasmid pGEM-UL9 in the presence of [35S]-methionine and incubated with glutathione agarose charged with 20 μM of either GST or GST/UL42. UL9 bound to the charged beads and eluted with GST/UL42, though very little associated with GST alone (Fig. 18, compare lanes 3 – 5 and 8 - 10). The aberrant mobility of the labeled band present in lane 9 was due to protein overloading at the position of UL9. This was caused by the similarity in apparent molecular weights of UL9 and GST/UL42 (93,600 and 91,000, respectively).
To demonstrate reciprocity of this interaction, a GST/UL9 fusion protein was created. Polymerase chain reaction (PCR) primers were selected to amplify the UL9 gene from KOS infected-cell DNA. Figure 19A demonstrates that PCR amplification produced a product of 3100 bp, the size expected for full-length UL9. The amplified UL9 gene was then introduced into the pCR2.1 TA vector downstream of the T7 RNA polymerase promoter. To insure the PCR product was the UL9 gene and that no nonsense mutations had been introduced during PCR amplification, the protein product of the amplified gene was synthesized by in vitro transcription/translation. Proteins were analyzed by SDS-PAGE and autoradiography (Fig. 19B). A predominant product of 93.6 Kd was produced in agreement with the migration of full-length UL9-expressed by recombinant baculovirus (not shown). The UL9 gene was subsequently cloned into the pGEX-3X GST fusion protein vector. Expression of the GST/UL9 was induced using IPTG and bacterial lysates were made. Expression of the GST/UL9 was confirmed by Coomassie blue staining of SDS-polyacrylamide gels (Fig. 20).

In initial affinity chromatography experiments with GST/UL9, analysis by Coomassie blue staining of the SDS-PAGE indicated a lack of full-length GST/UL9. In addition, a polypeptide of about 26 Kd eluted from the GST/UL9 affinity columns resulting, perhaps, from proteolytic cleavage of the GST/UL9 protein during incubation of the matrix with the bacterial extracts. These data suggested that the GST/UL9 fusion protein might be unstable under the binding conditions used. To confirm stability of the GST/UL9 protein, bacterial extracts containing GST/UL9 were incubated at 4°C for 0, 1, 2, 3, 4, 5, 6, 7, and 24 hr. Western blot analysis of the protein products at the various times during incubation, demonstrated full length GST/UL9 was present at prior to
binding (0 h, Fig. 21). However, full length GST/UL9 was absent from all other time points, suggesting that the GST/UL9 protein was unstable under these assay conditions (Fig. 21). In the absence of stable GST/UL9 fusion protein, subsequent protein-protein interaction and mapping studies were performed using GST/UL42 or various mutant GST/UL42 fusion proteins.

In order to increase sensitivity and to determine the relative portion of in vitro translated UL9 bound to each column, increasing amounts of GST fusion protein were used to charge the glutathione agarose. The amount of radioactivity, and thus IVTT-expressed UL9, present in the polypeptide bands was quantified using a PhosphorImager. Consistent with a direct interaction between UL42 and UL9, the amount of UL9 retained on a GST/UL42 affinity matrix increased as the concentration of GST/UL42 used for immobilization was increased from 200 to 1000 nM (Fig. 22).

These data indicate that UL9 and UL42 are capable of specific and stable physical association. Because UL42 and UL9 both bind to double-stranded DNA and UL9 also binds to single-stranded DNA (Elias et al., 1986; Gallo et al., 1988; Olivo et al., 1988; Gottlieb and Challberg, 1994; Abbots and Stow, 1995), it was possible that the association between UL42 and UL9 was mediated by mutual DNA binding. The addition of ethidium bromide (EtBr) has been shown to severely reduce the association of DNA-binding proteins with DNA, thereby allowing genuine protein-protein interactions to be distinguished from protein-DNA interactions on common DNA fragments (Lai and Herr, 1992). That the physical association of UL42 and UL9 was not dependent upon the presence of DNA was demonstrated by lack of effect of EtBr on coimmunoprecipitation
of UL42 and UL9 (Monahan et al., 1998). Therefore, all buffers for GST affinity chromatography included EtBr at a concentration of 100 μg/ml.

To map the regions of UL42 required for interaction with UL9, a series of mutant GST/UL42 fusion proteins was used for affinity chromatography. A diagram of the UL42 mutant proteins that were generated as GST fusion proteins and used for affinity matrix in GST affinity chromatography is shown in Fig. 31 (Chapter 6).

Glutathione agarose was charged with 1000 and 2000 nM of GST fusion protein. In vitro transcribed and translated UL9 protein was incubated with each affinity matrix, and fusion protein, along with any bound UL9 protein, was eluted from the matrix with 5 mM glutathione. Eluted proteins were analyzed by SDS-PAGE, autoradiography (Figure 23), and the amount of radioactivity present in the polypeptide bands was quantified using a phosphorimager (Table 7). UL9 protein was retained by all mutant UL42 fusion proteins tested (Figure 23 and Table 7). In addition, with increasing amounts of wild-type or mutant UL42 fusion protein used to charge the affinity matrix, increasing amounts of UL9 were retained on the column (data not shown). These data suggest the region of UL42 required for interaction with UL9 maps to residues 20 – 129, 164 – 202, or to multiple domains within UL42.

To examine which UL9 domain was responsible for complex formation with UL42, full length UL9 containing 851 amino acids (WT), the N-terminal residues 1 to 533 (9NT), and residues 1 – 10 linked to C-terminal residues 534 – 851 (9CT; Fig. 24), were expressed by in vitro coupled transcription/translation of plasmid DNA in the presence of [35S]-methionine. The first 10 N-terminal residues were added to the C-terminal portion in order to retain the same upstream sequence and translational
regulatory sequences, permitting approximately the same translational efficiencies for all plasmids (results not shown). Both the N- and C-terminal portions of UL9 used have been shown to form functional subdomains, capable of binding UL8 protein and ICP8 protein, respectively (Deb and Deb, 1991; McLean et al., 1994; Boehmer and Lehman, 1993; Boehmer et al., 1994).

Products of the translation were incubated with beads to which 2 μM of either GST or GST/UL42 were immobilized, as described above. The N-terminal, but not the C-terminal polypeptide, retained the ability to bind GST/UL42, while neither bound efficiently to the control GST columns (Fig 25). The results of this experiment indicate that the N-terminal polypeptide bound to GST/UL42 less efficiently than did the full-length UL9 while little, if any, 9CT was retained (Figure 25, compare lanes 10 and 11 with lanes 4 and 5).

To better determine the relative affinities of the wild-type and N-terminal portion of UL9 for UL42, binding of the polypeptides to glutathione agarose beads charged with increasing concentrations of GST or GST/UL42 was measured. Once charged, the beads were subdivided; incubated with the WT UL9, 9NT, or 9CT proteins; and washed. The proteins that eluted in 5 mM glutathione were then quantified. Figure 26 demonstrates that the 9NT protein bound four- to six-fold less efficiently than does the full-length UL9 when beads were charged with GST/UL42 at concentrations ranging from 200 to 2000 nM. The decreased binding of both WT UL9 and 9NT at high GST/UL42 concentrations was often observed and may be the result of crowding of immobilized GST/UL42 molecules, thus reducing their accessibility. The 9CT protein did not bind significantly to GST/UL42 even at the highest charging concentration used. However, the reduced
affinity of the 9NT protein suggested that at least a portion of the C-terminal residues facilitate or stabilize binding of UL9 to UL42.

UL9 has been shown to interact with ICP8 (Boehmer and Lehman, 1993; Boehmer et al., 1994) and UL8 (McLean et al., 1994). The C-terminal 317 amino acid residues of UL9 have been shown to be required for this interaction (Boehmer and Lehman, 1993; Boehmer et al., 1994). In contrast, the interaction of UL9 with UL8 requires the N-terminal 534 residues (McLean et al., 1994). More specifically, residues 132 to 210 have been shown to be essential for this interaction (N. Stow, personal comm.). Because the 9CT protein did not bind to GST/UL42, these results demonstrated that separate regions of UL9 are required for interaction with UL42 and ICP8. However, as both UL8 and UL42 bind to the N-terminal 534 residues, it was necessary to determine whether the regions of UL9 required for these interactions were separable.

To determine whether inclusion of C-terminal residues would increase the binding of 9NT, a C-terminal truncation encoding the first 591 amino acid residues was generated using PCR (UL9.591; Fig. 24). Extension to this site was chosen as residues 570 to 591 are predicted to form a pseudo-leucine zipper (Deb and Deb, 1991). Expression of a protein of the expected size (65 kDa) was demonstrated by in vitro transcription/translation of pGEM-UL9.591 plasmid (Fig. 27, lane 3). In addition, it was necessary to determine whether the region of UL9 required for interaction with UL8 was unique to that required for interaction with UL42. To this end, an N-terminal deletion mutant was created. Using PCR, the N-terminal 591 amino acids of the UL9 gene containing a deletion of sequences encoding amino acid residues 132 to 210 was amplified from purified baculovirus recombinant DNA harboring the same deletion
mutation (baculovirus recombinant kindly provided by N. Stow). The amplified fragment was introduced into the pCR2.1 downstream of the T7 bacteriophage RNA polymerase promoter creating TA-UL9ΔA.4.491 (Fig. 24). Expression of the expected 45 kDa protein was determined using in vitro transcription/translation followed by SDS-PAGE analysis and autoradiography (Fig. 27, lane 5).

To determine whether the additional 56 amino acid residues would increase the relative affinity of the 9NT protein for UL42 and if the UL8 and UL42 binding sites on UL9 were unique, binding of the polypeptides was measured to glutathione agarose beads charged with increasing concentrations of GST or GST/UL42, as indicated above. Figure 28 confirms that the 9NT protein bound less efficiently than the full-length UL9 when beads were charged with GST/UL42 at concentrations ranging from 200 to 2000 nM. Addition of 56 residues to the 9NT protein, restored its ability to bind to GST/UL42 although with some deficiency in binding compared to the full-length. In addition, UL9A4.591 was retained on the GST/UL42 column, and displayed the same relative affinity for GST/UL42 as the intact UL9.591 (Fig. 28). These data suggest that addition of 56 residues to 9NT restores additional binding capability to GST/UL42, although not to wild-type levels. In addition, these data suggest that regions of UL9 required for interaction with UL8 are unique from those required for interaction with UL42. Deletion mutations N-terminal and C-terminal to this region will be necessary to identify the particular region of UL9 required for interaction with UL42. A summary of wild-type and mutant UL9 binding to GST/UL42 is shown in Figure 29.

UL9 has been shown to specifically interact with ICP8 (Boehmer and Lehman, 1993; Boehmer et al., 1994) and with UL8 (McLean et al., 1994). And, for the first time,
a specific protein-protein interaction has been demonstrated between UL9 and the pol processivity factor, UL42 (this report; Monahan et al., 1998). The interaction was determined to be specific and independent of DNA.

The region of UL42 required for interaction with UL9 most likely does not exist as a linear domain in UL42 as determined by the ability of all of the GST/UL42 mutants tested to bind UL9. This, however, does not rule out the possibility that a linear domain required for binding UL9 is present upstream of residue 129 or downstream of residue 337 as deletions in this region were not tested. Alternatively, multiple domains of the UL42 protein may be involved in its interaction with UL9.

Since UL9 appears to be organized in two separate functional domains, it was necessary to determine whether either domain could bind UL42 independently. While no binding was detected with the C-terminal third of the protein (9CT), the N-terminal two-thirds of the protein (9NT) retained partial ability to bind UL42. Consistently, the 9NT bound 4- to 6-fold less efficiently than did full-length UL9. Interestingly, inclusion of the next 56 amino acid residues downstream of residue 534 (UL9.591) improved the efficiency with which the protein was bound, although wild-type binding was not restored. These data suggest the C-terminal residues are not completely dispensable for binding and may serve to stabilize the protein-protein interaction. Extension of the polypeptide further downstream of residue 591 will be necessary to determine sequences in the C-terminus required to restore wild-type binding.

Because these N- and C-terminal portions of UL9 appear to function independently, the N-terminal domain of UL9, which is essential for binding to UL42, most likely would be available when the C-terminal portion of UL9 is bound to
functional origins of replication and/or ICP8. In addition, the separable nature of the UL42 and UL8 binding sites on UL9 was demonstrated by the N-terminal 591 amino acids, lacking residues 103 – 279 (UL9Δ4.591), which bound as efficiently to UL42 as the intact 591 amino acids (UL9.591). Therefore UL9Δ4.591, which is incapable of interaction with ICP8 and UL8, contains residues sufficient for binding UL42. These data suggest domains of UL9 required for interaction with UL42 are unique to those required for interaction with UL8 or ICP8. In addition, these data indicate the presence of distinct domains of UL9 required for binding of UL42, UL8, and ICP8. Further deletions upstream of residue 103 and downstream of residue 279 will be necessary to further delineate regions of UL9 required for interaction with UL42.

Although independent domains of UL9 exist for binding of UL42, UL8 and ICP8, that all three proteins bind to UL9 at the same time is unknown. It is possible that binding of one or two of the proteins to UL9 excludes interaction with the others by steric hindrance or by inducing a conformational change that inhibits interaction with the other proteins. Coimmunoprecipitation experiments will be important to determine the ability of UL9 to bind UL42, UL8 and ICP8 at one time.

Functions of the interaction of UL9 with the aforementioned essential HSV-1 DNA replication proteins, including UL42, or with the host pol α primase (Lee et al., 1995) are unknown. However, the presence of so many interactions of UL9 with other proteins directly involved in DNA synthesis suggests the importance of these interactions in the ordered assembly, stabilization, and/or stoichiometry of proteins at functional HSV-1 origins of replication. This multiplicity of partners for UL9 might contribute to the negative effect of high concentrations of UL9 on the replication of HSV-1 DNA in
infected cells (Malik et al., 1992; Perry et al., 1993; Stow et al., 1993), because improperly assembled or aggregated UL9 could titrate out the necessary components of a functional replisome, including the UL42/pol complex.

Although no function for the UL42-UL9 interaction has been demonstrated to date, it is tempting to speculate that the UL42 protein acts to tether or recruit the viral DNA polymerase to a UL9-activated origin of replication. Immunofluorescence analysis has demonstrated that all of the seven essential viral DNA replication proteins are assembled in large globular-like “replication compartments” in cells productively replicating HSV-1 (deBruyn Kops and Knipe, 1988; Goodrich et al., 1990; Bush et al., 1991; Lukonis and Weller, 1996). It is interesting that only six of the replication proteins, exclusive of UL9, are necessary for ori-independent DNA synthesis in vitro (Skaliter and Lehman, 1994) and formation of large globular structures resembling, but not identical to, “replication compartments” (Uprichard and Knipe, 1997; Lukonis and Weller, 1997). However, since UL9 is required for viral DNA replication, these structures are not true “replication compartments” (Malik et al., 1991; Lukonis and Weller, 1996). These data suggest that UL9 may be required for efficient use of native viral genomes, perhaps by regulating the stoichiometry or configuration of a functional replisome.

Evidence supporting this hypothesis has been provided by immunofluorescence analysis in which fully functional pol-UL42 complex was required for the assembly of the replication proteins into large globular “replication compartments” regardless of whether the proteins were expressed transiently or during HSV-1 replication (Uprichard and Knipe, 1997, deBruyn Kops and Knipe, 1988; Goodrich et al., 1990; Bush et al.,
When HSV-1 pol activity was inhibited with phosphonoacetic acid (PAA) or acyclovir (ACG) in infected cells, ICP8, UL5, UL8, UL52, and pol colocalized in punctate structures in the nuclei (deBruyn Kops and Knipe, 1988; Goodrich et al., 1990; Bush et al., 1991; Lukonis and Weller, 1996), while both the UL42 and the UL9 proteins were more diffusely distributed throughout the nuclei (Goodrich et al., 1990, Malik et al., 1996). In addition, ICP8, UL5, UL52, and UL8 expressed together in transfected cells were necessary and sufficient to form similar punctate nuclear compartments and with the addition of either pol or UL42 the appearance of these prereplication compartments was unchanged (Uprichard and Knipe, 1997; Zhong and Hayward, 1997).

Taken together, the above results suggest that UL42 could serve a bridging function in vivo, pulling pol from nonproductive or prereplication sites to reorganized, functional, sites of viral replication. Because of UL42's ability to bind UL9, as well as UL9's ability to bind ICP8 and a member of the helicase/primase trimeric complex, this reorganized complex, perhaps with one or more host proteins, could form a stable assembly at functional origins of replication to initiate viral DNA synthesis. Analysis of the localization of UL42, pol, UL9, and ICP8 in cells infected with a UL42 mutant in which UL42 loses its ability to bind pol at the nonpermissive temperature will be important for testing this model.
<table>
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<tr>
<th>GST fusion protein&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Binding&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
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<tr>
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<td>d140</td>
<td>+</td>
</tr>
<tr>
<td>d129 – 163</td>
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<tr>
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<td>d274 – 288</td>
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<tr>
<td>d282 – 283</td>
<td>+</td>
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<td>N339</td>
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</table>

<sup>a</sup>Extracts were prepared from bacteria induced for expression of GST, wild-type or mutant GST/UL42

<sup>b</sup>Full-length UL9 was expressed by in vitro transcription/translation in rabbit reticulocyte lysates in the presence of [<sup>35</sup>S]methionine as described under Materials and Methods. Matrices were charged with 2 µM GST, GST/UL42, or mutant UL42 fusion protein and incubated with UL9. Proteins that eluted with 5 mM glutathione were subjected to SDS-PAGE and autoradiography and quantified by PhosphorImager.

<sup>c</sup>Background binding of GST was < 0.5%

**Table 7. Binding of UL9 to GST, wild-type and mutant GST/UL42**
Figure 15. **Diagram of UL9 functional domains.** Functional domains located within the UL9 protein are located as indicated above or below the UL9 open reading frame. The amino acid residues encompassing these regions are also indicated.
Figure 16. Stimulation of HSV-1 pol catalytic subunit by GST/UL42. GST/UL42 was partially purified by glutathione affinity chromatography and dialyzed. Serial 2-fold dilutions of GST/UL42 were used to stimulate the activity of pol in the presence of 125 mM KCl using activated calf thymus DNA as template as described previously (Monahan et al., 1993). Dots indicate the mean of two replicate samples, error bars indicate the range.
Prepare Cell Extracts

Calculate protein concentration by assaying GST activity

Bind fusion proteins to glutathione agarose

Remove unbound fusion proteins by washing

Incubate with IVTT expr. [35S] UL9 protein

Apply protein/agarose to mini-columns

Elute UL9 with glutathione

SDS-PAGE analysis

Quantify signal by PhosphorImage analysis

Figure 17. Diagram of GST affinity column chromatography. Cell extracts were generated from IPTG induced bacterial cultures. Amount of GST moiety present in each extract was quantified using the CDNB assay as described in Materials and Methods.
Figure 18. **Binding of UL9 to GST affinity columns.** Glutathione agarose was incubated with extracts prepared from bacteria induced to express GST or a fusion of GST with residues 20 – 456 of UL42 (GST/UL42; 20 μM). The GST- (lanes 3 – 5) and GST/UL42- (lanes 8 – 10) charged agarose beads were washed and subsequently incubated with [³⁵S]methionine-labeled UL9 prepared by coupled *in vitro* transcription/translation of plasmid pGEM-UL9. Bound proteins were eluted using 3 successive column volumes (E1 – E3) of 5 mM glutathione. Fractions were concentrated by lyophilization and subjected to SDS-PAGE using a 10 – 20 % polyacrylamide gel. Lanes 1 and 6, molecular weight marker. Lane 2 and 7, the proteins present in 1/10 of the volume of UL9-containing extracts subjected to affinity chromatography.
Figure 19. Amplification and IVTT expression of UL9. (A) The UL9 open reading frame (ORF) was amplified from KOS infected cell DNA as described under Materials and Methods. Amplified products were analyzed by 1 % agarose gel electrophoresis. Arrow indicates the correct product (3100 bp). (B) Amplified UL9 ORF was introduced into TA vector pCR2.1 downstream of the bacteriophage T7 RNA polymerase promoter. Proteins were prepared by in vitro transcription/translation with [35S]methionine. Products were subjected to SDS-PAGE analysis and analyzed by autoradiography. Arrow indicates in vitro transcribed and translated UL9 product.
Figure 20. Expression of GST/UL9. The UL9 open reading frame was introduced into the pGEX-2T as described under Materials and Methods. Bacterial cultures were induced to express GST/UL9. Bacteria were pelleted and total cell lysate was subjected to SDS-PAGE analysis and Coomassie blue staining. Arrows indicate GST and GST/UL9 proteins. Lanes 1 and 2, pGEX-2T (GST); lanes 3 and 4, GST/UL9.
Figure 21. Western blot detection of GST/UL9. Extracts were prepared from bacteria induced to express GST/UL9. Extracts were incubated end-over-end at 4° C for the indicated times. Proteins in the cellular extracts were separated by SDS-PAGE and electroblotted onto nitrocellulose. The filter was then probed with polyclonal antibody to UL9 (RH7). This antibody was prepared to a GST protein fused to the UL9 C-terminal sequences and is expected to react with UL9 and GST proteins. Immune complex on the filter was detected by [125I]-protein A. The arrows indicate full-length GST/UL9 and the GST moiety produced by breakdown.
Figure 22. Binding of UL9 prepared by *in vitro* transcription/translation to GST affinity columns. Full-length UL9 was prepared by coupled *in vitro* transcription/translation of plasmid pGEM-UL9 in the presence of \[^{35}\text{S}]\text{methionine}\) using rabbit reticulocyte lysates. Translation mixtures were incubated with GST (□) or GST/UL42 (△) affinity matrices which were prepared by charging with the indicated concentrations of GST or fusion protein. Bound proteins were eluted with 5 mM glutathione and separated by SDS-PAGE. The radioactivity in bands corresponding to UL9 was quantified using PhosphorImage analysis and used to calculate the relative amount of UL9 protein in the input that bound to and eluted from each matrix.
Figure 23. Binding of UL9 to wild-type and mutant GST/UL42. Full-length UL9 was expressed by in vitro transcription/translation in rabbit reticulocyte lysates in the presence of \([^{35}S]\)methionine as described under Materials and Methods. Lane 1 is the equivalent of 1/10 of the translation mixture used for binding to each matrix. Matrices were charged with 2 μM GST, GST/UL42, or mutant UL42 fusion protein and incubated with UL9. Proteins that eluted with 5 mM glutathione were subjected to SDS-PAGE and autoradiography. Arrow indicates UL9 protein.
<table>
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<tr>
<th>Protein</th>
<th>Deletion</th>
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<tr>
<td>UL9</td>
<td>-</td>
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<tr>
<td>9NT</td>
<td>d535-851</td>
</tr>
<tr>
<td>UL9.591</td>
<td>d592-851</td>
</tr>
<tr>
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Figure 24. **Diagram of wild type and mutant UL9 proteins.** Solid bars represent the UL9 open reading frame (ORF). Lines denote deletion of the indicated amino acid residues.
Figure 25. **Binding of N- and C-terminal UL9 subfragments to GST/UL42.** Full-length UL9 and polypeptides consisting of the N-terminal 533 amino acids (9NT) or C-terminal UL9 residues 534 – 851 linked to residues 1 – 10 (9CT) were expressed by *in vitro* translation in rabbit reticulocyte lysates in the presence of $[^{35}\text{S}]$methionine as described under Materials and Methods. Lanes 1, 7, and 13, the equivalent of 1/10 of the translation mixture used for binding to each matrix. Matrices were charged with 2 μM GST or GST/UL42 and incubated with UL9, 9NT, or 9CT, as indicated. Proteins that eluted with 5 mM glutathione were subjected to SDS-PAGE and autoradiography.
Figure 26. Binding of N- and C-terminal UL9 subfragments to various concentrations of GST/UL42. Full-length UL9 and polypeptides consisting of the N-terminal 533 amino acids (9NT) or C-terminal UL9 residues 534 - 851 linked to residues 1 - 10 (9CT) were expressed by \textit{in vitro} translation in rabbit reticulocyte lysates in the presence of \textsuperscript{35}Smethionine as described under Materials and Methods. Translated proteins corresponding to full-length UL9 (gray bars), 9NT (open bars), or 9CT (black bars) were incubated with affinity matrices charged with the indicated concentration of GST or GST/UL42. The values shown represent the amount which eluted from the GST/UL42 matrix less that which eluted from the GST matrix charged with the same concentration of protein. The amounts which bound to the control GST columns did not exceed 1.2 % for full-length, 0.75 % for 9NT, or 0.18 % for 9CT.
Figure 27. Expression of wild-type and mutant UL9 proteins. Proteins were expressed by *in vitro* translation in rabbit reticulocyte lysates in the presence of [*35*S]methionine as described under Materials and Methods. Products were analyzed by SDS-PAGE and autoradiography.
Figure 28. Binding of UL9, 9NT, UL9.591, and UL9Δ4.591 to GST/UL42. Full-length UL9 and polypeptides consisting of the N-terminal 533 amino acids (9NT), the N-terminal 591 amino acids (UL9.591), and the N-terminal 102 amino acid residues fused to residues 280 - 591 (UL9Δ4.591) were expressed by in vitro translation in rabbit reticulocyte lysates in the presence of [35S]methionine as described under Materials and Methods. Translated proteins corresponding to full-length UL9 (●), 9NT (■), UL9.591 (▲), or UL9Δ4.591 (♦) were incubated with affinity matrices charged with the indicated concentration of GST or GST/UL42. The values shown represent the amount which eluted from the GST/UL42 matrix less that which eluted from the GST matrix charged with the same concentration of protein. The amounts which bound to the control GST columns did not exceed 0.64 % for full-length, 0.48% for 9NT, 0.71 % for UL9.591, or 0.87 % for UL9Δ4.591.
Figure 29. **Summary of wild-type and mutant UL9 binding to GST/UL42.** Solid bars represent the UL9 open reading frame (ORF). Lines denote deletion of the indicated amino acid residues. The ability of each UL9 protein to interact with GST/UL42 was determined by GST fusion affinity chromatography as described in Figure 26.
A common theme among replicative polymerases is the association of the DNA polymerase catalytic subunit with subunits that increase processivity. Examples of such replicative polymerases and their processivity factors include eukaryotic DNA Pol δ and proliferating cell nuclear antigen (Prelich et al., 1987), Escherichia coli Pol III and the β subunit (Fay et al., 1982), and bacteriophage T7 Pol and E. coli thioredoxin (Tabor et al., 1987). The HSV-1 DNA polymerase is no exception as UL42 is its processivity factor. That the HSV-1 UL42 protein forms a heterodimer with HSV-1 DNA polymerase (pol; Crute and Lehman, 1989; Gallo et al., 1988; Gottlieb et al., 1990; Powell and Purifoy, 1977; and Vaughan et al., 1985), and increases the activity of pol in vitro (Gallo et al., 1989; Gottlieb et al., 1990; and Hernandez and Lehman, 1990) suggests a role of UL42 as a processivity factor. In addition, deletion of the C-terminal 27 amino acid residues of pol, shown to be dispensable for DNA polymerase activity, eliminated the ability of UL42 to interact with and stimulate pol in vitro and in vivo (Stow, 1993; Digard et al., 1993a; Tenney et al., 1993; Marsden et al., 1994; Digard et al., 1995). Taken together, these data suggest the ability of UL42 to complex with pol is of great importance during HSV-1 replication. This hypothesis is supported by genetic data, in this report and
others, that correlate the ability of mutant UL42 proteins to bind pol with the ability to support viral growth by either complementation of a UL42 null mutant (this report, Digard et al., 1993b; Tenney et al., 1993; Reddig et al., 1994) or isolation of UL42 deletion mutants (Gao et al., 1993).

The major essential in vivo role of UL42 in the infected cell is as the pol processivity factor. There is a consensus that the C-terminal third of UL42 is not required for activity in vitro or in vivo. However, most deletions in the remainder of the protein result in the simultaneous loss of the ability of UL42 to bind double-stranded DNA (ds DNA) and pol, and to stimulate pol activity in vitro. These UL42 mutant proteins also lose their ability to complement replication of the null virus.

In this study, the in vivo complementation assay was used to determine the ability of various UL42 deletion, insertion, and point mutants to complement the growth of UL42 null virus, CgalA42. The plasmids tested by in vivo complementation contained well-characterized lesions in the UL42 protein. These mutations previously were tested in vitro for their ability to bind ds DNA and pol, and to stimulate HSV pol activity. Briefly, affinity chromatography with glutathione-S-transferase (GST) fusion proteins of wild type and mutant UL42 was used to measure the degree of interaction of the fusion proteins with double-stranded DNA and pol. As a measure of UL42 mutant proteins to provide pol accessory function, the ability of the fusion proteins to stimulate the activity of pol in high salt buffer using activated calf thymus DNA as template was tested. In addition, The abilities of the mutant UL42 proteins to amplify ori-containing plasmids in vivo when the other DNA replication proteins were supplied by superinfection with the UL42 null mutant CgalA42 was observed. Although performed in vivo, the latter
experiment served only to test the ability of mutant UL42 proteins to support ori-dependent DNA replication, not productive viral replication as in the in vivo complementation assay. A summary of the in vitro and in vivo activities of each of the mutant UL42 proteins is found in Figure 30.

Generally, concordance was observed between the in vitro ability of UL42 to bind pol and its ability to complement the null virus replication. UL42 mutants d129 – 163, d137 – 142, d202 – 337, d256 – 282, and d274 – 282 failed both in their ability to bind pol in vitro and to complement the null virus. In addition, those UL42 mutants capable of binding pol in vitro to any extent, d140, R134D, T142A, i206, N459, and i459 were able to, at least partially, complement replication of the null virus. Taken together, these data suggest a strong correlation between the ability of UL42 to bind pol and its ability to function in vivo.

Discordance was observed between the in vitro and in vivo abilities of UL42 mutant d241 – 261. In vitro, this mutant displayed wild-type abilities to bind ds DNA, bind pol, and stimulate pol. However, mutant d241 – 261 was completely unable to complement growth of the null virus and allowed only a small amount of ori-dependent DNA replication in vivo compared to wild-type UL42. Digard et al (1993) found UL42 deletion mutant d242 – 250 retained wild-type ability to bind ds DNA and pol, and stimulate pol activity, although its ability to function in vivo was not tested. In addition, Hamatake et al (1993) demonstrated that trypsin digestion of UL42 in the presence of DNA generates two protease-resistant fragments: one comprised of amino acids 1 to 245 and one starting at residue 255. A complex of the two protease-resistant fragments was able to bind pol as well as stimulate its activity. Similar to our in vitro data for d241 –
261, these data suggest that regions of UL42 around residues 241 to 261 are dispensable for physical and functional interaction with pol in vitro. These data suggest that three regions within UL42 are required for activity in vitro: residues 137 – 142, 202 – 241, and 261 – 288. In contrast, the ability of UL42 to function in vivo appears to require sequences between residues 241 and 261 as deletion of these residues abrogates its ability to complement viral growth of the null mutant. It is possible that these sequences are involved in protein-protein interactions, such as to UL9 or other viral or host proteins, or in functions of UL42 as yet undetected.

Another mutant displaying discordance between the in vitro and in vivo activities was i206, an insertion mutant containing residues RGSA at codon 206. This mutant exhibited wild-type ability to bind pol, bound ds DNA but with an altered elution profile, and was able to stimulate pol, although not to wild-type levels. Despite these abilities, mutant i206 was only able to partially complement growth of the null virus (< 15%). Interestingly, Chow and Coen (1995) also described creation of an insertion mutant I206, in which the four amino acid sequence CIDA was inserted at codon 206. This mutant protein displayed a reduced affinity for ds DNA and a greatly reduced ability to stimulate pol, not unlike our i206 mutant. In addition, similar to our i206 mutant, their I206 mutant retained wild-type ability to bind pol. However, I206 was unable to support viral replication in a transient-complementation assay despite proper nuclear localization. This is similar to our i206 mutant that very weakly complemented the null mutant. Using the Robson-Garnier secondary-structure prediction algorithm (Garnier et al., 1978), these authors proposed that residues 206 to 209 lie in a flexible region of the UL42 protein and depending upon the amino acid residues inserted, the flexibility of the region was
effected. The reduced or complete lack of flexibility could then manifest in the ability of UL42 to function in vitro and in vivo. Perhaps the residual flexibility of our i206 protein, in the presence of other viral proteins in an infected cell, is capable of assuming a conformation that allows limited ability to function as a pol processivity factor.

Discordance between the in vitro and in vivo abilities was also observed for UL42 insertion mutant, i140, and deletion mutant d282 – 283. Interestingly, these mutant proteins lacked the ability to bind ds DNA and pol, and lacked the ability to stimulate pol in vitro. However, i140 and d282 – 283 demonstrated limited ability to complement growth of the null mutant (~ 11% and 23%, respectively). Perhaps, in vivo, these mutants assume a conformation in the presence of other viral and cellular proteins that they are unable to assume in vitro. It is also possible that the amount of UL42 present in vivo is able to overcome a threshold level which enables the mutant protein to interact with pol and provide at least partial ability to stimulate pol.

For the most part, the ability of the mutant proteins to support ori-dependent replication in vivo, reflected their ability to support growth of the null virus in the in vivo complementation assay. Interestingly, though, each of the low complementing plasmids (i140, i206, and d282 – 283) was found to support ori-dependent DNA replication at close to wild-type levels. These data suggest that the i140, i206, and d282 – 283 mutant proteins function adequately in vivo to replicate an ori-containing plasmid. However, this assay does not measure the quality of the DNA replicated. It is possible that the DNA produced in the ori-dependent replication assay contains a higher number of mutations or gaps in the DNA, neither of which would be detected by Southern blot analysis. Problems with the quality of DNA produced would, therefore, go undetected in the
aforementioned assay but would have been manifest in the *in vivo* complementation assay as the reduced ability to complement replication of the null mutant.

Overall, there is a general concordance between the *in vitro* and *in vivo* data suggesting the major essential role of UL42 in the infected cell is that of processivity factor. The ability to bind pol *in vitro* appears to correlate strongly with the ability of the protein to function *in vivo*. Discordance was noted only for those that yielded weak or no complementation of CgalΔ42. However, where discordance was observed, differences in conformation of the mutant UL42 proteins *in vitro* compared to *in vivo*, differences in the amounts of mutant proteins *in vitro* compared to *in vivo*, possible interactions with other viral or host proteins present *in vivo* but not *in vitro*, or functions of the UL42 protein other than stimulation of pol activity, cannot be ruled out. Structural information would be of great assistance in understanding how these mutations affect the protein’s local architecture and would shed more light on the different phenotypes of the mutants. In addition, recombinant viruses containing these mutations would provide insight into the role of these proteins under natural conditions of virus infection.

In this study, I have isolated two recombinant viruses, i140/UL42 and i206/UL42. As previously mentioned, by the methods used, UL42 mutant i140 was incapable of binding ds DNA or pol, or stimulating pol *in vitro*. UL42 mutant i206, however, displayed wild type ability to bind pol, bound ds DNA although with an altered elution profile, and stimulated pol, although not to wild-type levels. However, both mutations, i140 and i206, displayed partial ability to complement growth of the null virus (~11% and 13%, respectively). Despite only partial ability of each mutant to complement growth of the null virus, each recombinant virus was isolated from Vero cells suggesting
conformation of UL42 in the infected cell was maintained to support viral replication in a cell line nonpermissive for null viral growth.

It was hypothesized that since both the i140/UL42 and i206/UL42 viruses were isolated from Vero cells, the viruses might display a non-lethal replication deficiency such as that observed with the non-essential genes ICP0, ICP22, and ICP47 (Stow and Stow, 1986; Sacks and Schaffer, 1987; Everett, 1989; Cai and Schaffer, 1991; Sears et al., 1985; Longnecker and Roizman, 1986). However, a direct yield of progeny assay demonstrated that although no multiplicity-dependent effects were observed, both i140/UL42 and i206/UL42 viruses were deficient in the burst size produced at each multiplicity when compared to wild-type levels (4 PFU/cell, 1 PFU/cell, and 70 PFU/cell, respectively).

One possible reason for the reduced burst size observed between wild type and the mutant viruses was a delay in the production of progeny. A one-step growth curve revealed similar growth patterns of wild type and i140/UL42 suggesting an extended growth cycle was not responsible for the difference observed in the yield of progeny. Interestingly, though, initial viral progeny production for wild type virus began at 6 h p.i. while in Vero cells infected with i140/UL42, viral progeny production began at 8 h p.i. This may suggest a slower onset of DNA synthesis in i140/UL42 infected cells compared to wild-type infected cells. Perhaps a critical level of a viral function must be achieved in order to attain better DNA and viral production. A slightly elongated growth cycle was observed for i206/UL42. The elongated rise period suggests a slight delay in the production of progeny genomes important for the production of encapsidated viral progeny, possibly due to low UL42 production in the infected cell and/or to the inability
of i206 mutant protein to stimulate pol efficiently. Regardless, an extended growth cycle was not responsible for the reduced burst size observed for either mutant.

It was possible that the reduced burst observed in either case was due to low levels of UL42 in the infected cells. A quantitative immunoblot revealed the level of UL42 present in i140/UL42 infected Vero cells was 4-fold greater than that present in wild type infected cells. How could more i140 mutant UL42 protein than wild-type protein be expressed when cells were infected at the same multiplicity? It is important to remember that multiplicity of infection is based upon infectious units, not total particle count. For all animal viruses, the number of virus particles in any given preparation exceeds the number of infectious units. It is possible that the phenotype observed for i140/UL42 reflects an unusually high number of total viral particles compared to infectious particles. Therefore, to achieve the same multiplicity of infection between the i140/UL42 and the wild-type viruses, many more i140/UL42 total particles would be necessary. With the increased total particle count necessary to achieve i140/UL42 infection at a certain multiplicity, compared to the total particle count necessary for wild-type virus, the expression of viral proteins could be enhanced, therefore exceeding the ability of the viral and cellular replication machinery to support viral replication. This could result in an increased level of all viral expression, including UL42 (such as that observed), compared to wild-type virus, at the same multiplicity of infection and at the same time, a reduced burst size. It is possible that this high level of mutant UL42 protein present in the infected Vero cells reflects a threshold amount of UL42 required to permit limited physical and functional interaction between the mutant UL42 and pol. Perhaps no physical or functional interaction was detected in vitro because this threshold level was
not achieved. Since it is possible that the phenotype of the i140/UL42 virus reflects an unusually high total particle count compared to infectious units, it will be necessary to determine the total particle count compared to infectious particles present in i140/UL42 infected cells.

At least 4-fold less UL42 protein was observed in the i206/UL42 infected Vero cells compared to wild-type levels. This reduced expression could reflect lower UL42 mRNA levels, shorter half-life of the i206/UL42 mRNA, or shorter half-life of the UL42 protein itself. The reduced level of mutant i206 UL42 protein in Vero infected cells may be responsible for the delayed growth cycle and reduced progeny production. It is interesting to note Johnson et al (1991) observed wild-type levels of viral DNA synthesis and infectious virions in V9 cells infected with the null virus, CgalΔ42, in the presence of less than 1% the level of UL42 produced in a wild-type infected cell. These data suggest only small quantities of wild-type UL42 are required for efficient HSV-1 replication. However, the UL42 present in i206/UL42 infected Vero cells is not wild type and may require a higher threshold amount of mutant UL42 protein to efficiently functionally interact with pol. In addition, in vitro studies demonstrated that the i206 mutant protein displays a reduced affinity for DNA as suggested by its altered elution profile. This, too, may manifest itself as a reduced yield of progeny in vivo as processivity of the pol on the DNA template may be affected. Regardless, these studies indicate that both mutant UL42 proteins are being efficiently expressed in the infected cell.

The reduced burst size observed for the recombinant viruses could be due to a problem with the quantity of DNA produced resulting from a reduced yield of total viral DNA due to inefficient stimulation of pol or from an increased threshold level of mutant
UL42 protein. Alternatively, the reduced burst size could reflect a problem with the quality of the DNA produced due to a high mutation frequency and low pol fidelity, or from gapped progeny genomes resulting from inefficient pol processivity. It is also possible UL42 plays an additional role in the infected cell in addition to that of processivity factor. To further understand the phenotypes of the i140/UL42 and i206/UL42 viruses, it will be necessary to examine possible deficiencies in total DNA synthesis or synthesis of full length viral DNA, resulting from the mutations introduced into the UL42 protein. This can be done by in vivo labeling of newly synthesized DNA with $[^3]{H}$thymidine and Southern blot analysis of DNase I resistant viral DNA, respectively. The yield problem could also be a reflection of the inability of the mutant proteins to stimulate pol to wild-type levels. This can be resolved by determining the pol activity present in extracts of cells infected with i140/UL42 and i206/UL42 viruses as described in Reddig et al. (1994).

**Domains important for UL42 and UL9 protein-protein interaction.** UL9 has been shown to specifically interact with ICP8 (Boehmer and Lehman, 1993; Boehmer et al., 1994) and with UL8 (McLean et al., 1994). And, for the first time, a specific protein-protein interaction has been demonstrated between UL9 and the pol processivity factor, UL42 (this report; Monahan et al., 1998). The interaction was determined to be specific and independent of DNA.

The region of UL42 required for interaction with UL9 most likely does not exist as a linear domain in UL42 as determined by the ability of all of the GST/UL42 mutants tested to bind UL9. This, however, does not rule out the possibility that a linear domain required for binding UL9 is present upstream of residue 129 or downstream of residue
337 as deletions in this region were not tested. Alternatively, multiple domains of the UL42 protein may be involved in its interaction with UL9.

Since UL9 appears to be organized in two separate functional domains, it was necessary to determine whether either domain could bind UL42 independently. While no binding was detected with the C-terminal third of the protein (9CT), the N-terminal two-thirds of the protein (9NT) retained partial ability to bind UL42. Consistently, the 9NT bound 4- to 6-fold less efficiently than did full-length UL9. Interestingly, inclusion of the next 56 amino acid residues downstream of residue 534 (UL9.591) improved the efficiency with which the protein was bound, although wild-type binding was not restored. These data suggest the C-terminal residues are not completely dispensable for binding and may serve to stabilize the protein-protein interaction. Extension of the polypeptide further downstream of residue 591 will be necessary to determine sequences in the C-terminus required to restore wild-type binding.

Because these N- and C-terminal portions of UL9 appear to function independently, the N-terminal domain of UL9, which is essential for binding to UL42, most likely would be available when the C-terminal portion of UL9 is bound to functional origins of replication and/or ICP8. In addition, the separable nature of the UL42 and UL8 binding sites on UL9 was demonstrated by the N-terminal 591 amino acids, lacking residues 103 – 279 (UL9Δ4.591), which bound as efficiently to UL42 as the intact 591 amino acids (UL9.591). Therefore UL9Δ4.591, which is incapable of interaction with ICP8 and UL8, contains residues sufficient for binding UL42. These data suggest domains of UL9 required for interaction with UL42 are unique to those required for interaction with UL8 or ICP8. In addition, these data indicate the presence
of distinct domains of UL9 required for binding of UL42, UL8, and ICP8. Further deletions upstream of residue 103 and downstream of residue 279 will be necessary to further delineate regions of UL9 required for interaction with UL42.

Although independent domains of UL9 exist for binding of UL42, UL8 and ICP8, that all three proteins bind to UL9 at the same time is unknown. It is possible that binding of one or two of the proteins to UL9 excludes interaction with the others by steric hindrance or by inducing a conformational change that inhibits interaction with the other proteins. Coimmunoprecipitation experiments will be important to determine the ability of UL9 to bind UL42, UL8 and ICP8 at one time.

Functions of the interaction of UL9 with the aforementioned essential HSV-1 DNA replication proteins, including UL42, or with the host pol α primase (Lee et al., 1995) are unknown. However, the presence of so many interactions of UL9 with other proteins directly involved in DNA synthesis suggests the importance of these interactions in the ordered assembly, stabilization, and/or stoichiometry of proteins at functional HSV-1 origins of replication. This multiplicity of partners for UL9 might contribute to the negative effect of high concentrations of UL9 on the replication of HSV-1 DNA in infected cells (Malik et al., 1992; Perry et al., 1993; Stow et al., 1993), because improperly assembled or aggregated UL9 could titrate out the necessary components of a functional replisome, including the UL42/pol complex.

Although no function for the UL42-UL9 interaction has been demonstrated to date, it is tempting to speculate that the UL42 protein acts to tether or recruit the viral DNA polymerase to a UL9-activated origin of replication. Immunofluorescence analysis has demonstrated that all of the seven essential viral DNA replication proteins are
assembled in large globular-like "replication compartments" in cells productively replicating HSV-1 (DeBruyn Kops and Knipe, 1988; Goodrich et al., 1990; Bush et al., 1991; Lukonis and Weller, 1996). It is interesting that only six of the replication proteins, exclusive of UL9, are necessary for ori-independent DNA synthesis in vitro (Skaliter and Lehman, 1994) and formation of large globular structures resembling, but not identical to, "replication compartments" (Uprichard and Knipe, 1997; Lukonis and Weller, 1997). However, since UL9 is required for viral DNA replication, these structures are not true "replication compartments" (Malik et al., 1991; Lukonis and Weller, 1996). These data suggest that UL9 may be required for efficient use of native viral genomes, perhaps by regulating the stoichiometry or configuration of a functional replisome.

Evidence supporting this hypothesis has been provided by immunofluorescence analysis in which fully functional pol-UL42 complex was required for the assembly of the replication proteins into large globular "replication compartments" regardless of whether the proteins were expressed transiently or during HSV-1 replication (Uprichard and Knipe, 1997, deBruyn Kops and Knipe, 1988; Goodrich et al., 1990; Bush et al., 1991; Zhong and Hayward, 1997). When HSV-1 pol activity was inhibited with phosphonoacetic acid (PAA) or acyclovir (ACG) in infected cells, ICP8, UL5, UL8, UL52, and pol colocalized in punctate structures in the nuclei (deBruyn Kops and Knipe, 1988; Goodrich et al., 1990; Bush et al., 1991; Lukonis and Weller, 1996), while both the UL42 and the UL9 proteins were more diffusely distributed throughout the nuclei (Goodrich et al., 1990, Malik et al., 1996). In addition, ICP8, UL5, UL52, and UL8 expressed together in transfected cells were necessary and sufficient to form similar
punctate nuclear compartments and with the addition of either pol or UL42 the appearance of these prereplication compartments was unchanged (Uprichard and Knipe, 1997; Zhong and Hayward, 1997).

Taken together, the above results suggest that UL42 could serve a bridging function *in vivo*, pulling pol from nonproductive or prereplication sites to reorganized, functional, sites of viral replication. Because of UL42’s ability to bind UL9, as well as UL9’s ability to bind ICP8 and a member of the helicase/primase trimeric complex, this reorganized complex, perhaps with one or more host proteins, could form a stable assembly at functional origins of replication to initiate viral DNA synthesis. Analysis of the localization of UL42, pol, UL9, and ICP8 in cells infected with a UL42 mutant in which UL42 loses its ability to bind pol at the nonpermissive temperature will be important for testing this model.

**Summary.** This research supports the hypothesis that the main essential role of UL42 *in vivo* is that of a processivity factor. However, the ability of UL42 protein to function as such *in vitro* or *in vivo* appears to be separable from its ability to bind DNA. In light of this research, it will be necessary to modify the current model of UL42 as a pol processivity factor where UL42 acts as a tether between pol and ds DNA. Instead, since the ability of UL42 to bind ds DNA appears to be dispensable, it is possible that UL42 confers a conformational change upon pol that results in increased processivity and stimulated activity. However, this remains to be investigated.

In addition, this research has demonstrated a specific protein-protein interaction between UL42 and UL9. Although a functional interaction has not been demonstrated, it is tempting to speculate, in view of the immunofluorescence data, an important role of
UL42 in the formation of replisomes at a functional origin of replication and is an important feature of the model in Figure 31. This could be a role of UL42 in addition to that of a processivity factor. However, this remains to be investigated.

It is likely that, similar to prokaryotic and eukaryotic systems, protein-DNA and several specific protein-protein interactions are important for the formation of a functional replisome and subsequent replication of the HSV-1 genome. Several of these interactions have already been discussed such as UL42/pol, ICP8/pol, UL42/UL9, UL9/host pol α, UL9/ICP8, UL9/UL8, and UL5/UL8/UL52. In addition to its interactions with UL42 and ICP8, pol has also been shown to physically interact with UL8 (Marsden et al., 1997) although no functional interaction has been detected. ICP8 has been shown to stimulate the DNA helicase activity of the DNA helicase-primase in a species-specific manner (Crute and Lehman, 1991; Tanguy et al., 1996) in a process that requires UL8 (Tanguy et al., 1996). Also, a UL8/ICP8 protein-protein interaction appears to be important for utilization of ICP8-coated DNA templates for primase activity (Tanguy et al., 1996). Together these data support the existence of a multiprotein complex that functions in the replication of the HSV-1 genome. Based on these data, a model of a functional replisome at an HSV-1 origin of replication is presented in Figure 31.

Many protein-protein interactions are shown in Figure 31. Presumably, UL9 and ICP8 interact to unwind the A + T rich region within the origin of replication. It is possible that the initial primer laid for initiation of viral DNA synthesis is mediated by the interaction of UL9 with the host pol α primase (not shown in this model). Once laid and leading strand synthesis has begun, it is possible the helicase/primase complex acts to
lay primers for lagging strand synthesis while unwinding the DNA ahead of the progressing fork. The physical interaction between pol and UL8 may serve to keep pol in close proximity to the helicase/primase complex for lagging strand synthesis. Interaction of UL42 with UL9 may serve to recruit pol to the functional origin of replication. Additionally, ICP8 may serve to stimulate the pol holoenzyme via its interaction with the pol subunit (not shown in this model). In this model, the replisome is shown to contain two polymerase holoenzyme units, however, this is speculation as this has not been demonstrated in the literature.

This model assumes that UL42 can interact with both pol and UL9 simultaneously. However, concurrent interaction has yet to be demonstrated. One possible method to determine whether UL42 can bind to both pol and UL9 at once is by coimmunoprecipitation. In this experiment pol, UL42, UL42/pol complex, and UL9 would be purified from insect cells infected with the appropriate recombinant baculovirus constructs. Purified proteins would be mixed *in vitro* and resulting complexes immunoprecipitated with antibody to either UL42 or UL9. The immunoprecipitated complexes could then be separated by size exclusion column chromatography. Detection of a high molecular weight protein complex reacting with antibodies against pol, UL42 and UL9 would indicate UL42 was capable of interaction with UL9 and pol simultaneously. These data would support the proposed model.

Alternatively, BIAcore 2000 technology could be used to determine whether UL42 could bind both pol and UL9 at the same time. First, purified pol, UL42, and UL9 could be bound to different flow cells of the BIAcore chip (using bovine serum albumin [BSA] to detect any nonspecific protein-protein interactions) and the binding of
pol, UL9, and UL42 in solution to the immobilized proteins could be measured by passing increasing concentrations of pol, UL9, and UL42 over the immobilized proteins. In addition to detecting a UL42/pol interaction, this experiment would confirm the interaction between UL42 and UL9 and determine whether pol can interact directly with UL9 in the absence of UL42.

Next, the same experiment would be done. However, instead of pol, a purified complex of pol/UL42 would be immobilized on the chip. The binding of UL9, UL42 and UL42/pol complex in solution by each of the immobilized proteins or protein complexes could then be measured by passing increasing concentrations of these proteins across those immobilized on the chip. A similar or lower affinity of UL9 for pol/UL42, than for UL42 alone, and the inability of pol to interact directly with UL9 would indicate UL42 is capable of interaction with both UL9 and pol simultaneously and would support the proposed model.

Another yet unanswered question posed by the presented model is whether UL9 can interact with UL42, UL8 and ICP8 simultaneously. This question could be answered in a manner similar to that proposed above. Purified UL9, UL42, UL8, and ICP8 proteins could be mixed in vitro and resulting complexes coimmunoprecipitated with antibodies against all of the proteins. The precipitated complexes could then be separated size exclusion chromatography. Western blot analysis with antibodies against all four proteins would determine which proteins were present in higher molecular weight complexes.

The entire replisome could potentially be purified from virus infected cells. Alternatively, the replisome could be purified from insect cells transfected with an ori-
containing plasmid and superinfected with recombinant baculovirus constructs encoding all seven of the HSV-1 replication proteins. Size exclusion chromatography could be used to isolate high molecular weight complexes. Non-denaturing PAGE and Western blot analysis could be used to identify proteins present in the high molecular weight complexes. In addition, denaturing PAGE analysis and coomassie blue staining could be used to determine the stoichiometry of the proteins present at a functional origin of replication. This would be important for reconstitution of a functional replisome \textit{in vitro}.

In conclusion, it is likely that the numerous protein-protein interactions detected among the HSV-1 replication proteins are important for formation of replisomes at a functional HSV-1 origin of replication. UL9 and UL42 most likely play key roles in the formation of functional replisomes, probably by regulation of the correct stoichiometry. Further investigation into the roles of UL42 and UL9 in replisome formation, such as those presented here, will be important to understand HSV-1 genome replication.
### UL42 Mutants

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* Indicates altered binding properties

*b* These mutants have detectable ODR activity but at less than 30% WT level

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**Figure 30. Summary of *in vitro* and *in vivo* functions of mutant UL42 polypeptides.**

Dashed lines represent deletions within the open reading frame (ORF) of UL42. Arrowheads below the ORF mark deletion of single amino acid residues at the indicated position. Arrowheads above the ORF denote in-frame insertion, at the indicated positions. "X" on the ORF indicate locations of point mutations. The ability of each mutant UL42 gene to function *in vivo* was determined by its ability to complement the replication of CgalΔ42 as indicated in Table 4. The *in vitro* function of each mutant was determined by its ability to physically associate with pol and DNA by GST fusion column chromatography. The ability of each GST fusion protein to stimulate *in vitro* transcribed/translated pol activity was tested under high salt conditions with activated calf thymus DNA as template, as described previously (Monahan et al., 1998).
Figure 31. Model of a functional HSV-1 origin of replication. ICP8, the single-stranded DNA binding protein, coats the single-stranded A+T-rich region between the binding sites of UL9 within an HSV-1 origin. Homodimers of UL9 bind regions within the origin and oligomerize to distort the origin sequences. Protein-protein interactions are shown between ICP8/UL9, UL9/UL42, UL42/pol, UL5/UL8/UL52, and pol/UL8. Not shown are known interactions between pol and ICP8 and UL9 and DNA pol α. Thick lines represent double-stranded DNA; open boxes represent box 1 and box 2 within an HSV-1 origin; thin lines represent A+T-rich region within an HSV-1 origin.
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