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# CHARACTERIZATION OF THE HSV-1 POLYMERASE ACCESSORY FACTOR UL42

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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1998

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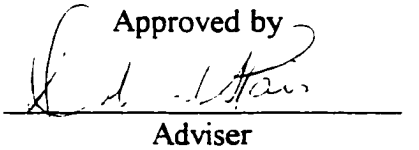

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CHARACTERIZATION OF THE HSV-1 UL42 POLYMERASE ACCESSORY FACTOR UL42

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The Ohio State University, 1998

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*Herpesviridae* represent a large family of animal viruses of substantial public health importance. The most extensively studied member of the family, herpes simplex virus type 1 (HSV-1), requires seven viral-encoded genes for origin-specific DNA replication. The UL42 gene, encoding a 65 kDa protein, represents one of these genes. Although the UL42 gene product has no inherent catalytic activity, it can bind to double-stranded DNA. Furthermore, it binds to the 140 kDa viral DNA polymerase (pol) at a 1:1 stochiometry, and increases the processivity of pol. To better understand the role of UL42 in DNA replication and to identify the mechanism by which UL42 modifies pol activity, experiments were performed to analyze three biochemical activities ascribed to the UL42 protein: DNA binding, pol binding, and the ability to stimulate pol activity. Toward that end, mutations spanning the entire UL42 open reading frame were evaluated for their effects on these functions.

Glutathione -S-transferase (GST)-UL42 fusion proteins were used to provide quantitation of these biochemical activities. It was demonstrated that nearly all of the

UL42 mutations yielded proteins in which both DNA binding and pol binding activities were abrogated. However, the differential activities of two mutant proteins that retained pol accessory function suggest that UL42 protein may increase the processivity of pol by two independent mechanisms. These mechanisms suggest that UL42 can independently stimulate pol activity by tethering of pol to the primer/template and by altering the conformation of pol. The results demonstrated that DNA binding by UL42 is not absolutely necessary for pol accessory function *in vitro*. More importantly, the data suggest that binding of UL42 to pol is necessary but not sufficient for the formation of a fully processive pol. Because a complete separation of the ability of UL42 to bind pol and DNA was not achieved, it is possible that most of the N-terminal two-thirds of UL42 the protein is required for a conformation of UL42 protein which is important for all three activities of UL42 examined.

Experiments were also conducted to determine the correlation between the *in vitro* and *in vivo* activities of UL42. A modified transient transfection study was used to quantify the ability of UL42 protein to amplify plasmids containing an HSV-1 origin of replication. Cells transfected with an HSV-1 origin-containing plasmid and a plasmid containing wild-type or mutant UL42 were infected with a UL42 deleted HSV-1 virus (Cgal Δ42). The results showed that there is an excellent correlation in the ability of UL42 mutant proteins to function *in vitro* and *in vivo*. Surprisingly, a deletion of amino acids 241-261 which had no effect on pol accessory function *in vitro*, provided no pol accessory function in infected cells. These results suggest that UL42 may provide other essential functions in infected cells in addition to acting as a pol accessory factor. Taken together, the results of these studies suggest that UL42 may represent a new class of pol accessory proteins that stimulates pol activity through its association with both pol and DNA.

To Martha Flemings and Family

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# CHAPTER 1

## INTRODUCTION

The *Herpesviridae* comprise a large family of viruses that infect virtually all vertebrates. Human herpesviruses are among the most intensely investigated of all viruses. The human herpesviruses include herpes simplex virus type-1 (HSV-1), herpes simplex virus type-2 (HSV-2), human herpes virus 6 (HHV-6), human herpes virus 7 (HHV-7), human herpes virus 8 (HHV-8), human cytomegalovirus (HCMV), varicella-zoster virus (VZV) and Epstein-Barr virus (EBV) (reviewed in Roizman et al., 1996). Human herpesviruses are prevalent pathogens and are, therefore, of substantial public health importance. HSV-1, for example, is the cause of fever blisters (herpeslabialis) (reviewed in Fields et al., 1996), while HSV-2 causes severe genital infection (reviewed in Fields et al., 1996). EBV is associated with Burkitt's lymphoma and infectious mononucleosis (reviewed in Fields et al., 1996) while VZV causes chicken pox in children and shingles when reactivated in adults (reviewed in Fields et al., 1996). More importantly, herperviruses that produce benign infections in normal individuals can cause debilitating or life-threatening infections in an immunocompromised host.

The *Herpesviridae* have similar structures in that the linear double stranded DNA genome is surrounded by an electron-opaque core contained within an icosahedral capsid. An amorphous tegument surrounds the capsid and an outer envelope exhibits spikes on its surface to comprise the virion ( Roizman and Furlong, 1997).

HSV-1 has been the most extensively studied of the *Herpesviridae*. Its 152 kilobase-pair double stranded genome has been sequenced entirely and more than 75 open reading frames have been identified ( McGeoch et al., 1988; Roizman et al., 1997). HSV has a G + C content of 68% for HSV-1 and 69% for HSV-2 (Berker and Dym, 1968; Kief et al., 1971; McGeoch et al., 1988). As shown in figure 1, the HSV-l genome has 2 covalently linked segments, the unique long ($U_L$) and unique short ($U_S$) regions, which are separated by inverted repeats, ($IR_L$ and $IR_S$, located internally). The genome is flanked by terminal repeats ($TR_L$ and $TR_S$). The L and S components can invert with respect to each other yielding four linear isomeric forms of the viral DNA that are generated in approximately equimolar quantities during each round of replication (Hayward et al., 1975).

**HSV-1 DNA replication.**

The mechanism of HSV -1 viral DNA replication is not fully understood; however, several details have been elucidated. It is known that the input linear double stranded DNA genome adopts an "endless" configuration shortly after infection that is consistent with circularization (Garber et al., 1993; Poffenberger and Roizman, 1985). The circularized genome presumably acts as a template for origin-dependent theta replication. Evidence in support of early theta replication includes the observation that topoisomerase II is required for the initial phases of the infectious cycle, suggesting that decantenation of early replication products may be an essential step in HSV replication (Hammarsten et al., 1996).

The prevalence of endless DNA until the late stages of HSV-1 infection suggests a rolling circle mechanism for HSV-1 DNA replication. Several lines of evidence support this idea. Restriction enzyme digestion of high molecular weight HSV-1 DNA isolated from infected cells revealed head-to-tail unit-length concatemers (Serverini et al., 1994; Zhang et al., 1994). Consistent

with this observation, high molecular weight HSV-1 DNA labeled during pulse-chase experiments lacks free ends, a finding that suggested that the DNA consisted of head-to-tail concatomers (Jacob et al., 1979). Further evidence supporting a rolling circle mechanism of replication by HSV-1 is provided by the presence of head to tail concatamers of plasmid DNA containing an HSV-1 origin of replication in cells coinfected with HSV-1 (Stow et al., 1982). Taken together, these results strongly support the idea that HSV-1 replicates its DNA at some point via a rolling circle mechanism.

Electron microscopic (EM) studies revealed that replicating HSV-1 DNA contains highly branched secondary structures (Shlomai et al., 1976; Friedman et al., 1977). More recently, pulse-field gel electrophoresis (PFGE) and two-dimensional gel electrophoresis have also been used to confirm that HSV-1 DNA replication intermediates consist of highly branched DNA structures (Serverini et al., 1994; Serverini et al., 1996). Weller et al (1996), using pulse field gel electrophoresis (PFGE) showed that restriction enzyme digestion of HSV-1 DNA replication intermediates with single restriction enzyme cutters resolved discrete monomer length DNA.

A plausible first step in HSV-1 DNA replication would involve the binding of UL9 as a homodimer to a high affinity (box I) origin binding site on one or more HSV-1 ori, (Koff et al., 1991; Rabkin and Hanlan, 1991; Hazoda et al, 1991). The protein:protein interaction between UL9 homodimers allows cooperative binding to the low affinity binding sites in box I and III at the ori (Rabkin and Hanlon, 1991). Because UL9 possesses helicase activity *in vitro* and the conserved motifs are important for viral DNA replication *in vivo* (Brackner et al. 1991; Fierer and Challberg, 1992; Stow et al, 1992), UL9 may work to unwind a limited amount of the DNA at the origin to allow entry of the remainder of the proteins. UL9 interacts specifically with ICP8 (UL29), the single stranded DNA binding protein, and is believed to recruit the HSV-1 single-stranded DNA binding

protein, ICP8, to stimulate local unwinding at the origin (Makhov et al., 1996). Gustafsson et al, (1995) confirmed this association by demonstrating that the C-terminal region of ICP8 forms a complex with UL9 in the absence of DNA. ICP8 also has been found to stimulate the helicase and DNA-dependent ATPase activity of UL9 (Fierer and Challberg, 1995; Dodson and Lehman, 1993; Boehmer et al., 1993). Taken together, these results suggest that ICP8 may stimulate DNA unwinding at the ori by maintaining the single stranded conformation of unwound DNA (Boehmer et al., 1993). The observation that the UL9 and ICP8 are physically associated in the presence of ds DNA, but not on single stranded DNA (Gustafsson et al., 1995), suggests that this complex dissociates after unwinding at the ori. The 317 amino acid carboxyl end of UL9 binds to DNA at 2 sites to loop and distort intervening A-T rich sequences (Koff et al., 1991). UL9 has been shown to induce structural changes in DNA at the ori as demonstrated by the hypersensitivity to DNase 1 (Elias et al., 1990). On the basis of these and other studies, it is proposed that the interaction between UL9 dimers bound to boxes I and II leads to the bending, looping, and distortion of the A + T rich region. The amino terminus mediates the dimerization between UL9 monomers (Elias et al., 1992).

Following the local unwinding of A-T rich sequences at the ori, it is predicted that like other systems, eukaryotic and prokaryotic, unwinding of DNA sequences ahead of the replication fork and priming of single stranded regions of DNA ensue. During HSV-1 DNA replication, the unwinding activity is provided by the helicase/primase complex (UL5, UL8, UL52). The physical interaction between UL9 and the UL8 components of the helicase primase provides a means to bring these components to the origin (McLean et al., 1994) and may serve as the mechanism by which the helicase-primase complex is recruited to the advancing replication fork. An interaction between

ICP8 and UL8 also is needed to stimulate the DNA helicase activity of the DNA helicase/primase in a species-specific manner (Crute and Lehman, 1989; Le Gac et al, 1996). Another possible priming mechanism is suggested by the binding of UL9 to human DNA pol α primase (Lee et al., 1995). Any of these mechanisms would allow unwinding and priming at the replication fork.

The next possible step in DNA replication would involve leading and lagging strand DNA synthesis. In the replication of Eschericia coli *(E. coli)* or SV40, either an asymmetric dimeric polymerase ( *E. coli)* or two different DNA polymerases, DNA pol α-primase and DNA pol δ, are needed to promote the leading and lagging strand synthesis required for semidiscontinious DNA replication (Komberg et al., 1992). Dimerization of HSV-1 pol has not been reported. However, the formation of a pol:UL42 complex allows highly processive DNA replication compared to pol alone.

**Essential HSV-1 DNA Replication Genes.**

The use of temperature-sensitive mutants identified essential HSV-1 genes and revealed several complementation groups with defects in DNA synthesis (Schaffer et al., 1973, Aron et at., 1975; Stow et al., 1978; Parris et al., 1978; Chu et al., 1979; Parris et al., 1980; Weller et al., 1983; Matz et al., 1983). The development of a transient DNA replication assay Wu et al ( 1986), facilitated the identification of genes essential for HSV-1 DNA replication. Cells transfected with plasmids containing various fragments of the HSV-1 genome and a HSV-1 origin (ori)-containing plasmid supplied all of the *trans* activating activity necessary to replicate plasmids. Using this procedure, Wu et al (1988), demonstrated that only 7 open-reading frames (ORF) were sufficient for ori-dependent DNA replication: UL5,UL8 ,UL9 ,UL29 ,UL30 ,UL42 and UL52. *Spodoptera frugiperda* (Sf9) cells infected with recombinant baculovirus expressing these same seven essential DNA replication genes were able to support replication of ori-containing plasmids (Stow et al.,

1993) demonstrating that any cellular functions required for HSV-1 origin-dependent DNA replication were conserved between insect and mammalian cells. The known functions of these gene products are summarized in Table 1.

The HSV-1 DNA polymerase catalytic subunit is a 136 kDa protein encoded by the UL30 gene (Quinn and Mc Geoch, 1985; Purifoy et al., 1977; Jofe et al., 1977; Gibbs et al., 1985; Hall et al., 1986). DNA polymerase purified from HSV-1 infected cells exists as a heterodimer with the UL42 gene product. Early studies of the heterodimeric pol reported that polymerization by pol is stimulated by salt with an optimal activity seen at 100 mM NaCl. HSV-1 pol has sequence similarity to other viral and cellular α type DNA polymerases including T4 DNA polymerase, *Saccharomyces cerevisiae* DNA pol δ, *E coli* pol I, and human DNA pol α-primase (Digard and Coen, 1990; Blanco et al., 1991; Hall et al., 1995). HSV-1 DNA pol possesses 3'-5' exonuclease activity which serves as a proofreading function to ensure high fidelity DNA replication. The 3'-5' exonuclease proofreading activity of pol contains three sequence motifs that align with the exonuclease motifs I, II, and III of *E coli* DNA polymerase I (Blanco et al., 1991; Hall et al., 1995; Derbyshire et al, 1991). Although the C-terminal 227 residues of pol have been shown to be sufficient for binding to UL42 (Digard and Coen, 1990; Digard et al., 1993), a defining region of UL42 sufficient for pol binding or DNA binding has not been determined. Examination of UL42 has shown that the C-terminus of pol (residues 1209-1235) is required for association with UL42 and stimulation by UL42 (Stow et al., 1993; Digard et al., 1993; Marsden et al., 1994; Digard et al, 1995).

Replication genes UL5,UL8, and UL52 (Crute et al., 1989; Dodson et al., 1989; Golddrick et al., 1993) form a stable trimeric complex of 440 kDa that has helicase/primase activity (Chadler et al., 1990; Crute et al., 1989). The holoenzyme consists of a 1:1:1 association of the 3 proteins in

HSV-1 infected cells or in insect cells infected with recombinants expressing each of these proteins. The helicase-primase complex has a molecular weight of 270 kDa (Dodson et al., 1991). UL5 and UL52 were found to have helicase/primase activity (Chalder and Stow, 1990) and the addition of UL8 in vitro does not alter this activity (Dodson and Lehman, 1991). Sherman et al (1992), later demonstrated that UL5 alone had low level DNA-dependent ATPase activity. Klinedinst and Challberg (1994), found that a recombinant baculovirus which expressed mutant UL52 protein was deficient in primase activity. UL5 has homology to other helicases (Walker et al., 1982). UL8 is believed to stabilize the primer to the template (Sherman et al., 1992) and stimulate primer synthesis by the UL5/UL52 complex by as much as 3 fold (Tenney et al., 1994; Le Gac et al., 1996; and Tenney et al. 1995). UL8 has no effect on DNA-dependent ATPase activity Le Gac et al (1996), however it is required for optimal DNA helicase and primase activity in the presence of ICP8 (Le Gac et al., 1996). Stow et al (1992), demonstrated that UL8 was required for the localization of UL5 and UL52 into pre-replication sites based on immunoflouresence analysis. UL8 interacts with each of the helicase/primase components UL5 and UL52 as judged by the ability of cells co-transfected with each of these pairs to transport both into the nucleus Sherman et al (1992).

The UL29 gene (ICP8) encodes a 120 kDa protein (Conley et al., 1981; Weller et al., 1983; Gao et al., 1989) that binds cooperatively (Reyerhan, 1938) and preferentially to single stranded DNA (Powell et al., 1981; Weller et al., 1983; Lee and Knipes., 1985; Powell and Purifoy, 1976). ICP8 stimulates DNA unwinding by UL9 (Boehmer et al., 1993) while also increasing pol activity on circular DNA (Orberg and Schaffer, 1987). ICP8 plays a role in the multiple protein:protein interactions that affect activities of pol, DNA helicase-primase, and UL9. ICP8 also appears to be essential for the interaction between DNA pol and UL9 (Boehmer et al., 1993; Gao and Knipe, 1991).

ICP8 can reduce the melting temperature of synthetic polynucleotides and can destabilize helix DNA to unwind short regions of duplex DNA in an ATP independent manner ( Powell et al., 1976; Boechmer and Lehman, 1993). ICP8 also plays a role in the assembly of HSV-1 replication proteins into pre-replication sites that initially were thought to be precursors for DNA replication (Bush et al., 1991).

**Literature Review of UL42.**

The UL42 gene, located in the unique long region of the HSV genome (McGeoch et al., 1988), encodes a 488 amino acid (Marcetti et al.,1988; McGeoch et al, 1988) 65 kilodalton replication protein (Johnson et al., 1991; Parris et al., 1988; Marchetti et al, 1988) that is expressed with β kinetics (Goodrich et al., 1990). The gene encoding this protein was first shown to map between coordinates 0.574 and 0.682 (Marsden et al., 1987). The gene was then mapped more precisely to an ORF within the Hind III L fragment using *in vitro* translation of hybrid-selected mRNA and specific antibodies to the protein and peptides (Parris et al., 1988). Johnson et al (1991) found that mutant virus with deletions of UL42 failed to make viral DNA or produce infectious viral progeny. However, replication of a UL42 null mutant could be complemented in *trans* in cells transfected with plasmids encoding UL42. The ability of UL42 to bind with high apparent affinity to DNA with no sequence specificity and in a non cooperative manner (Gallo et al., 1988; Vaughan et al, 1985) is independent of its interaction with pol (Gallo et al., 1988). Moreover, mutants with temperature-sensitive lesions in UL42 have been characterized to have no viral DNA synthesis and such mutations lead to a reduced replication of origin-containing plasmids in the presence of the other 6 HSV-1 replication proteins (Marchetti et al., 1988). UL42 and pol interact to form the catalytic core for HSV-1 DNA elongation. UL42 and pol were first found to physically interact( Powell and

Purifoy, 1977;Vaughn et al., 1985) and were co-purified from infected cells (Gallo et al 1988; Crute and Lehman 1989; Gottlieb et al 1990). The UL42/pol interaction has also been seen in heterologous systems (Digard et al., 1990; Hernandez and Lehman, 1990; Gottleib et al., 1990) where they form a heterodimer with a predicted 1:1 stochiometry. Hamatake et al (1993), demonstrated that the $K_{association}$ of pol +UL42 was $1.2 \times 10^8$ $M^{-1}$ using poly (dA): oligo ($dT_{20}$) template. UL42 has been found to functionally stimulate pol activity in high salt on activated calf thymus DNA template (Gallo et al., 1989; Monahan et al., 1993; Reddig et al., 1994 ) and to increase the processivity of pol on singly primed M13 DNA (Gottleib et al., 1990; Hernandez and Lehman, 1990). UL42 and pol also co-localize to the nucleus within replication compartments (Goodrich et al., 1990).

Hamatake et al (1993), defined 2 proteolytic fragments, 28 kDa (representing aa 1-248) and 8 kDa (representing residues aa 255-315), that together retained the ability to coimmunoprecipitate with pol and stimulate its activity. These results were in contrast to previous observations in which residues 1-248 of UL42 were insufficient for pol accessory function. This suggested that the 8 kDa peptide fragment , which could not be separated from the 28 kDa fragment during purification, was also required for increased pol processivity. The fact that these fragments retain activities suggests that the functional domains of UL42 form a well-ordered structure that do not have to be covalently linked. Monahan et al, (1993) determined that UL42 mutants that lacked similar regions of UL42, region I (aa deletions 129-163) and region II (aa deletions 202-337), failed to precipitate with pol or stimulate its activity on activated calf thymus DNA.

Owisianka et al (1993), synthesized 96 overlapping pentapeptides spanning the entire 488 amino acids of UL42 and tested their ability to inhibit the activity of pol:UL42 on singly primed M13 template, to bind to ds DNA, and their ability to inhibit pol activity of other polymerases. Results

demonstrated that several regions of UL42 bound DNA and most likely represent or correspond to a region of UL42 responsible for DNA binding. Most of the peptides including residues 64-78,89-102, 229-243, and 279-293 also inhibited the production of full length DNA in the M13 processivity assay. All of the peptides that maintained DNA binding activity bound nonspecifically to mammalian polymerase α and the Klenow fragment of *E coli* DNA polymerase I. The limited specificity of peptides to these polymerases make them unsuitable as compounds for antiviral drug development.

Digard et al (1993), made an extensive set of mutations ( C-terminal and N-terminal deletions, and insertions) spanning the entire UL42 gene to map the domains of UL42 necessary for pol binding (by coimmunoprecipitation assay (co-ip)), DNA binding (on ds DNA cellulose),and stimulation of pol activity (on singly primed M13 template) by expressing plasmids representing UL42 mutants by in vitro transcription/translation (IVTT). Results demonstrated that the pol binding domain of UL42 resides between residues 20 and 340. Partial pol binding was observed when truncated at residues 307 and 269 of UL42. DNA binding activity of UL42 was completely abolished when truncated at residue 340. Of the internal deletions tested, the smallest deletions (residues 9-20,242-250) were the only to maintain all pol accessory functions. These results and the observation that mutants that knocked out one UL42 activity (DNA binding, pol binding or pol stimulation) failed in the others, suggests that mutations used in this study could affect the folding of protein and affect UL42 pol accessory function. Insertion mutations throughout UL42 had no effect on pol accessory function with the exception of an insertion mutation at 160 which yielded protein failed to bind pol or stimulate pol activity (Digard et al., 1993). Results demonstrated that mutants that failed to stimulate pol *in vitro*, also failed to complement with a HSV-1 null UL42 mutation *in vivo*. The observation that deletion mutations at residues 307 and 269 bound pol slightly but failed to bind ds DNA suggests that

the 2 activities of UL42 are separable.

Monahan et al (1993), used C-terminal, N-terminal, and internal UL42 deletions to analyze pol binding (by coimmunoprecipitation), and pol stimulation (by $^{3}H$ TTP incorporation into gapped calf thymus DNA) and demonstrated that deletions in 2 non-overlapping regions of UL42 abrogated UL42 activity. Co-expression of pol and UL42 by *in vitro* transcription/translation (IVTT) and coimmunoprecipitation with an antibody specific for UL42 determined that deletion of residues 129-163 (region I) maintained partial pol binding while proteins with deletion of residues 202-337 (region 2) failed to bind pol. Both mutants failed to stimulate pol activity. These results suggest that pol binding alone was not sufficient for pol stimulation since the region I mutant protein could not stimulate pol activity. Monahan also observed that an insertion at residue 206 slightly affected pol stimulation while an insertion of 2 prolines at residue 140 inhibited pol stimulation. A more subtle mutation (deletion of residue 140) restored 50% pol stimulation (compared to wild-type) suggesting that region I of UL42 is sensitive to mutations, resulting in protein that abrogates or reduces pol accessory function. Reddig et al (1994), reported that there is a good correlation between the activities of region I and region II mutants (described by Monahan et al., 1993) *in vitro* and *in vivo*. Most of the mutants that failed to stimulate pol activity *in vitro* also failed to complement a null HSV-1 UL42 virus.

Chow and Coen 1995, identified 2 mutants of UL42 with reduced DNA binding but wild-type pol binding. IVTT expressed UL42 mutant protein of i206 (four aa insertions at aa residue 206) and i203 (four aa insertions at aa residue 203) failed to bind ds DNA. Coimmunoprecipitation of IVTT-expressed UL42 mutants with purified recombinant pol demonstrated that i203 and i206 bound pol. All other mutants failed to stimulate pol activity on poly (dA) :oligo ($dT_{20}$) template or complement

a null UL42 HSV-1 virus. Results suggested that i203 and i206 loss of pol accessory function *in vitro* and *in vivo* is due to the loss of DNA binding.

**Protein:Protein Interactions.**

It is becoming increasingly apparent that the fundamental mechanisms of DNA replication have been highly conserved during evolution. In most cases, accessory proteins may modify the properties of the DNA polymerase to allow optimal processive catalytic activity. Conservation has been observed in the functional domains among many DNA replication components from a variety of species. For example, HSV-1 DNA pol exhibits sequence similarity to other viral and cellular DNA polymerases, particularly the α-like pols including human DNA pol α-primase, *Saccharomyces cerevisiae* DNA pol δ, *E. coli* DNA pol I, and bacteriophage T4 DNA pol (Digard and Coen, 1990, Blanco et al., 1991, Hall et al., 1995).

As predicted for most complex and simple replication systems that involve multiple subunit enzymes, there are several lines of evidence which suggest that protein:protein and protein:DNA interactions are important for HSV-1 DNA replication. These interactions include UL9:UL9 homodimers (Fierer et al., 1992), UL9:DNA (Elias et al., 1986; Olivo et al., 1988; Hazuda et al., 1991), UL9:UL42 (Monahan et al., 1998), pol (UL30):UL42 (Gallo et al., 1988; Hernandez and Lehman, 1990; Gottlieb et al., 1990), UL5:UL8:UL52 (Crute et al., 1989; Dodson and Crute et al., 1989), UL5:UL52 (Goldrich et al., 1993), pol:ICP8 (Ruyechan and Weir, 1984), UL42:ds DNA (Gallo et al., 1988; Marsden et al., 1987).

Further evidence of the protein:protein interactions among the HSV-1 DNA replication proteins has been observed using immunoflourescence studies. Early after infection, the 7 HSV-1 replication proteins are distributed throughout the nucleus (Gao et al., 1989; Olivo et al., 1989; Gao

et al., 1992; Calder et al., 1992; Marsden et al, 1996). As early as 2 hours post infection, ICP8 is found to localize to so called pre-replication sites (deBruyn and Knipe, 1988) and localizes UL30 (pol) to these sites (Bush et al., 1991). Knipe et al (1996) found that the addition of DNA synthesis inhibitors to cells infected with HSV-1 mutant viruses causes redistribution of ICP8 from a diffuse to a puntate pattern but only in cells that were in S-phase. It has also been demonstrated that ICP8/UL9 and the UL5/UL8/UL52 (helicase-primase) together are required for localization to pre-replication sites when cells were transfected with plasmids expressing these proteins (Liptak et al., 1996) or infected with virus defective in one of these viral proteins (Lukonis and Weller 1996). Liptak et al (1996) showed that UL5,UL8, and UL52 are sufficient for the localization of ICP8 into pre-replication sites. At the same time, these studies showed that a functional pol/UL42 complex was required to develop active replication compartments (Liptak et al 1996). Using mutant viruses of each of the HSV-1 replication proteins, Knipe et al (1996) found that UL9 and the helicase/primase proteins are required for the transport of ICP8 to pre-replication sites. Conversely, Hayward and Zhong (1997), showed that UL9 was not required for the transport of ICP8 into pre-replication sites.

The knowledge that the assembly of various complexes including protein:protein and protein:DNA interactions are critical for HSV-1 DNA replication, suggests that interference of any of these interactions may inhibit DNA replication. The targeting of protein:protein interactions may be a useful strategy since more than one protein is required for replication so it would be hard for single resistant strains to develop. The interest in protein:protein interactions has grown from observations that HSV ribonucleotide reductase is specifically inhibited by a nonapeptide corresponding to the small subunit of the enzyme (Coen et al., 1986; Dutia et al., 1989). The peptide acted by competing for a site on the large subunit to which the smaller one binds and so inhibits

normal association of the 2 subunits (Coen et al., 1986, Dutia et al., 1989, Darling et al., 1990, McClements et al., 1988, Luzzi et al., 1994). These results suggested that in principle, any biological process that depends on protein:protein interaction between 2 subunits could be blocked by compounds that interfere with that interaction.The interaction between pol and UL42 with an association constant of $1 \times 10^8 M^{-1}$ (Hamatake et al., 1993) and the requirement of this interaction for viral replication has led to growing interest in UL42 as a potential novel antiviral drug target using compounds that can interfere with heterodimerization. It is also possible that antiviral agents that mimic the DNA binding region of UL42 may also interfere with viral DNA replication. This would make a UL42 DNA binding site a good target. Such an antiviral agent could be HSV specific and not affect cellular functions.

**Polymerase Accessory Proteins.**

As stated earlier, UL42 like other pol accessory proteins, increases the processivity of pol (Table 2). Processivity requires a physical association of the accessory protein and pol to prevent the dissociation of pol from the substrate during catalysis and during the relative movement between the enzyme and the DNA template. Mammalian pol δ interactions with PCNA or the *E. coli* pol III with the β subunit facilitate lagging strand DNA synthesis by acting as sliding clamps to keep pol associated with DNA. Although the overall functions for pol accessory proteins are similar, the mechanism by which they increase the processivity of pol's may be different for different species (Table 2).

The *E. coli* β subunit binds to pol III in a DNA-independent manner with the assistance of a protein γ complex and ATP. The crystal structure of β protein (Kong et al., 1992) revealed that the β subunits adopts a ring structure around duplex DNA behind the primer template. β requires

a clamp loader γ complex (composed of 5 subunits) the latter of which is capable of rapid assembling of multiple clamps to DNA. Pol removes γ from the β subunit ring (Naktining et al., 1996) to allow 2 subunits of β to bind pol and tether it to DNA to increase processivity. Elegant experiments showed β subunit bound tightly to nicked circular plasmid but β dissociated upon linearization of the plasmid by sliding over the ends of the DNA (Stukenberg et al., 1991; Yao et al., 1996). Results suggested that the β subunits bound DNA in a topical fashion by encircling DNA rather than through a direct interaction with DNA. These results have been confirmed by X-ray crystallography (Kong et al., 1992).

Eukaryotic pol processivity factor, PCNA, is a functional homolog of the β subunit of *E. coli* pol III, as is gene 45 of T4 bacteriophage. PCNA increases the processivity of eukaryotic pol δ and pol ϵ. Like β, PCNA requires a clamp loader protein (RF-C) and ATP to assemble onto DNA, although PCNA assembles as a trimer that structurally resembles the β dimer. Eukaryotic polymerase δ binds and initiates processive DNA replication and recycles PCNA from DNA (Yao et al., 1996). Clamp loaders RF-C and the γ complex of pol III also are homologous (O'Donnel et al., 1993; Collman et al., 1995). Duplex DNA is predicted to pass through the toroidal processivity factors (β and PCNA) with little or no contact with DNA. This sliding does not slow down the movement of the polymerase.

In the T4 bacteriophage system, the processivity factor gp45 is loaded onto the DNA by the clamp loader gp44/62 complex in an ATP-dependent manner. This in turn confers processivity on the gp 43 DNA polymerase (Younger et al., 1992). The gp 45 protein exists as a trimeric complex (Jarvis et al., 1989) that has a ring-like structure like the assembled β and PCNA factors. Like the accessory proteins listed above, gp45 increases processivity with no contact with DNA (Table 2).

In the eukaryotic and prokaryotic systems discussed above, pol accessory proteins provide their function through their ring-shaped structure which allows them to slide along the DNA template with little or no contact with the DNA. At the same time, these proteins require ATP and co-factors in order to be loaded onto the DNA (Table 2).

Thioredoxin of *E. coli* uses a different mechanism to increase the processivity of T7 g5 polymerase protein. Unlike the accessory proteins listed above, thioredoxin does not bind to DNA. Thioreoxin association with DNA is ATP-independent and requires no clamp loader. Thioreoxin binds tightly to T7 DNA polymerase in a 1:1 stochiometry (Modrich and Richardson, 1975; Modrich and Richardson, 1976) and converts a weakly processive gene 5 protein into a highly processive enzyme that can polymerize thousands of nucleotides without dissociation (Huber et al., 1987; Marians et al., 1992; Tabor et al., 1982). DNA synthesis in the absence of thioredoxin was much less productive and highly prone for frameshift errors in homopolymeric runs (Kunkel et al., 1994; Kroutil et al., 1996). Thioredoxin increases pol activity on ds DNA several hundred fold while having little effect on the activity on single-stranded DNA (Hori et al., 1979; Alder and Modrich ,1979; Tabor and Richardson, 1987). The high affinity binding of thioredoxin also increases 3'-5' exonuclease activity of the pol (Tabor et al., 1987; Huber et al, 1987). Recent studies with supressor mutations in the pol binding and exonuclease binding domains of thioredoxin has led to the hypothesis that thioredoxin binds to both domains to form a clamp that binds DNA (Himawan and Richardson, 1992).

UL42 is distinguished from other pol accessory proteins (with the exception of thioredoxin) in that UL42 binds to ds DNA, does not require ATP or co-factors for the physical association with pol or DNA, and is not likely to form a torus that surrounds ds DNA (Table 2). A similar ds DNA binding pol accessory protein, BMRF1, preferentially binds ds DNA and increases the processivity

of EBV pol BALF5 (Tsurumi, 1993; Tsurumi et al., 1993; Dorsky et al., 1993). Pol accessory function has also been determined for the UL44 protein of HCMV (Ertl and Powell, 1992). At the same time, there is a correlation between the loss of DNA binding activity of BMRF1 and UL44 and the loss of pol stimulation (Kiehl and Dorsky, 1995; Weiland et al., 1994). The relative ability of UL42 to bind DNA suggests that the mechanism of processivity for UL42 may be different from that of β, PCNA or gp 45 that simply tether pol to DNA to increase processivity.

As stated earlier, UL42 varies in activity from many other pol accessory proteins by its ability to bind ds DNA without cofactors or other replication proteins. This characteristic has led to the hypothesis that UL42 tethers HSV-1 DNA pol to the template to increase the processivity of pol. Evidence in support of a tethering mechanism for UL42 activity was supplied by Gottlieb et al (1994). Using a mobility shift assay on a primer-template which contained both single and double-stranded regions of DNA, they determined the affinity of pol or UL42 alone and together for single and double stranded DNA. Challberg et al, (1994) demonstrated that UL42 increased pol binding to the 3' end of the primer template by 10 fold. DNA footprinting results demonstrated that pol bound the primer-template at the single to double strand junction protected 18 bp of upstream single-stranded DNA and 14 bp of double-stranded DNA. These experiments demonstrated that UL42 extends the protection of the double stranded portion of DNA (than seen with pol alone) while not affecting the single-stranded protected region of the template. This provided support for the idea that an increase in processivity for pol in the presence of UL42 is related to DNA binding of free UL42 and that the role of UL42 in the DNA pol complex is to increase the affinity of HSV DNA pol for 3' end of a priming DNA strand by positioning pol to the 3' end of the primer template. These results provide a model in which UL42 increases the processivity of pol by acting as a tether or clamp

(Gottleib et al., 1994).However, these results do not explain the mechanism by which UL42's DNA binding ability is overcome to allow sliding of the pol:UL42 comlex.

Little is known about the exact mechanism by which UL42 increases the processivity of pol. The mechanism by which DNA binding proteins like UL42 slide along the DNA will likely require detailed structural information of the protein. To determine the mechanism by which UL42 acts as a pol accessory factor, several approaches have been used to determine the domains of UL42 responsible for binding to pol and DNA. Several labs have demonstrated that the N-terminal 339 residues of UL42 are required for its pol-accessory function (Monahan et al., 1993; Gibbs et al., 1991; Tenney et al., 1993; Digard et al., 1993; Gao et al., 1993). The binding domain was defined to the N-terminal 315 residues (Tenney et al., 1993).

Evidence suggest that DNA binding activity, the physical association with pol, and the functional stimulation of DNA pol might reside in different regions of UL42 ( Digard and Coen, 1990; Digard et al., 1993; Monahan et al., 1993; Gibbs et al., 1991; Tenney et al., 1993; Digard et al., 1993; Gao et al., 1993; Reddig et al., 1994; Chow and Coen, 1995). In order to define the mechanism of UL42 pol accessory function, it is important to separate UL42 DNA and pol binding activities of UL42.These activities may determine whether UL42 tethers pol to DNA or by its physical association with pol.

Several problems are evident in the assays used to identify UL42 functional domains and the mechanism by which UL42 increases the processivity of pol. As stated by Digard et al (1993), the large size of UL42 deletions used in their assays could account for the loss of pol accessory function of mutants by allowing misfolding of the proteins. Inconsistencies in the the activity of UL42 mutants to stimulate or bind to pol in some cases could be due to variabilities in protein expressed using

IVTT. For example, UL42 deletion mutant, d129-163, which was found to bind pol by Coen et al (1995), was found not to bind to pol by Monahan at al (1993). The difference in the observed activity probably reflects a difference in protein expression and some lack of specificity in immunoprecipitation. In both studies, lack of specificity of UL42 antibody was reported and may have affected the characterization of distinct mutants. This lack of specificity in coimmunoprecipitation may also explain the partial pol binding observed with mutants deleted at residues 307 and 269 (Digard et al., 1993). More specifically, these assays do not allow for the quantitation of the individual mutant activities which may assist in separating the individual activities of UL42. A problem arises in that most of the mutations tested ( linker-insertions and amino acid deletions) to date retain all or none of their pol accessory functions (pol binding, DNA binding, pol stimulation). A plausible explanation could be that the insertions or deletions in certain regions of UL42 destabilize the structural integrity of UL42. To circumvent this problem, more subtle mutations are required.

My hypothesis suggests that the 3 activities of UL42 (pol binding, DNA binding, and pol stimulation) involved in pol accessory function are located concurrently within a defined region of UL42.

PCR mutagenesis was used to make site-directed mutations within conserved residues of UL42. I have analyzed these UL42 mutants for pol binding, DNA binding, and pol stimulation by expressing them as GST-fusion proteins and utilizing high affinity chromatography to assist with the assays. Because the effects that mutations have *in vitro* may not be a reflection of the actions of mutants *in vivo*, I used an origin-dependent DNA replication assay to determine the ability to UL42 mutants to replicate an HSV-1 origin-containing plasmid.

Taken together, these results will be essential in identifying the regions necessary for UL42 pol accessory functions. Moreover, characterization of these mutants in vitro and in vivo will greatly facilitate identification of the mechanism by which UL42 provides pol accessory function in HSV-1 DNA replication.

**Figure 1. Structure of the herpes simplex virus type 1 genome.** Diagrammatic representation of the HSV-1 genome. The positions of the a,b,and c repeats within the terminal repeats ($TR_L$ and $TR_R$) and internal repeats ($IR_L$ and $IR_S$), and the positions of the DNA replication origins ($ori_S$ and $ori_L$) are as indicated. The positions and directions of transcription of the seven essential DNA replication genes are indicated by arrows. The shaded area shows the composition of a unit-length a sequence, consisting of direct repeats (DR) 1,2, and 3, and unique (U) domains b and c. Abbreviations: U, unique; $_b$, terminal; $_n$, variable number of copies; I, inverted orientation; $_L$, long: $_S$, short. Not to scale.

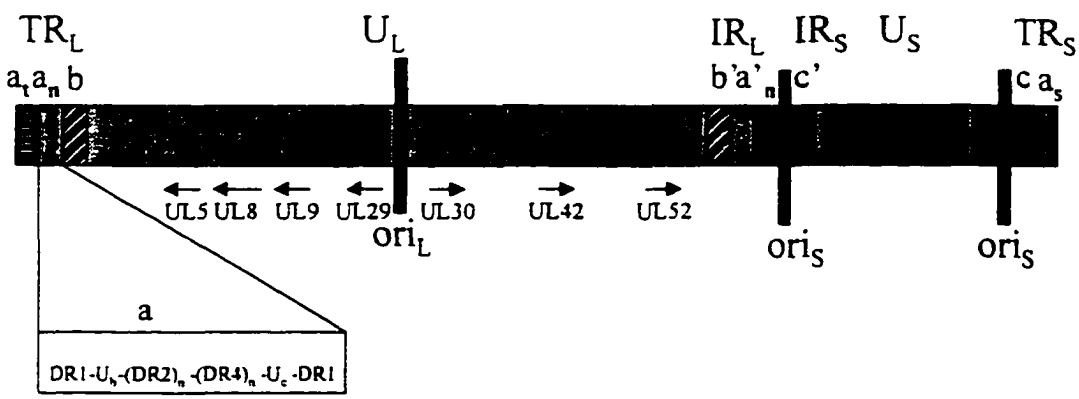

TR_L
a_t a_n b
U_L
IR_L
b'a'_n
IR_S
c'
U_S
TR_S
c a_s
UL5 UL8 UL9 UL29 UL30 UL42 UL52
ori_L
ori_S
ori_S
a
DR1-U_b-(DR2)_n-(DR4)_n-U_c-DR1

**Figure 1.**

| Essential HSV-1 DNA Replication proteins | Gene | Size (kDa) | Activities | References |
|---|---|---|---|---|
| DNA polymerase | UL30 (pol) | 136 | DNA polymerase<br>3'-5' exonuclease | Knopf et al., 1979; O'Donell et al., 1987. |
| Pol accessory protein | UL42 | 65 | ds DNA binding<br>DNA pol processivity factor | Gallo et al., 1989; Vaughn et al., 1985; Gottlieb et al., 1990;Hernandez and Lehman, 1990; Monahan et al., 1993; Reddig et al., 1994. |
| Origin-binding protein | UL9 | 94 | Origin-binding<br>3'-5' DNA helicase | Olivo et al., 1988; Elias et al., 1988; Hernandez et al., 1991; Brackner et al., 1991; Fierer and Challberg, 1992; Stow et al., 1992 |
| Single-stranded DNA binding protein | UL29<br>(ICP8) | 128 | Single-stranded DNA binding protein<br>Stimulates DNA pol<br>Stimulates helicase-primase<br>Stimulates UL9 helicase | Baylis et al., 1975; Powell and Purifoy, 1976; Knipe and Lee, 1985; Gustafssan et al., 1995; Fierer and Challberg, 1995; Dodson and Lehman, 1993; Boehmer et al., 1993. |
| DNA helicase-primase | UL5 | 99 | 5'-3' helicase<br>DNA-dependent ATPase | Walker et al., 1992<br>Sherman et al., 1992 |
| | UL8 | 80 | Stimulates primer synthesis ICP8 and UL9 nuclear localization | Sherman et al., 1992; Tenney et al., 1992; Stow et al., 1996. |
| | UL52 | 114 | DNA primase | Klinedinst and Challberg, 1994. |

**Table 1. Essential HSV-1 DNA Replication protein.**

# Polymerase Accessory Proteins

| | Increase processivity | Require ATP | Req clamp loader | # of subunits | Contacts DNA |
|---|---|---|---|---|---|
| β-subunit | + | + | + | 2 | - |
| PCNA | + | + | + | 3 | - |
| Gene 45 | + | + | + | 3 | - |
| thioredoxin | + | - | - | 1 | + |
| UL42 | + | - | - | 1 | + |

**Table 2. Polymerase Accessory Proteins**

# CHAPTER 2

## MATERIALS AND METHODS

**Growth of cells and viruses.** Baby hamster kidney (BHK) cells and African green monkey kidney (Vero) cells were cultivated in Dulbecco modified minimum essential medium (Flow Laboratories, McLean, Va) supplemented with 5% and 7.5% fetal bovine serum, respectively, 100 units of penicillin/ml, 100 µg streptomycin/ml, and 0.075% $NaCO_3$ for closed vessels, or 0.225% $NaCO_3$ for open vessels, as previously described (Gallo et. al., 1988). The Sf9 insect cell line used to propagate each of the baculovirus strains was kindly provided by Dr. Fred Hink (The Ohio State University, Columbus, OH). Sf9 cells were propagated in TNM-FH (Sigma Chemical Company, St. Louis ,MO), 10% lactose hydralase (Difco Laboratories, Detroit, MI) 100 units penicillin per ml, 100 µg of streptomycin sulfate per ml, and 10% fetal bovine serum (Flow laboratories, Detroit, MI) at 27°C and were passaged weekly at a 1:10 ratio.

The HSV-1 parental (WT) strain used was KOS (Smith, 1964). Stocks of HSV-1 were prepared in Vero cells by low multiplicity of infection passage (Parris et al., 1978). The UL42 deletion mutant (CgalΔ 42) has been described in detail (Johnson et al., 1991). Recombinant baculoviruses ( *Autographica californica* nuclear polyhedrosis virus) AcNPV which express the HSV-1 DNA replication genes UL42, UL29, UL5, UL8, UL52, and UL30

were the kind gifts of Mark Challberg (National Institute of Health, Beshesda, MD), Robert Lehman (Stanford University, Palo Alto CA), and Nigel Stow (MRC Virology Unit, Glasgow,Scotland). Stocks of recombinant baculoviruses were prepared by infecting subconfluent flasks of insect cells at a low moi (1 pfu/cell) and harvesting supernatant at 4-5 days post infection.

**Production of UL42 Recombinant Baculovirus**

**Recombinant baculovirus production**. Production of recombinant baculovirus was performed using the BaculoGold™ System ( PharMingen, San Diego, CA, USA). Five micrograms of pVL1392/UL42 or mutant UL42 DNA was mixed with 0.5 μg of BaculoGold DNA and transfected using the $CaPO_4$ technique into 60 mm plates according to instructions of the manufacturer. Briefly, 750 μl of TNM-FH + 10% FBS was added to plates with a 1 ml solution containing pVL1392/UL42 DNA in 25 mM Hepes (pH 7.1), 140 mM NaCl, and 125 mM $CaCl_2$. After 4 hours, the supernatant was removed and the cells refed with TNM-FH + 10% FBS. Cells were incubated at 27°C for 5 days, pelleted by low speed centrifugation for 10 minutes, and the virus-containing supernatant collected and stored at 4°C.

**Plaque assay of recombinant baculoviruses.** Sf9 cells were seeded into 60 mm plates at 2 x $10^6$ cells/plate (to provide 70% confluency) in 4 ml of complete media and allowed to adhere at 27°C for 30 minutes. The cells were then infected with dilutions ($10^{-1}$ -$10^{-8}$) of virus-containing supernatant (250 μl of virus in serum free TNM-FH media) at 27°C for 1 hour. After 1 hour, the supernatant was removed and the cells overlayed with a 1% low melting point agarose solution containing TNM-FH +10% FBS at 27° C, according to

instructions of the manufacturer (Pharmigen San Diego, CA). Plates were stained with neutral red 4 days post infection and plaques counted the next day. The virus titer (pfu/ml) was calculated as the average # of plaques/plate x 1/dilution x 1/0.25 ml.

**Plaque purification**. In some cases, single isolated plaques were picked into individual wells using a sterile Pasteur pipet and incubated at 27°C for 6 days. Cells were harvested by centrifugation at 1,000 rpm in a Beckman TJ-6 table top centrifuge for 10 minutes and the supernatant collected and stored at 4°C. Stored pellets were subjected to Western blot analysis to monitor UL42 protein expression.

**Amplification of Recombinant Baculovirus.** Cells (2 x $10^7$) were seeded into a 150 cc flask to 70% confluency in 25 ml of TNM-FH complete media. Cells were infected with virus at a moi of 1 pfu/cell for 1 hour at 27°C. Twenty five milliliters of fresh media was added to the cells and they were further incubated at 27°C for 6 days. The titer of the virus was determined by plaque assay in Sf9 cell monolayers.

**Preparation of crude cell extracts.** Baculovirus- infected Sf-9 Cells were harvested 72 hours post- infection and centrifuged at 3,000 rpm in a Beckman TJ-6 centrifuge for 10 minutes. Cells were resuspended in 2X lysis buffer (0.8 M NaCl, 40 mM Tris HCl (pH 8.2), 2 mM EDTA, 2mM BME 0.5 mM PMSF) and lysed by sonication. Cellular debris was removed by centrifugation at 13,00 x g at 4°C for 15 minutes. A cocktail of protease inhibitors 1 μg/ml aprotinin, leupeptin, pepstain and β-lactalbumin (Sigma, St. Louis, MO.) were added to the sample and the extract was stored at -80°C.

**Coimmunoprecipitation.** Nuclear extracts of Sf9 cells infected with recombinant baculovirus labeled metabolically with [$^{35}$S]-methionine were prepared and dialyzed against 1X B2 buffer ( 10 mM Tris-HCl (pH8.0) 10% glycerol) and stored at -80° C. Metabolically labeled proteins were subject to SDS-PAGE analysis to identify radiolabeled proteins. Immunoprecipitation was performed as described by Monahan et al (1993) with some modifications. Aliquots containing approximately equal amounts of radioactivity of recombinant proteins were mixed in a final volume of 100 µl with B2 buffer ( 10 mM Tris-HCl (pH8.0) 10% glycerol) and an equal volume of 2X Zweigs buffer ( 0.2 M Tris-HCl ( pH 8.0) 20% glycerol 1% Nonidet NP-40, 1% Sodium Deoxycholate, 0.4 mM PMSF). Samples were cleared by incubation at room temperature with 0.1 volume of a 50% (wt/vol) slurry of protein A-Sepharose CL-4B (Sigma, St. Louis, MO.) in 1X Zweigs buffer containing 0.02% sodium azide and centrifuged at 6500 x g for 30 seconds. Supernatants were mixed with 5 µl of antiserum overnight at 4°C. Immune complexes were recovered by the addition of 20 µl of protein-A-Sepharose for 1 hour at 4°C. The Sepharose beads were washed 4 times with buffer containing 0.1 M Tris HCl ( pH 8.0), 0.5 M NaCl and 1% 2-mercaptoethanol, 0.004% bromophenol blue. Bound protein was eluted by boiling for 3 minutes in 25 µl of dissociation buffer (0.125 M Tris-HCl (pH 6.8),0.2% SDS, 2% glycerol 5% 2-mercaptoethanol, 0.004% bromophenol blue) and analyzed by SDS-PAGE as described previously.

**Western blot analysis.** Cell pellets were resuspended in 500 µl of 2X lysis buffer and disrupted by sonication. A 25 µl aliquot was subjected to SDS-PAGE and transferred to nitrocellulose electrophoretically for 6 hours at 450 m Amps as previously described (Towbin et al., 1979). Nitrocellulose filters were blocked in TBS (Maniatis et al 1992) containing 3%

gelatin and 0.02 % sodium azide, washed with TBS, and probed with antibody diluted in TBS containing 1% gelatin. The blot was washed in TBS and incubated with 3 μCi [$^{125}$I] Staphylococcus aureus protein A (specific activity 63 mCi/mg; ICN Biochemicals, Inc., Irvine, CA) diluted in TBS with 1% gelatin as previously described (Gallo et al., 1988), and exposed to Kodak X-OMAT film at -80°C.

**Antibodies.** The mouse monoclonal antibody (mAb) 6898, reactive to an epitope which maps to amino acids 363 to 369 of UL42, was provided by Howard Marsden (University of Glasgow, Glasgow, Scotland). Polyclonal antibody (pAb) 834 was prepared by immunizing rabbits with a promiscuous T cell epitope fused to UL42 residues 360 to 377 as previously described (Monahan et al., 1993). A synthetic peptide corresponding to residues 1216 to 1224 of HSV-1 pol (UL30) was coupled to BSA and injected into rabbits to produce the pol specific antiserum 771 (Monahan et al.,1993). Peptides 834 and 771 were synthesized at the Peptide and Protein Engineering Laboratory at the Ohio State University, Columbus, OH).

**Plasmids and cloning**. Plasmids were maintained in E. coli host strains DH5α, JM101 or JM109. Large scale preparation of plasmid DNA was performed using Qiagen column chromatography (Qiagen, StudioCity, CA, USA) according to instructions of the manufacturer. Recombinant plasmids were constructed by standard methods (Maniatis et al., 1982) and will be described in detail below. All restriction enzymes were purchased from BRL (Bethesda Research Laboratories) except BseRI which was purchased from New England Biolabs(New England Biolabs, Beverly, MA).

Plasmids pT085 (a gift of Nigel Stow) and pON114 (a gift of Ed Mocarslic) were used for the origin-dependent DNA replication assay. HSV-1 pol and UL42 were subcloned

into phagemid vector pTZ19U downstream of the T7 RNA pol promoter in the sense orientation (Monahan et al., 1993). Plasmid pGEM2-702 contains full length pol sequences, while pTZ7-7.1 contains pol lacking the first 67 amino acids, as previously described (Dorsky et al.,1988). Plasmid pLBN19A, which encodes full length UL42 (Gallo et al., 1989), was used as the wild-type construct from which all mutants of UL42 were derived. Mutants i206, i140, d129-163, d202-337 and d140 were described by (Monahan et al.,1993), while mutants d137-142 and d274-288 were described elsewhere (Reddig et al.,1994). Mutants d256-282 and d282,83 were created by Exo III digestion of the UL42 SstI fragment followed by insertion of Bam HI (12 mer) linkers and re-cloning of fragments into PLBN19A parental vector (minus the Sst I fragment).UL42 mutations cloned into the parental phagemid vector (pTZ19U) are described by the mutant created. For example deletion of amino acids 129-163 is shown as d129-163.

**a. Cloning of PCR products .**

PCR amplification of UL42 or UL42 mutants was performed as described (Chapter 2). Eighty microliters of the PCR reactions were electrophoresed on a 1% low melting point agarose gel at 90 volts for 90 minutes. The bands corresponding to UL42 wild type or mutant sequences were excised and purified using glass beads (Bio 101 Inc., Vista, CA) according to the instructions of the manufacturer. Ten nanograms of each purified DNA was mixed with 50 ng PCR™ II Vector (Invitrogen, San Diego, CA, USA), 10µl of 10X ligation buffer (Invitrogen, San Diego, CA, USA), and 1 µl of T4 DNA ligase in a total volume of 20 µl . Ligations were incubated for 14 hours at 14°C. One microliter of this ligation mixture was used to transform One Shot™ *E. coli* cells (Invitrogen, San Diego, CA, USA) following

the protocol supplied by the manufacturer. Transformed cells were cultured on L-broth ( LB) plates containing 50 μg/ml ampicillin. Immediately before use, agar plates were spread with 25 μl of a 40mg/ml stock of 5-Bromo-4chloro-β-D-galactosidase (X-gal) in formamide. Cultures were grown at 37°C overnight and white colonies were selected. To verify the correctly sized insert, DNA was isolated from 3ml of LB (containing 50 μg/ml ampicillin) using the Wizard™ miniprep DNA purification system (Promega, Madison, WI, USA) according to instructions of the manufacturer. DNA was digested with EcoR1 (GIBCO, BRL Gaithersburg, MD) and electrophoresed on a 1% agarose gel to separate the insert from the vector sequences. Orientation of inserts was confirmed by Hind III/Nru I digestion (GIBCO, BRL Gaithersburg, MD) followed by agarose gel electrophoresis.

**b.Cloning of UL42 sequences into baculovirus transfer vector**.

Baculovirus transfer vector pVL1392 (PharMingen, San Diego, CA) was digested with EcoR1 (GIBCO, BRL Gaithersburg, MD) at 37°C for 2 hours, treated with calf intestine phosphatase (CIP) at 37°C for 15 minutes, subjected to electrophoresis in 1% LMP agarose, and the appropriate band excised and purified with glass beads. UL42 genes cloned into the TA vectors were released by EcoR1 digestion and the DNA fragment purified through LMP agarose as indicated above. The UL42 fragment was ligated into 1 μg of (CIP treated) pVL1392 using a 3:1 insert to vector ratio for 18 hours at 14° C. DNA was transformed into competent *E. coli* strain DH5α cells (GIBCO, BRL Gaithersburg, MD), according to the instructions of the manufacturer, and colonies screened for the correctly sized insert as indicated above. Plasmids containing inserts are preceded by the vector name pVL1392 followed by a slash and the relevant name of the UL42 mutation. For example,

pVL1392/d129-163 plasmid is the baculovirus transfer vector with UL42 sequences deleted for amino acids 129-163 while TA/d129-163 contains the same UL42 sequences cloned into the TA vector.

**c.Cloning of UL42 mutants into GST.**

The UL42 open reading frame (encoding amino acid residues 20-456 was cloned downstream of the glutathione-s-transferase (GST) gene which is under control of the *tac* promoter. The MluI fragment within the UL42 ORF was flanked with Bam HI linkers (New England Biolabs, Beverly, MA) and cloned into the Bam HI site of plasmid pGEX-2T (Pharmacia Biotech, Piscataway, NJ), containing the glutathione-S-transferase gene. This construct (GST/UL42) when induced with isopropyl-β-D-thiogalactopyranoside (IPTG) results in the expression of an N-terminal glutathione S-transferase (GST) fused to residues 20-456 of UL42. Deletion mutant d202-337 was expressed as a GST fusion protein by introducing the MluI fragment encoding this deletion mutation into pGEX-2T. The N-terminal 339 residues of the UL42 protein were expressed as a GST fusion protein by introducing the MluI to SmaI fragment of the UL42 gene into pGEX-2T. Mutant, i206, and deletion mutants d274-288, d256-282, d241-261, d282-3 were cloned into the GST vectors by replacing the internal Sst I fragment within the GST/UL42 vector with the Sst I fragment of the UL42 mutants cloned into the parental vector pLBN19A. For Region I UL42 mutants, BseRI fragments from TA vectors were cloned into the 600 bp CIP-treated BseRI fragment of GST/UL42 vector. Plasmids encoding GST fusion proteins were propagated in JM109 or DH5α cells and given the designation GST/d129-163, which describes amino acid deletion 129-163 cloned into the GST-containing vector pGEX-2T.

**PCR amplification of HSV-1 UL42 DNA**. The UL42 containing plasmid, pLBN19A (Gallo et al., 1989), was used as a template for PCR amplification. Plasmid sequences were digested with Hind III (GIBCO, BRL Gaithersburg, MD), electrophoresed on a 1 % low melting point agarose gel at 90 volts for 90 minutes (separating a 2.2 kb UL42 containing fragment from vector sequences), and the band was isolated and purified with glassmilk.

The optimized PCR mixture contained the following components: 1X PCR buffer (10 mM Tris-HCL pH 8.4, 50mM KCl, 1.5 mM MgCl), 250 μM of each of 4 dNTP's (dATP, dCTP, dGTP, dTTP), 2.5 units of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT, USA), 250 p moles of each primer, 5 % dimethyl sulfoxide (DMSO),and 100 ng of linearized DNA template. The PCR mixtures were overlayed with 1 drop of mineral oil (Sigma Chemical, St. Louis, MO, USA) in 0.5 ml Gene-Amp reactions (Perkin-Elmer) and subjected to cycling conditions. The "hot start" procedure was accomplished by adding the Taq polymerase to each reaction tube only after the block temperature had risen to 94°C for 5 minutes during the initial denaturing step. Thermal cycling conditions consisted of 40 cycles of a 1 minute denaturing step at 94°C, 30 seconds at 55°C (annealing step), and 2.5 min. elongation step at 72°C. The last cycle included a 10 minute extension step at 72°C. All reactions were performed in a Perkin Elmer DNA Thermal Cycler model # (Norwalk, CT, USA). Primer selection was made with the assistance of the Oligo 3.0 Software (Oligo National Bioscience, Inc., Plymouth, MO, USA).. Varying concentrations of $MgCl_2$ (0-5 mM), DNA 25-250 ng), primers (1.5 -250 pmoles) 1 & 2 (Table 2), and DMSO (0-20%) were used in the standard PCR reaction mixture (stated above) in order to identify the optimal conditions for amplifying UL42 (see Chapter 3).

**Site-directed mutagenesis of UL42.** An overlap extension PCR method (Ho et al., 1989) was used to introduce site-specific substitutions within the UL42 open reading frame. Two separate PCR reactions were required. PCR reaction 1 contained buffer A with 250 μM of each dNTP, 2.5 units of Taq polymerase, 50 ng pLBN19A (prepared as previously described), 5% DMSO, and 250 pmoles of each of 2 sets of primers (Figure 13A). For example, for the proline to glutamic acid mutation at amino acid 133, primer 1(outer) and 13 (inner mutagenic) were used in one reaction while primer 2 (outer) and 14 (inner mutagenic) were used in a separate PCR reaction. PCR was conducted with the following cycle parameters: denaturation at 94°C for 1 minute; annealing at 62°C for 30 seconds; and extension at 72°C for 2.5 min. Following 25 rounds of amplification, the PCR products were electrophoresed on a 1% low melting point agarose gel, the bands representing the left and right portions of the UL42 gene were extracted,and genecleaned (as stated previously) and the DNA concentration determined. In a second PCR reaction, 10 ng of each of the PCR fragments was mixed and amplified under the conditions of the first PCR reaction with 250 pmoles of the outside primers 1 and 2 (see Table 2). UL42 mutants R135D (Arginine-Aspartic acid), and T142A (Threonine-Alanine) used primer combinations (7 and 8), and (9 and 10) respectively as described in Table 2. The PCR product were gel purified as indicated above and cloned into a TA cloning vector as described above. Mutations were confirmed by sequencing with appropriate primers according to the protocol of the Sequence™ Version 2.0 DNA sequencing kit (USB Amersham Life Sciences, Cleveland, OH).

**DNA sequencing.** Mutations in plasmids containing UL42 DNA inserts were confirmed by sequencing. Forty microliters of Wizard miniprep DNA was denatured using the alkaline method (Maniatis et al., 1992) and the products were sequenced according to the protocol of the Sequenase™ Version 2.0 DNA Sequencing Kit (USB Amersham Life Sciences, Cleveland, OH). Universal forward primer (Primer 3) and universal reverse primer (Primer 4) were used to identify the 5' and 3' end of cloned PCR fragments into PCR II vector.

***In vitro* transcription/translation.** *In vitro* transcription/translation reactions were performed as previously described ( Monahan et al, 1993) with some modifications. Plasmids were linearized downstream the UL42 open reading frame (ORF) and run-off transcripts were generated with T7 RNA polymerase (Promega, Madison, WI, USA) for 1 hour at 40°C (according to the instructions of the manufacturer). Reactions were then DNase treated, phenol/chloroform extracted,ethanol precipitated and the RNA resuspended in water. RNA concentrations were determined spectrophotometrically. Fifty to 100 ng of transcript was translated in rabbit reticulocyte lysate (RRL), (Promega Biotech, Madison, WI) for 1 hour at 30 °C according to instructions of the manufacturer. For some experiments, [$^{35}$S] - L-methionine (specific activity 1000 to 1200 Ci/mmol, Amersham, Arlington, Heights Il) was added to translation reaction mixes to a final concentration of 1 μCi /ul. In some cases, proteins were expressed by coupled *in vitro* transcription/ translation using plasmid DNA and the TnT kit (Promega Biotech, Madison, WI) for 90 minutes at 30°C according to instructions of the manufacturer. Labeling in the coupled system was as in the above reactions. Translated proteins were analyzed by SDS-PAGE and fluorographed as previously described.

**Preparation of GST-fusion proteins**. For induction of fusion proteins, an overnight culture of E. coli carrying the GST fusion plasmid was diluted 1:10 into fresh L broth containing 50 μg/ml ampicillin. The culture was incubated at 37°C with vigorous shaking until $OD_{600}$ of 0.6 was reached. IPTG was then added to a final concentration of 0.1 mM and cultures were incubated at 37° C with shaking. After 12 hours, the culture was collected by centrifugation for 30 minutes at 3,000 x g and the bacterial pellets were stored at -20° C. Under the IPTG inducible promoter, GST/UL42 was synthesized to high levels (6-12 μg/ml of culture). The cells were thawed and suspended in TED buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT) containing PMSF, lysed with 20 μg/ml lysozyme, 0.1% Triton-X 100, and the DNA was sheared by sonication (Sonifier Cell Disruptor, Branson Co., Danbury , CT). The lysate was subjected to centrifugation at 10,000 x g for 15 min at 4°C and the supernatant fraction containing the fusion protein was retained and stored at 4°C.

GST activity was determined using the transferase-mediated reaction between GSH and 1 chloro-2,4-dinitrobenzene (CNDB method) according to the directions of the manufacturer (Pharmacia Biotech, Piscataway, NJ). Reactions were initiated by the addition of 10 μl of protein and the formation of the conjugate between GSH and 2,4-dinitrobenzene was measured spectrophotometrically at 340 nm. A standard curve of the change in absorbance for known concentrations of commercially prepared GST (Sigma Chemical Company, St. Louis ,MO) was prepared and used to calculated the concentration of mutant forms of UL42 protein, expressed as GST fusion proteins.

**Affinity chromatography.** Glutathione-agarose beads (Sigma Chemical Company, St. Louis ,MO.) were charged with various concentrations of GST or GST fusion proteins (as determined by CNDB method) overnight at 4 °C in TED buffer containing 0.1 M NaCl and 50 mg/ml bovine serum albumin. Fusion proteins immobilized to 500 µl of a 50% v/v slurry of glutathione agarose beads in TED + 0.1 M NaCl + 50 mg/ml BSA were collected by centrifugation at 3,000 x g for 10 min. and subsequently washed 4 times with 10 volumes of TED + NaCl buffer. *In vitro*-expressed or metabolically labeled pol (1.0 µCi/µl L-[$^{35}$S]-methionine) was mixed with the beads charged with GST or GST fusion proteins for 3 hours at 4 ° C with shaking. The 500 µl suspension was loaded into a disposable pipette tip plugged with glass wool, washed with 1 ml of TED + 0.1 M NaCl + 50 mg/ml BSA, and eluted with 5 mM glutathione. Three 250 µl elutions were collected lyophilized , suspended in 2X loading buffer (0.8 M NaCl, 40 mM Tris HCl (pH 8.2) 2 mM EDTA, 2mM BME 0.5 mM PMSF), analyzed by SDS-PAGE electrophoresis, embedded with EN$^3$HANCE (DuPont, Boston, MA) as previously described (Monahan et al., 1993) and flourographed. Radioactive bands representing the full length pol were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, Ca).

A similar procedure was followed to obtain partially purified GST or GST/UL42 proteins for biochemical analysis except that proteins eluted were dialyzed and stored at -80° C. Eluted proteins were dialyzed against B-2 buffer (20 mM Tris-HCl, pH 7.5, 50 mM EDTA, 10% glycerol, 2 mM BME) and stored in aliquots at -80 °C. Protein concentration was determined using the Bradford method and the CNBD assay (as previously described) and proteins were analyzed by SDS-PAGE followed by Coomassie blue or silver staining.

**DNA binding assay.** The ability of GST/UL42 mutant proteins to bind to double-stranded DNA was determined using a $^{32}P$ labeled 112 base pair piece of DNA probe. Plasmid pCRII (Invitrogen, San Diego, CA) was cut with Bam HI + EcoRI, and end-filled with Klenow enzyme (Gibco-BRL) in the presence of [$^{32}P$]-dATP. The 112 bp and 3800 bp DNA fragments resulting from restriction digestion were separated through agarose gel electrophoresis. The [$^{32}P$]-labelled 112 bp fragment was excised and purified using glassmilk GST/UL42 mutant proteins (0.25 mg/ml) were immobilized to glutathione agarose beads as described above, suspended in an equal volume of TED + 0.1 M NaCl + BSA, and loaded into 1 ml disposable pipette tips containing a glass wool plug (0.25 ml of 50% v/v beads per column). The glutathoine agarose beads, to which GST/UL42 proteins were bound, were washed with 8 column volumes (500 µl) of Tm (20 mM Tris-HCl, pH 8.0; 5 mM $MgCl_2$) + 50 mM NaCl. An end-filled $^{32}P$ labeled 112 bp fragment of pCRII was added to the column at room temperature. After 1 hr at room temperature, unbound oligonucleotide was removed by washing the column with 500 ul of Tm + 50 mM NaCl. Columns were subjected to successive washes with 500 µl (4 column volumes) of Tm containing 100 mM, 250 mM, 500 mM and 1M NaCl. Remaining GST fusion proteins were eluted by a final wash with 5 mM glutathione. Radioactivity in each elute was determined by liquid scintillation spectrometry. The amount of radioactivity released with each salt elution was expressed as a percentage of the total radioactivity loaded per column.

**DNA polymerase assays**. HSV-1 pol activity was measured as the incorporation of [$^{3}H$]dTTP (43 to 82 Ci/mmol; ICN or Amersham) into trichloroacetic acid-insoluble radioactivity at 37 °C using maximally activated calf thymus DNA as a template as described

previously (Dorsky et al.,1988, Gallo et al.,1989, and Monahan et al.,1993). To measure stimulation of pol activity, UL42 synthesized by IVTT or as a partially purified GST- fusion proteins was used to determine pol activity in buffer containing 125 mM KCl, 100 mM Tris (pH 8.0), 4 mM $MgCl_2$, 5 mM dithiothreitol, 5 mg bovine serum albumin, and each of the other three deoxynucleotide triphosphates at 0.2 mM in a volume of 100 µl. Pol activity was determined in the absence or presence of UL42 protein. Reactions were initiated by the addition of the pol and GST/fusion protein to the remaining assay reaction mixture at 37 °C for 30 min. Fold stimulation was calculated as the ratio of pol activity in the presence of UL42 to that in the absence of basal pol activity. In some cases, IVTT UL42 and pol were translated separately and mixed for 5 min. At 37° C prior to initiation of reactions.

**Assay for origin-dependent DNA replication**. Origin-dependent DNA replication (ODR) was assayed on BHK cell monolayers in 35 mm petri dishes. Cells (2 X $10^5$) were co-transfected using 5 µg of lipofectase (GIBCO, BRL Gaithersburg, MD) with origin-containing plasmid pT085 (0.4 µg) (Stow et al.,1983 ) and 1µg of TA plasmids (under control of the T7 promoter) representing wild-type or mutant UL42 genes . After 4 hours at 37 °C, the supernatant was replaced with new media. Cells were infected 12 hours later with HSV-1 CgalΔ42 at a moi of 10 pfu/cell and harvested at 24 hours post- infection. Total cellular DNA was extracted using the Easy-DNA Kit (Invitrogen, San Diego, Ca) and one-fourth of the total DNA was cleaved with EcoR1 and Dpn1 (GIBCO, BRL Gaithersburg, MD). The products of digestion were separated by electrophoresis on a 1% agarose gel and subjected to southern blot analysis as described below. Blots were probed with labeled vector sequences as described below. Washed filters were subjected to autoradiography and

quantitation of replication products was performed on a Molecular Dynamics Phosphorimager. The linear amplified plasmid band (pT085) was quantified versus the total amount of vector sequences in each lane. Amplification was calculated as the radioactivity of amplified vector sequences/ counts of total vector sequences x 100. Amplification of vector sequences were normalized to 100% for wild-type UL42.

ODR in Sf9 cells was performed as described by Stow et al. (1992) with some modifications. Monolayers of Sf9 cells ($2x10^5$ cells/plate) were transfected with 0.4μg of pT085 (origin-containing plasmid) using a lipofectase-mediated procedure (GIBCO, BRL Gaithersburg,MD) according to instructions of the manufacturer. After 4 hours at 27° C, the supernatant was replaced with new media. Twelve hours later, cells were infected with a cocktail containing recombinant baculovirus expressing the 7 genes required for HSV-1 ODR at a moi of 5 pfu/cell of each virus for 1 hour 27° C. The inoculum was replaced with 3 ml of new media and incubated at 27° C. Cells were harvested at 50 hours p.i. and total cellular DNA was prepared as described above.

**Southern blot analysis.** Sf9 cells were seeded at 1 x $10^6$ cells/well in a 6 well (35 mm diameter/well) plate in 3 ml of TNM-FH complete media. Cells were infected with recombinant virus at a moi of 10 pfu/cell and incubated at 27°C for 48 hours. Cells were harvested by centrifugation at 1,000 rpm for 10 minutes, total cellular DNA was extracted by the Easy DNA method (Invitrogen, San Diego,CA), and one fourth of the DNA was cleaved with 10 units of EcoR1 (GIBCO, BRL Gaithersburg, MD). The DNA fragments were separated by electrophoresis on a 1% agarose gel at 90 volts for 1 hour, denatured with 1.0 M NaOH/ 0.5 M NaCl, neutralized with 1.5M NaCl/1M Tris (pH 7.4), and immobilized to

nytran membrane (Schliecher and Shuell,Keene,NH ) by the capillary transfer method (Maniatis et al., 1992). Blots were baked for 2 hours at 80°C to fix DNA to the membrane. Blots were incubated in hybridization buffer (1X SSC, 5X Denharts , 0.1% SDS) for 2 hours at 65°C. Hybridization was performed overnight at 65 °C in hybridization buffer ( modified to 6X SSC) containing denatured UL42 probe sequence. The probe was prepared by labeling a 756 base pair UL42 Pst I fragment to a specific activity of approximately $1.2 \times 10^8$ cpm/μg by using the random prime technique and a Oligolabeling kit (Pharmacia Biotech, Piscataway, NJ) according to instructions of the manufacturer. The fragment was purified over an aqua select sephadex G-50 spun column (5' Prime-3' Prime, Inc.,Boulder ,Co ), denatured at 95°C for 10 min. with 100 μg of salmon sperm DNA, and quenched on ice. After hybridization, membranes were washed in 1X SSC for 15 minutes at room temperature and at a final stringency of 0.5X SSC containing 0.1% SDS at 65°C for 15 minutes and exposed to Kodak X-OMAT film.

# CHAPTER 3

## PCR AMPLIFICATION OF UL42 MUTANTS

In order to understand and study the role of UL42 in HSV-1 DNA replication, it was necessary to design a system to express large amounts of mutant and wild-type UL42 protein. A PCR-based system was used to facilitate the cloning of UL42 mutants into the baculovirus genome for expression. For expression purposes, primers designed for PCR removed 300 bp of upstream untranslated sequence of cloned UL42 in the vector pBLN19A (Chapter 2).

### Optimization of PCR for amplification of UL42

The HSV-1 DNA sequence has a high overall G+C content (approximately 68%), with some regions approaching 80% (Berker and Dym, 1968; Kieff et al., 1971; McGeoch et al., 1988). In order to overcome many of the problems that occur with amplifying high G+C containing DNA, the parameters of the PCR reaction were examined. Standard PCR conditions (Chapter 2) were modified using increasing $MgCl_2$. Amplification of a 1518 bp UL42 band (devoid of 300 bp untranslated UL42 sequence) was observed with the addition of 1.5 mM $MgCl_2$ (Figure 2, lane 3 ). Concentrations above 1.5 mM $MgCl_2$ resulted in an inhibition of amplification (Figure 2 , lanes 2-6). The 1518 bp band observed with 5.0 mM $MgCl_2$ was not present in repeat experiments (data not shown).

The use of dimethyl sulfoxide (DMSO) (Ted et al.,1991) and formamide (Sarkar et al.,1990) in PCR reaction enhances the amplification of high G/C DNA in many instances by removing secondary and tertiary structures within the molecules to be amplified. Amplification of UL42 was inhibited in reactions that included formamide (data not shown). PCR reactions with DMSO ranging from 0-20% revealed that 5% and 10% DMSO yielded amplified UL42 DNA. Reactions which lacked DNA (Figure 3, lane 1) yielded no amplification, while DMSO concentrations above 10% inhibited amplification (Figure 3, lane 6). In experiments without DMSO, amplification of UL42 was achieved only with conditions that included high amounts of template (1μg) and annealing temperatures below 42°C. These conditions also resulted in amplification of non-specific PCR products (data not shown). Thus, the use of DMSO greatly enhanced the amplification of UL42 and increased the specificity of amplification.

As indicated above, the addition of DMSO to 5% lowered the amount of template required to achieve amplification. Titration of template amount revealed that 25 ng of UL42 DNA could be amplified but was maximal with 250 ng of DNA template under these reaction conditions (Figure 4).

I next determined the effect of increasing primer concentration on UL42 PCR amplification. Primer concentrations were optimal at 30 pmoles of each primer 1 and 2 (Table 2) (Figure 5, lane 5) and higher amounts had little effect on the amount of full length product when 100 ng of template was included (Figure 5, lanes 6-8). No false-priming or the production of non-specific amplified products was observed under these conditions which included 5% DMSO. An annealing temperature as low as 52°C was sufficient to allow

specific amplification of UL42. Annealing UL42 primer to template at temperatures above 52°C significantly decreased amplification of UL42 DNA (Figure 6 ) but did somewhat increase specificity.

**Amplification of UL42 mutants**

In order to provide a system that would allow the rapid cloning of UL42 mutations into a variety of vector systems, it was important that the conditions for amplification of wild-type sequences be sufficient for other UL42 mutations cloned into phagemid vectors. Results from the amplification of UL42 mutations d129-163, d202-337, d274-288 together with wild-type UL42 are shown in Figure 7. By using the conditions stated earlier: 1X PCR buffer (10 mM Tris-HCL pH 8.4, 50mM KCL, 1.5 mM $MgCl_2$), 250 uM of each of 4 dNTP's (dATP, dCTP, dGTP, dTTP) 2.5 units of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT, USA), 250 p moles of each primer, 5 % DMSO, and 100 ng of linearized DNA template, it was possible to amplify sequences representing the UL42 mutations shown in Figure 7. These results demonstrate that the specificity of the optimal PCR reactions for wild-type UL42 sequences remained constant for various constructs of UL42 and provided a useful protocol for the production of UL42 genes lacking 5' untranslated sequences (Figure 8).

**PCR-directed mutagenesis of UL42**

The protein encoded by UL42 has several regions of high homology with pol accessory proteins from other alphaherpesviruses including, pseudorabies virus, equine herpesvirus, and varicella-zoster virus (Berthomme et al, 1995). Berthomme's alignment was refined using the CLUSTALV multiple alignment program. Analysis of this alignment identified several conserved amino acid residues located within region 1 of UL42 (Figure 9a),

described by (Monahan et al., 1993). Using Chou-Fasman and Robson-Garnier algorithms, Monahan predicted that this region formed a helix-coil- helix motif (Figure 9b ). Based on these features, I selected residues in the helix and the coil regions throughout this domain for mutagenesis (Figure 9b ). I predicted that mutations that affect the integrity of the helix-coil-helix motif would impair UL42 pol accessory function. A PCR-directed strategy (Figure 10) was used to produce overlapping PCR fragments containing mutations of amino acids 133, 135, and 142 (Figure 11). These fragments were purified, denatured, and re-annealed in a second PCR reaction with outside primers (as described in Chapter 2) to produce full length mutant DNA products (Figure 12 ). There was variability in the amount of full length amplified product produced for each mutation (Figure 12) that most likely resulted from differences in the efficiencies of the mutagenic primers. Mutations at amino acid residues 135 and 142 were confirmed by sequence analysis. However, mutations at amino acids 133 and 160 were not created primarily because of the high G+C DNA flanking those residues.

**Confirmation and initial characterization of PCR-directed UL42 mutations.**

In vitro transcription/translation (IVTT) is an efficient and well-documented system for the study of UL42:DNA polymerase interactions (Gallo et al., 1989; Coen et al., 1995; Monahan et al., 1993; Dorsky and Crumpacker., 1988). Previous reports have shown a 4-7 fold stimulation of pol activity when pol and UL42 transcripts were co-translated in rabbit reticulocyte lysates (RRL) compared to the activity of pol translation products (Gallo et al., 1993). This system has proved useful for defining domains of UL42 required for its functional interaction with pol (Monahan et al., 1993), though the sensitivity of this system to screen mutations is often hindered by low or inconsistent levels of expression.

In order to limit the variability of expression of mutant UL42 proteins used in the pol stimulation assay, linearized TA/UL42 plasmids (Chapter 2) were transcribed with T7 RNA polymerase as described in Chapter 2. One hundred nanograms of RNA encoding each of the mutant proteins was translated with RRL supplemented with 1μCi/μl of [$^{35}$S]-L-methionine in order to ensure that mutant UL42 RNA's were equally translated with similar amounts of RNA. Results showed that most of the mutants tested had similar levels of translated protein, though defective translation was observed for P133G and d256-288 mutations (Figure 13). Expression of these mutants were later confirmed by IVTT (data not shown). A doublet band was evident for most products at lower expression levels. The doublet had no affect on the ability of UL42 to stimulate pol activity (data not shown). The lower mobility (top) band resulted from an initiation AUG codon within the TA vector that added 16 amino acids in frame to the UL42 ORF. Plasmids containing mutations i140 and d140 yielded similar protein expression levels as those mutants shown in Figure 13 (data not shown).

The ability of pol alone to incorporate [$^{3}$H]dTTP into gapped calf thymus DNA template was defined as basal pol activity. Pol stimulation assays compared the amount of pol activity with pol accessory protein (UL42) to the basal pol activity. In high salt conditions (125 mM), UL42 has been shown to stimulate pol activity 4-7 fold (Gallo et al., 1989).

In order to determine the effect that UL42 mutations have on the ability of UL42 to stimulate pol activity, a plasmid containing the pol gene, pT7-7.1 (as described in Chapter 2) and TA vectors containing UL42 mutants were translated in separate reactions and mixed at 37°C for 5 minutes. Reactions for the assay of pol activity were initiated by the addition of the translation mixtures and reactions were incubated at 37°C for 30 minutes. Pol activity

was measured as TCA precipitated counts on filter discs. In these experiments, the wild-type UL42 translated product stimulated pol activity 5-fold over the basal pol level. Pol activity in the presence of R135D and i140 was indistinguishable from that in the presence of wild-type UL42, while mutants T142A and d137-142 failed to stimulate pol activity (Figure 14 a ). Mutants d202-337, d274-88 and d282,3 all failed to stimulate pol activity, while d241-61 d256-82, and d270 stimulated pol at or above wild-type UL42 levels (Figure 14 b). The activity of mutants d256-82, d270, T142A and i140 is different from the results observed with the same mutants expressed as gst fusion proteins. As seen with the IVTT of the proteins (Figure 13), variability in protein expression often occurs and could affect the quantification of the results from this assay. The differences in pol stimulation of these mutants probably reflect the low basal pol stimulation activity which was approximately 500 counts for IVTT-expressed basal pol protein as compared to the 5,000 used with the gst pol stimulation assay. For these reasons, this experiment was only performed once and gst pol stimulation assay which allowed control over the amount of protein used in the pol stimulation assay was favored. These results are consistent with previous results that demonstrate that there are at least 2 separable domains of UL42 which are required for pol accessory function. More importantly, the ability of PCR-derived wild-type and mutant UL42 proteins to stimulate pol activity suggests that amplification of UL42 did not cause mutations in the gene that affected pol stimulation. At the same time, these results demonstrate that UL42 is sensitive to mutations and that these mutations severely impair UL42's ability to stimulate pol activity on activated calf thymus DNA template.

| Primer # | Oligonucleotide (5'-3') | Length | Primer Description |
|---|---|---|---|
| 1 | ccagcccgcgttggatgac | 20 | UL42 5' upper primer used in amplification of entire (1.5 kb) UL42 sequence |
| 2 | ccgggatggtgcgagttg | 20 | UL42 3' lower primer used in amplification of entire (1.5 kb) UL42 sequence |
| 3 | atcacgggccaggccgagttc | 22 | upper mutagenic primer changing amino acid 133 from proline to glutamic acid of UL42 |
| 4 | gaaactcggcctgcccgtgat | 22 | lower mutagenic primer changing amino acid 133 from proline to glutamic acid of UL42 |
| 5 | ccccgttgacacgctggtt | 20 | upper mutagenic primer changing amino acid 135 from arginine to aspartic acid of UL42 |
| 6 | ctgacccagcgtgtcaaacg | 20 | lower mutagenic primer changing amino acid 135 from arginine to aspartic acid of UL42 |
| 7 | ggacgtcgtcgcccatatgc | 20 | upper mutagenic primer changing amino acid 142 from threonine to alanine of UL42 |
| 8 | cgcatatgggcgacgacgtc | 20 | lower mutagenic primer changing amino acid 142 from threonine to alanine of UL42 |
| 9 | ctgatgaaggacgaactgacga | 22 | upper mutagenic primer changing amino acid 160 from arginine to aspartic acid of UL42 |
| 10 | tcgtcagttcgtccttcatcag | 22 | lower mutagenic primer changing amino acid 160 from arginine to aspartic acid of UL42 |
| 11 | aacagctatgaccatg | 16 | M13 reverse universal sequencing primer |
| 12 | gtaaaacgacggccag | 16 | M13 forward universal sequencing primer |
| 13 | aggcccagctcaccaaggt | 20 | Hpa I sequencing primer for residues 187 C-terminal |
| 14 | gcgtgtcgtccagcacc | 17 | Not I seq. primer allows seq through a.a 248 of UL42 C-terminal |
| 15 | ggaccacagagaag | 15 | α Not I seq. primer allows seq through a.a 248 of UL42 N-terminal |
| 16 | gaggacgactccgatgc | 17 | Nru I 65k seq. primer |
| 17 | gacctacgtcgggtggag | 18 | Nde I seq. primer through a.a 140 of UL42 N-terminal |
| 18 | gctcgtcagttcgcg | 15 | α Nde I seq. primer through a.a 140 of UL42 C-terminal |
| 19 | gaccagccagtccag | 16 | α Pst 65k seq. primer through a.a 281 of UL42 N-terminal |
| 20 | gtggctgaccgcgtc | 15 | α Sst I seq. primer through a.a 337 of UL42 N-terminal |

**Table 3. PCR and Sequencing Primers**

**Figure 2. Amplification of UL42 with increasing magnesium.** PCR reactions were performed in a 100 µl reaction which contained various amounts of $MgCl_2$, 1X PCR buffer, 5% DMSO, 250 µM of each of 4 dNTP's, 250 pmoles of each primers 1 and 2, 100 ng of DNA, and 2.5U of Taq polymerase. A denaturing temperature of 94°C for 1 min, followed by an annealing step at 55°C for 30 seconds, and an elongation temperature of 72°C for 2.5 minutes was used for a total of 40 cycles.

**Lanes:**

1. Lambda marker
2. No DNA template (n.d.)
3. DNA + 1.5 mM $MgCl_2$
4. DNA + 2.0 mM $MgCl_2$
5. DNA + 3.0 mM $MgCl_2$
6. DNA + 4.0 mM $MgCl_2$
7. DNA + 5.0 mM $MgCl_2$

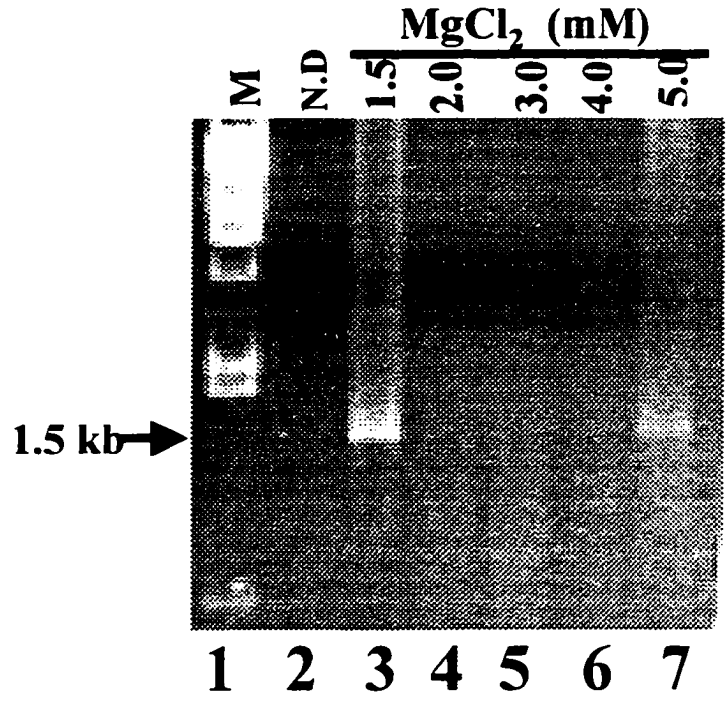



**Figure 2.**

**Figure 3. Amplification of UL42 with increasing DMSO.** PCR reactions were run as described in Figure 3 except DMSO concentration varied.

**Lanes:**
1. No DNA template (n.d.)
2. DNA + 1% DMSO
3. DNA + 5% DMSO
4. DNA + 10% DMSO
5. DNA + 20% DMSO

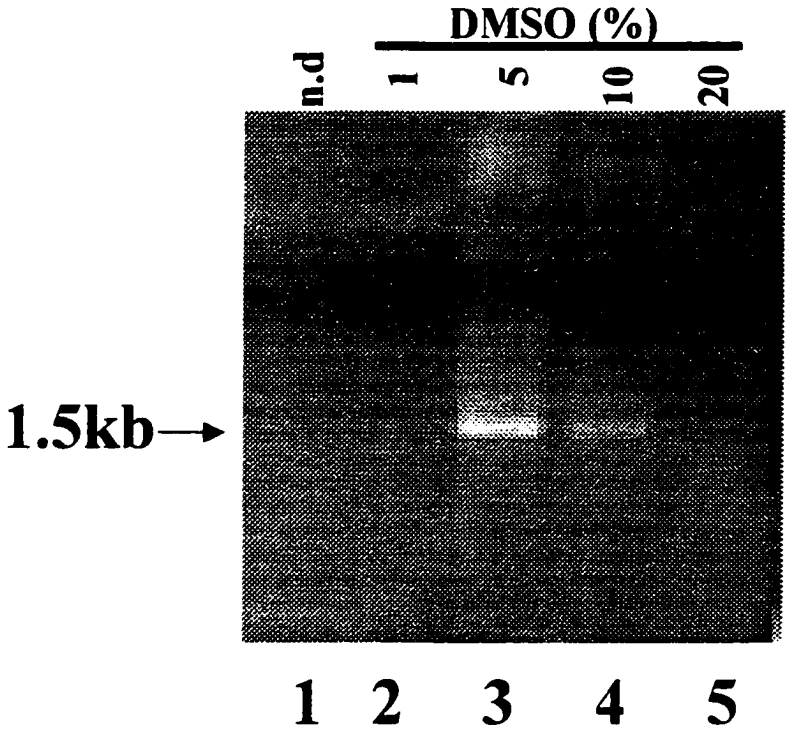



**Figure 3.**

**Figure 4. Amplification of UL42 with increasing template (pLBN19A).** PCR reactions were run as described in Figure 3 with 5% DMSO. The amount UL42 (2.2 kbp) template varied. Arrow denotes 1.5 kb amplified UL42 DNA.

**Lanes:**

1. Lambda marker
2. 25 ng DNA
3. 75 ng DNA
4. 125 ng DNA
5. 250 ng DNA

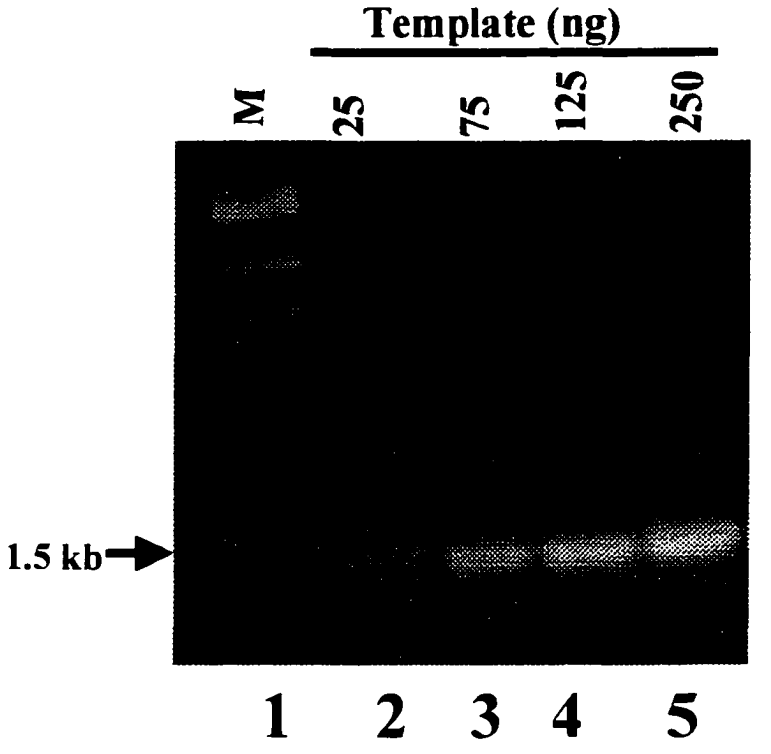



**Figure 4.**

**Figure 5. Amplification of UL42 with increasing primer concentration.** PCR reactions were run as described in Figure 3 (with 100 ng template) with the exception of increasing amounts of primer 1 and 2. (appendix, Table 1).

**Lanes:**

1. Lambda marker
2. DNA + 1.5 pmoles primer 1 and 2
3. DNA + 7.5 pmoles primer 1 and 2
4. DNA + 15 pmoles primer 1 and 2
5. DNA + 30 pmoles primer 1 and 2
6. DNA + 45 pmoles primer 1 and 2
7. DNA + 60 pmoles primer 1 and 2
8. DNA + 75 pmoles primer 1 and 2

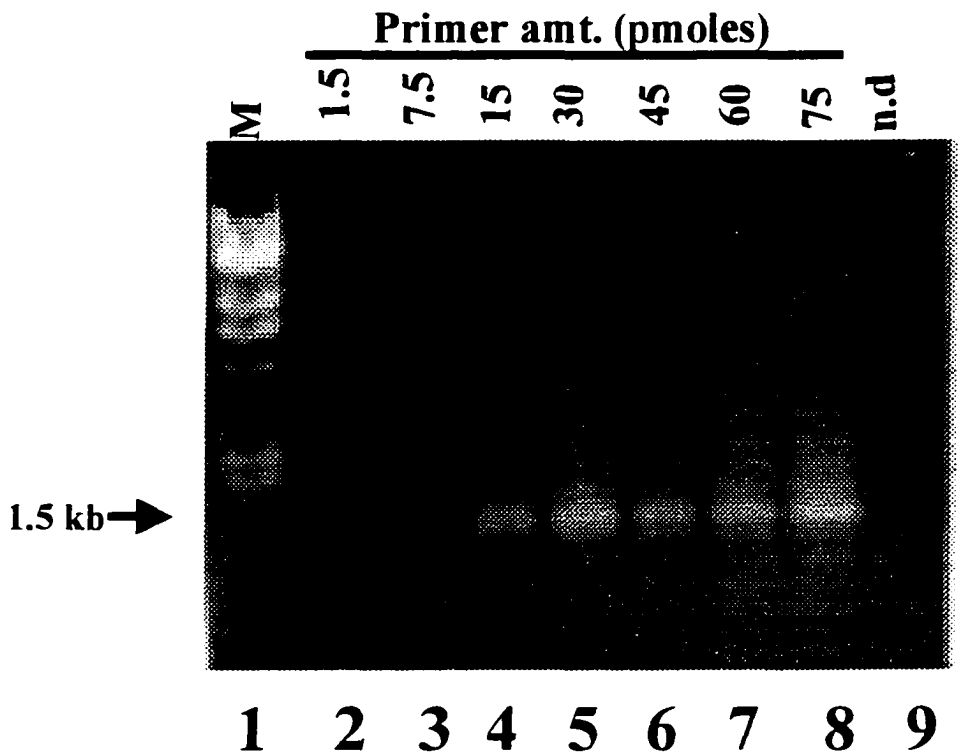



**Figure 5.**

**Figure 6. Amplification of UL42 with increasing annealing temperatures.** PCR reactions were run as described in Figure. 3 with various annealing temperatures. Arrow denotes 1.5 kb amplified UL42 DNA.

**Lanes:**
1. Lambda marker
2. 42°C
3. 52°C
4. 57°C
5. 62°C
6. 72°C
7. No DNA template

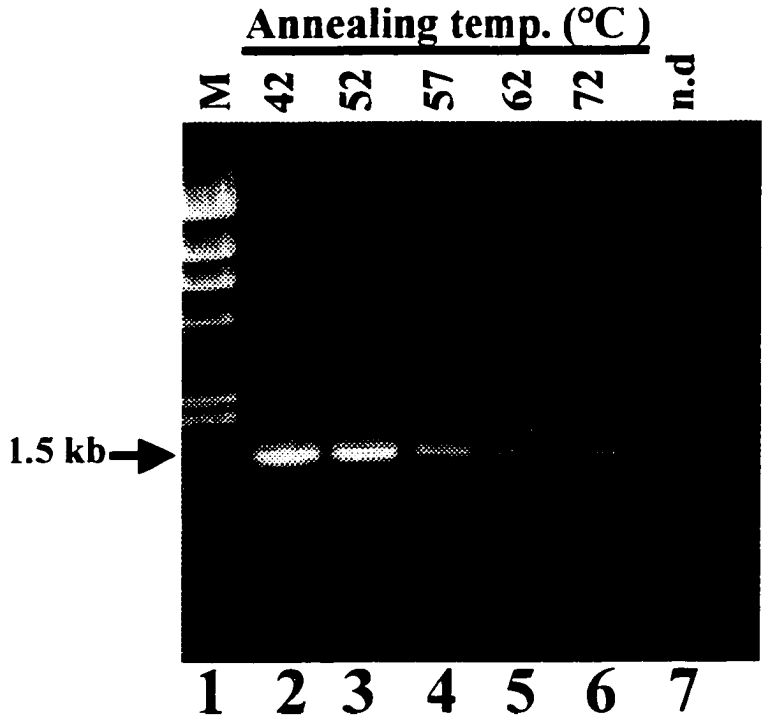



**Figure 6.**

**Figure 7. Amplification of UL42 mutants.** PCR reactions were performed as described in Figure 3 but with optimal PCR parameters; 5% DMSO, 1.5 mM $MgCl_2$, 125 ng DNA, 250μM of each of 4 dNTP's and 50 pmoles of primers 1 and 2. Cycling conditions were 94°C for 1 minute, 52°C annealing for 30 seconds and 72°C elongation for 40 cycles. One -fifth of the reactions were run on a 1% agarose gel and stained with ethidium bromide.

**Lanes:**

1. Wild-type UL42
2. d129-163
3. d202-337
4. d274-288
5. No DNA template

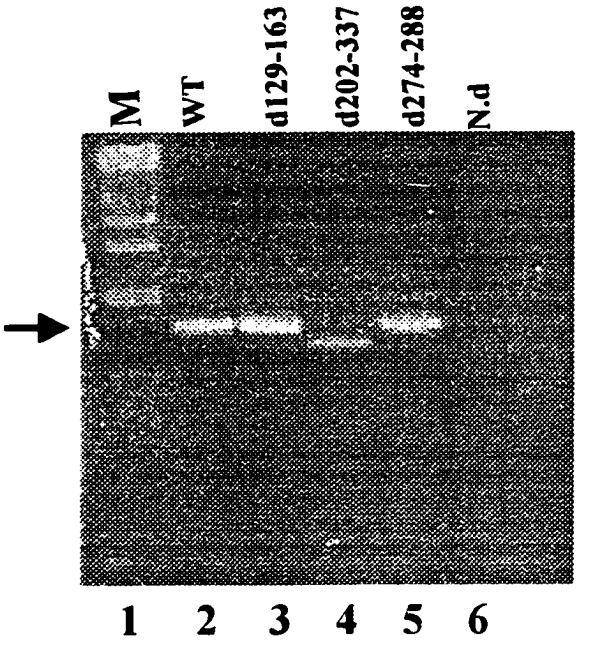



**Figure 7.**

**Figure 8. Schematic diagram of UL42 mutations.** Insertion mutations (i) were made by inserting in-frame linkers at convenient restriction sites and deletion mutations were constructed as described in chapter 2. Point mutations were constructed by overlapping PCR mutagenesis as described in chapter 2.

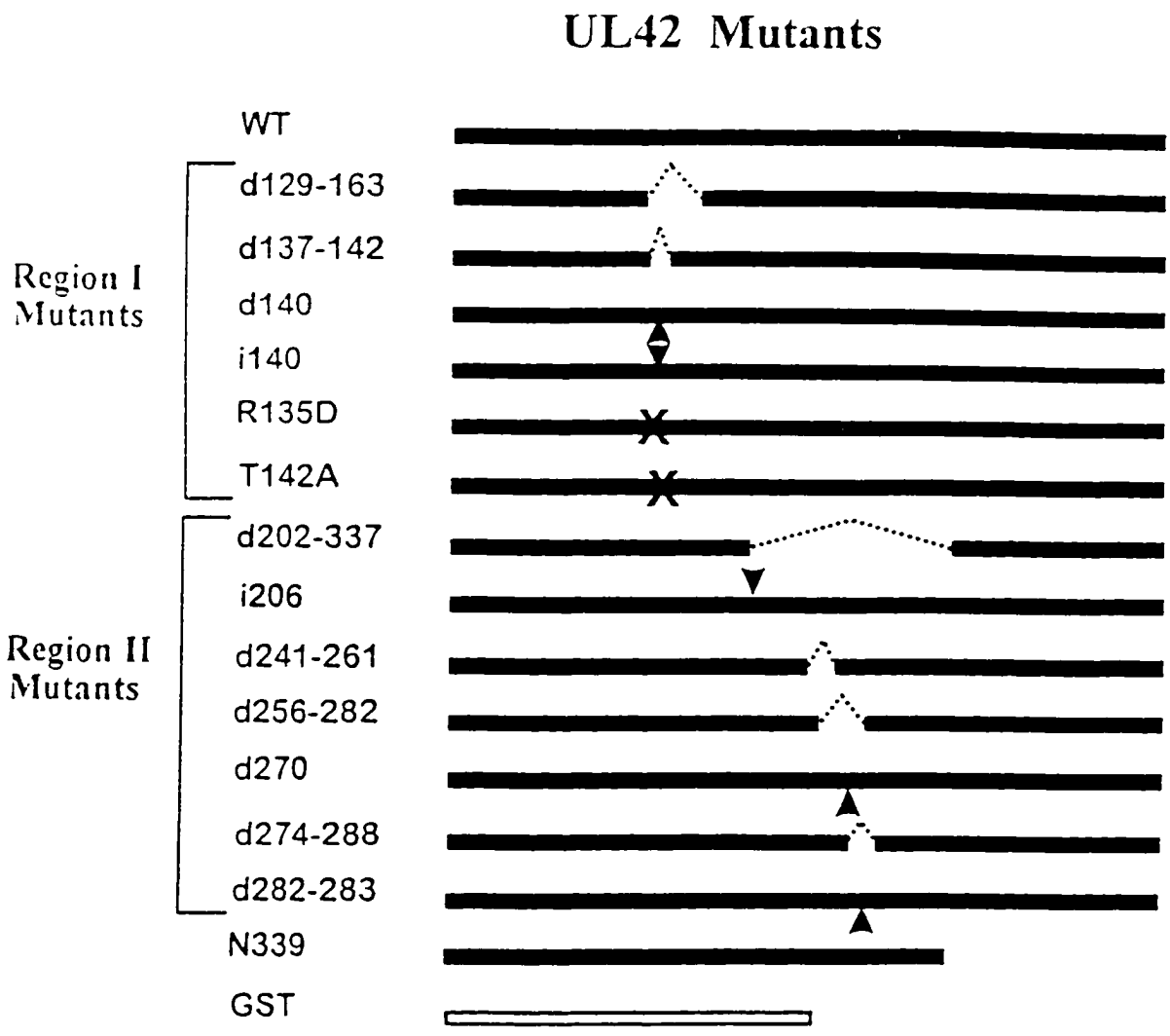
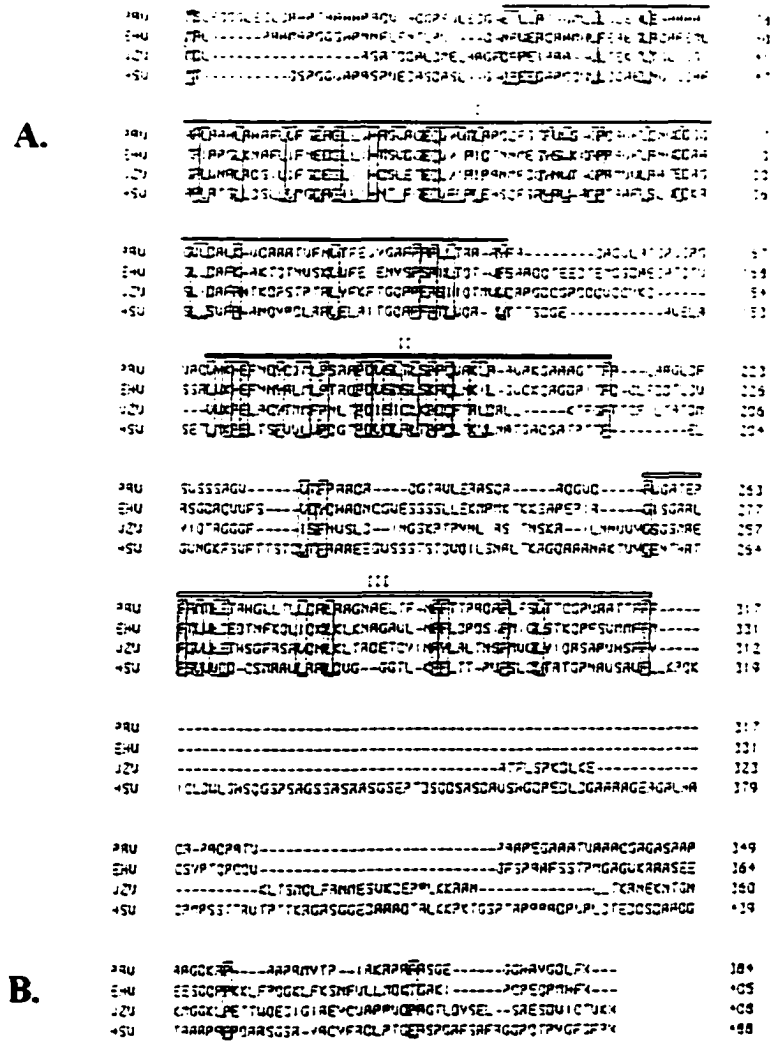

UL42 Mutants
WT
d129-163
d137-142
d140
i140
R135D
T142A
d202-337
i206
d241-261
d256-282
d270
d274-288
d282-283
N339
GST
Region I Mutants
Region II Mutants

**Figure 8.**

**Figure 9a. Amino acid alignment of 3 conserved domains of pol accessory proteins (PAP) from 4 alphaherpesviruses.** Pseudorabies virus (PRV) PAP was aligned with its counterparts from HSV-1 (UL42 gene), VZV (gene 16), and EHV-1 (gene 18), using the multiple alignment CLUSTALV computer program (Berthomme et al.,1995). **b. Predicted secondary structure of UL42 region I as described by Monahan (unpublished).**




# Predicted Secondary Structure Of UL42 in Region I

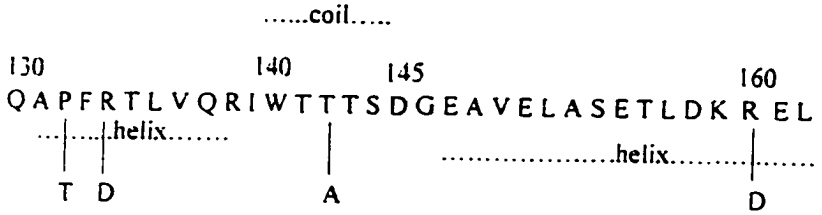



**Figure 9 .**

**Figure 10. Two-step PCR mutagenisis of UL42**. Combining two separate PCR products with overlapping sequences into one new full length longer UL42 product. The overlapping (inside primers) are shown containing an amino acid change within UL42.

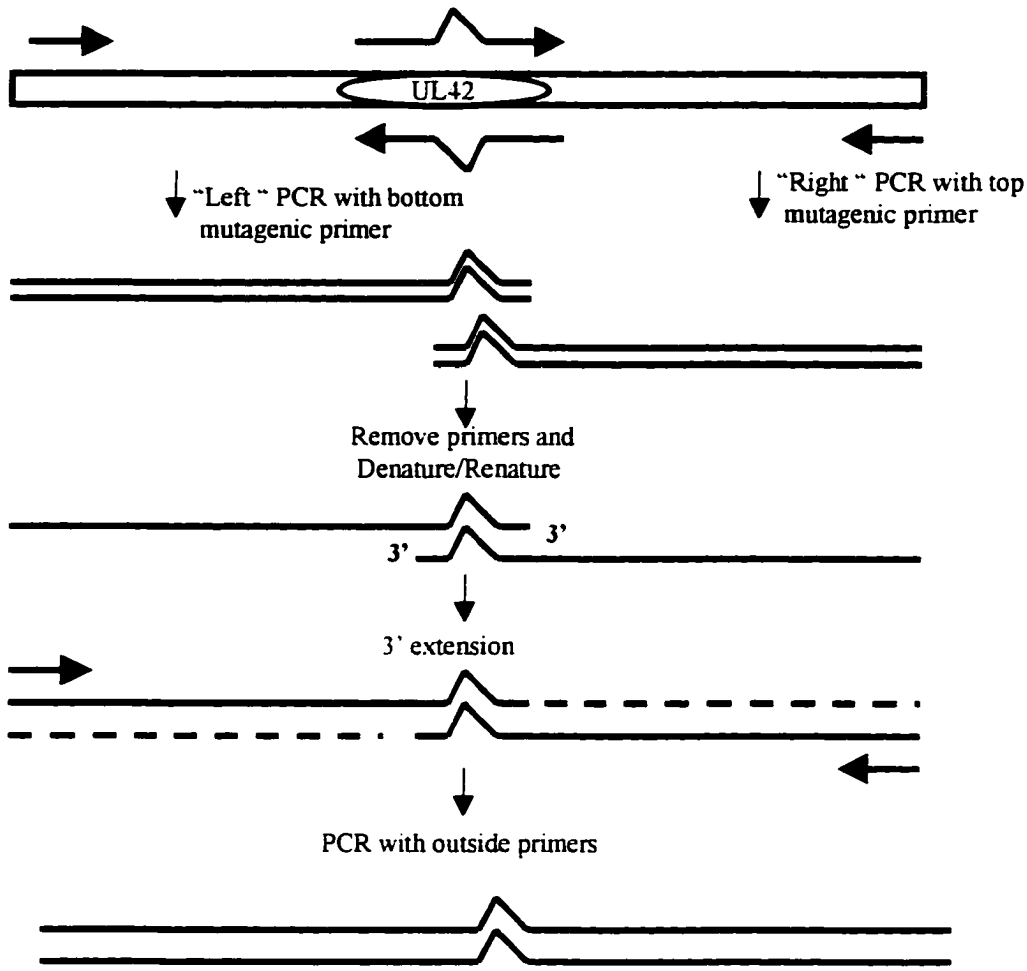

UL42
"Left " PCR with bottom mutagenic primer
"Right " PCR with top mutagenic primer
Remove primers and Denature/Renature
3'
3'
3' extension
PCR with outside primers

**Figure 10.**

**Figure 11. Two step PCR-directed mutagenesis.** PCR reactions with mutagenic primers for region 1 (Table 2) were performed as described in Chapter 2.

**Lanes:**
1. DNA Marker (M) Lambda Hind III digested
2. DNA + primers 1 and 133L
3. DNA + primers 2 and 133R
4. DNA + primers 1 and 135L
5. DNA + primers 2 and 135R
6. DNA + primers 1 and 142L
7. DNA + primers 2 and 142R
8. DNA + primers 1 and 160L
9. DNA + primers 2 and 160R
10. No DNA template (n.d)

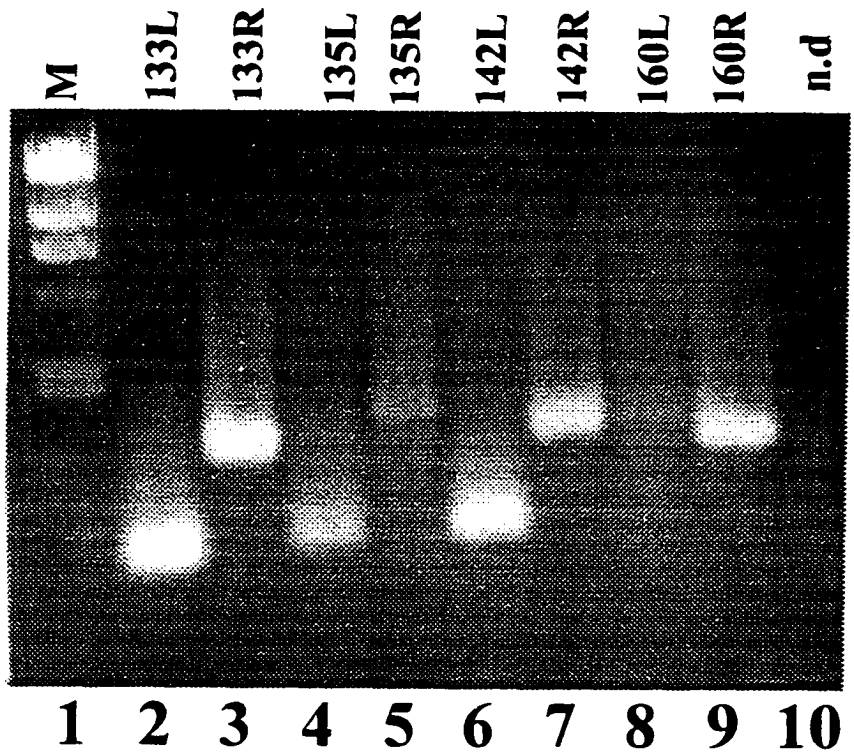



**Figure 11.**

**Figure 12. Two step PCR-directed mutagenesis.** A second mutagenic PCR reaction was performed by combining overlapping sequences to produce full length UL42 (Chapter 2).

**Lanes:**
1. Marker
2. P133G left and P133G right DNA
3. R135D left and R135D right DNA
4. T142A left and T142A right DNA
5. R160D left and R160D right DNA
6. No DNA template

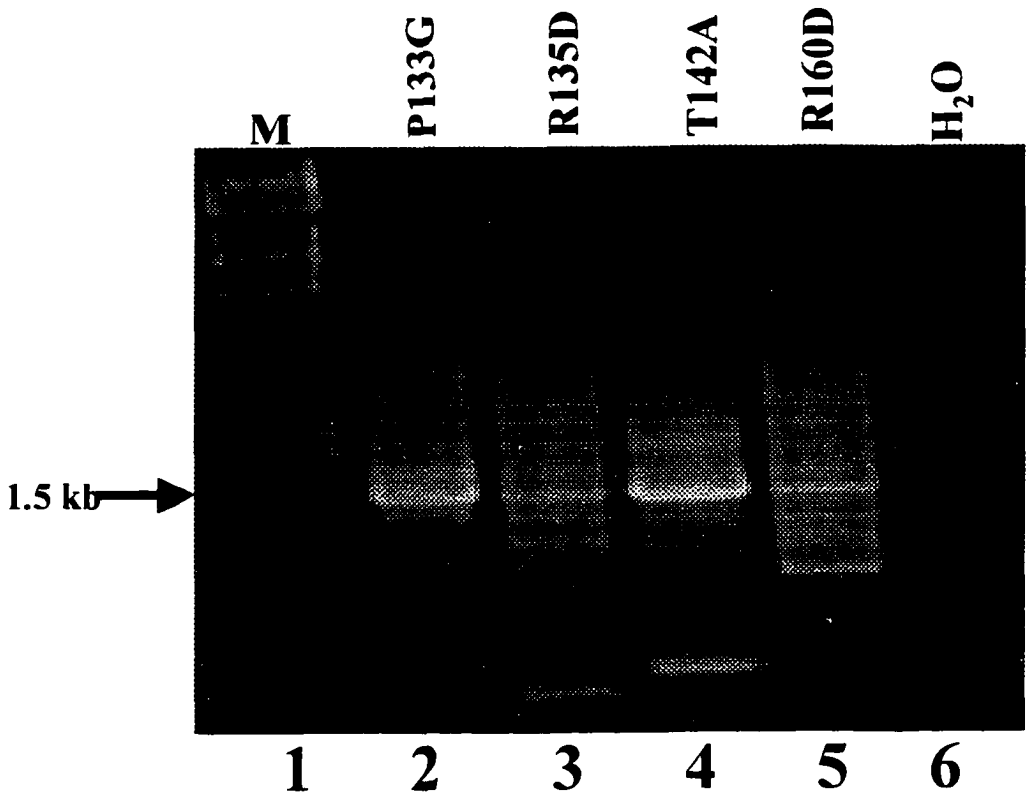



**Figure 12.**

**Figure 13. *In vitro* transcription and translation of UL42.** TA vectors containing UL42 mutations were linearized and transcribed with T7 RNA polymerase. The resulting mRNA was translated in RRL in the presence of $^{35}S$-labeled methionine as described in Chapter 2. An autoradiogram of an SDS-PAGE is shown.

**Lanes:**
1. Marker
2. WT (wild-type)
3. d129-163
4. d202-337
5. d256-282
6. d137-142
7. d274-288
8. P133G
9. R135D
10. T142A
11. D160U
12. Pol
13. No plasmid

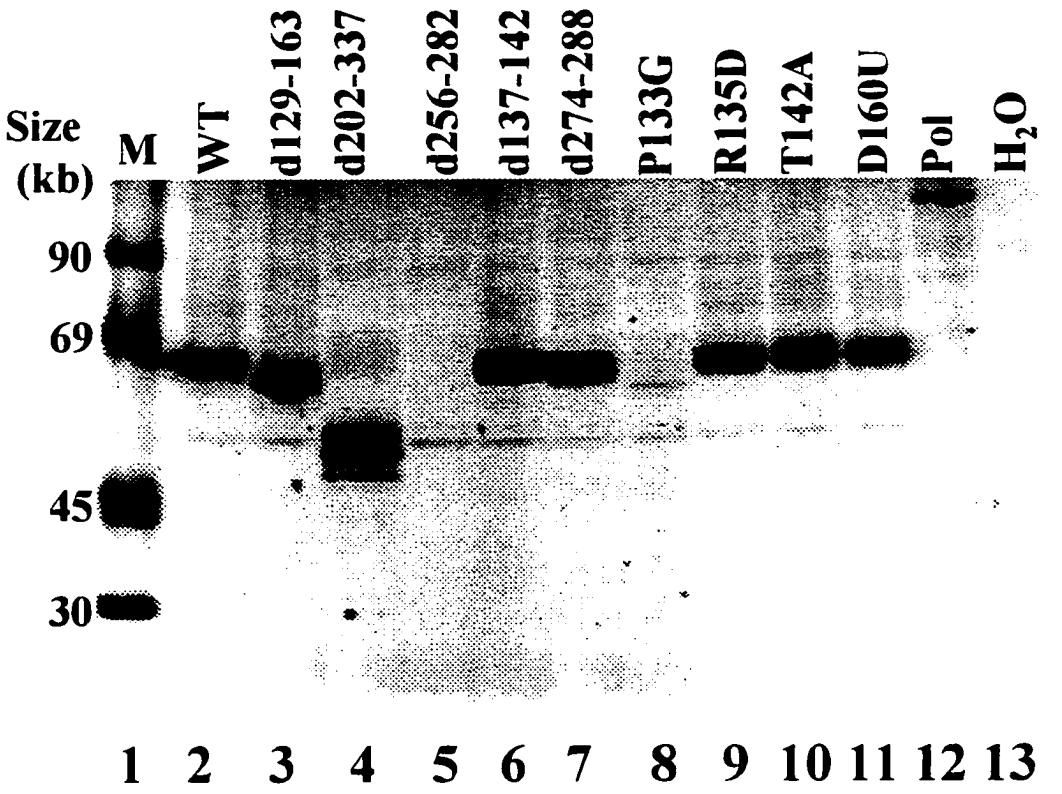

Size
(kb)
M
WT
d129-163
d202-337
d256-282
d137-142
d274-288
P133G
R135D
T142A
D160U
Pol
H2O
90
69
45
30
1 2 3 4 5 6 7 8 9 10 11 12 13

**Figure 13.**

**Figure 14. Stimulation of pol activity by IVTT expressed UL42 wild-type or mutant plasmids.** Linearized TA vectors containing UL42 mutations were transcribed and translated. Translated proteins were mixed with IVTT pol and assayed for the incorporation of [$^3$TTP] into activated calf thymus DNA. A) Results for Region I mutants B) Results for Region II mutants.

Reactions

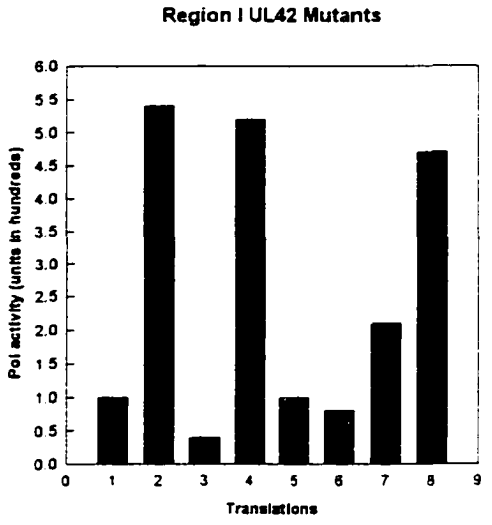
A. Region I UL42 mutants

1. pol
2. R135D
3. T142A
4. wild-type
5. d129-163
6. d137-142
7. d140
8. i140

B. Region II UL42 mutants

1. wild-type
2. d202-337
3. d256-282
4. d274-288
5. d241-261
6. d282,3
7. d270
8. pol

A


Region I UL42 Mutants
Pol activity (units in hundreds)
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9
Translations

B

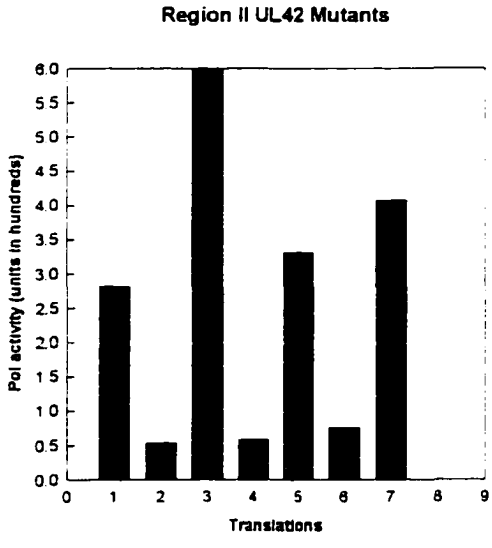

Region II UL42 Mutants
Pol activity (units in hundreds)
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 1 2 3 4 5 6 7 8 9
Translations

**Figure 14.**

# CHAPTER 4

## *IN VITRO* CHARACTERIZATION OF UL42 MUTANT PROTEINS

UL42 has been shown to provide accessory function to the HSV-1 pol catalytic subunit by increasing the processivity of pol. We hypothesized that several activities of UL42 would be required for it to provide this accessory function, including the ability of UL42 to physically bind pol, the ability of UL42 to bind DNA, and the ability of UL42 to stimulate pol activity in high salt. A major goal of my studies was to define the domains on UL42 required for the physical interaction with the pol catalytic subunit.

Coimmunoprecipitation with antiserum to pol or UL42 has been a commonly used technique to determine the domains of UL42 required for the physical association with pol (Digard et al., 1993; Digard et al., 1990; Tenney et al., 1993; Monahan et al, 1993). Although the ability of antibody specific for one of these proteins to co-immunoprecipitate the other is a qualitative measure of the ability of pol and UL42 to interact, such studies are limited by the avidity of antibodies and the effect of antibody binding on the conformation of the protein complex. Both limitations would prohibit the use of immunoprecipitation to quantify the physical interaction between pol and wild-type or mutant UL42 proteins. In my studies with baculoviruses expressing pol and UL42 and mutants (d129-163, d202-337, d274-288), all of the mutants tested were able to precipitate pol with a mAb 6898 or pAb 834 to UL42. At the

same time, UL42 antibody was able to precipitate a non-specific β-galactosidase protein at levels similar to pol with wild-type UL42 (data not shown). These results demonstrate problems associated with the use of coimmunoprecipitation of abundantly expressed protein as a method to quantify the physical interaction between UL42 and pol.

As an alternative method to quantify pol/UL42 interaction, I used glutathione-S-transferase (GST) fusion proteins to characterize the binding of mutant and wild-type UL42 to pol. An advantage of the gst system is that it does not rely on the specificity and avitidy of antibodies which in previous studies allowed non-specific binding of β-galactosidase to UL42 (data not shown). In addition, GST-fusion proteins were used to determine the ability of DNA to bind to mutant UL42 proteins and the ability of these proteins to stimulate the activities of pol under high salt conditions (Figure 15). We anticipated that affinity chromatography with GST-fusion proteins would be a more sensitive and quantitative method to measure the relative affinities of wild-type and mutant UL42 proteins for pol and DNA. The affinity chromatography of GST- fusion proteins also would provide a reasonably facile way to purify protein for use in enzyme assays with pol. It was previously shown that a GST fusion with UL42 (residues 20-456) provided stimulation of pol activity indistinguishable from the full-length UL42 protein expressed by IVTT (Monahan et al., 1998) Therefore for the purposes of this dissertation, the fusions containing UL42 (residues 20-456) will be referred to as GST-WT UL42. Mutations in UL42 were introduced into the context of this construct (as described in Chapter 2).

**Pol binding by mutant forms of GST/UL42**

To define the essential domains of UL42 required for pol binding and to provide a means for better quantitation of relative binding ability, increasing concentrations of GST or GST/UL42 mutant fusion proteins (isolated from whole cell E coli extracts (Chapter 2)), from 1 to 10 μM, were incubated with a 50% slurry of glutathione agarose. In this concentration range, most of the fusion protein is expected to be bound by glutathione agarose since the highest concentration used represents less than one-fifth of the available glutathione (according to the manufacturer). Once immobilized, the fusion proteins were incubated with [$^{35}$S]-methionine labeled pol in the presence of 100 μg/ml of EtBr. The addition of EtBr in pol binding reactions ensure that contamination of the extracts by DNA would not affect pol binding by UL42 (which might be expected by pol accessory proteins that tether pol to DNA). EtBr was included to demonstrate that binding of pol to UL42 is DNA independent (Lao et al., 1992; Monahan et al., 1998). GST- fusion proteins (whether or not bound to pol) were eluted with 5 mM glutathione in 4 sequential 1 column volumes. The first three elutions were analyzed by SDS-PAGE. In order to ensure that the amount of pol bound reflected differences in binding affinities of UL42 for pol and not differences in binding affinities of the GST/UL42 fusion proteins to glutathione agarose, gels of pol elutions were stained with Coomassie blue following electrophoresis. These results demonstrated that elution of pol with the GST fusion protein following the addition of glutathione was a measure of pol:UL42 interaction (Figure 16). The total pol which eluted at different concentrations of GST or GST-fusion proteins was determined by phosphorimager analysis (Figure 17). The amount of pol bound was calculated as a

percentage of the total pol loaded per column. Wild-type GST/UL42 bound approximately 27% of the total pol loaded per column while GST alone bound pol at 1 to 2% of the pol input. GST-fusions of region I mutants d129-163, i140 and d140 bound less pol than wild-type GST: UL42 at each of the charging concentrations (1, 2, or 10 μM) of GST proteins (Figure 17). Mutant d137-142 failed to bind significant amounts of pol (data not shown). However, the point mutations introduced into this region had a less profound effect on pol binding. GST:T142A retained approximately 50% of the pol bound by the GST:UL42 at 2 and 10 μM charging concentration. The other point mutation tested, R135D, retained 1/3 less pol than wild-type UL42. These results suggest that region I of UL42 is essential for the physical interaction with pol, especially since site-directed point mutations in this region severely impaired the pol/UL42 physical interaction (Table 4).

Steve Monahan demonstrated that the binding of pol by GST fusions with many of the region II mutants (d202-337,d256-282,d282,3) was severely impaired (data not shown) and resembled the profile shown for d274-288 (Figure 18). These mutant proteins bound pol at less than that bound by wild-type GST/ UL42. The region II insertion mutant i206, and deletion mutant d241-61, bound pol at nearly wild-type levels at the charging GST concentration(10 μM) tested (Figure 18). However, at lower charging concentrations, significantly less pol bound to the mutant fusion proteins compared to GST/wild-type UL42. The lower level of binding at limiting concentrations of GST/UL42 protein may indicate a somewhat reduced affinity of pol for these proteins. Mutant N339 bound pol at 50% the level observed with wild-type GST/UL42. These results suggest that the region of UL42 downstream of amino acid 261 and upstream amino acid 241 are essential for pol binding.

At the same time, these results suggest that a region of UL42 which may include amino acid 206 may affect pol binding to a lesser extent than the downstream region. Taken together, the results suggest that there are at least 2 separate regions of UL42 that are involved in the physical association with pol since mutations in each of these regions impair the ability of UL42 to bind pol (Table 4). However, it is not clear whether these regions participate in the actual binding to pol or whether they are required to induce a conformation on UL42 which is necessary for high affinity binding to pol.

**Binding double stranded DNA to mutant forms of UL42**

UL42 binds double-stranded DNA in an apparently sequence independent manner. To determine the ability of GST/UL42 mutant proteins (5 μM) to bind to double-stranded DNA, immobilized GST fusion proteins were incubated with (50,000 cpm) a 112 bp end- labeled DNA fragment prepared from vector sequences. In earlier studies, Monahan found that GST/UL42 bound a 112 bp fragment 3 times more effectively than a 3800 bp fragment (unpublished results). Affinity columns containing these proteins were washed with 4 column volumes of buffer containing increasing concentrations of NaCl (50-1000 mM), and the radioactive DNA released at each salt concentration was quantified by liquid scintillation counting. DNA released was expressed as the percentage of total counts per minute (cpm) loaded per column. Gst fusion proteins were not affected by increasing salt concentration (data not shown).

Approximately 90% of the labeled DNA loaded onto the GST-WT UL42 column was retained after initial washing with 50 mM NaCl (Figure 19). By contrast, columns containing immobilized GST retained only 10% of labeled DNA. Approximately 80% of the DNA

which bound to the wild-type GST:UL42 column was eluted with 500 and 1000 mM NaCl. All of the Region I mutants tested (d129-163, d137-142, d140, R135D, and T142A) failed to bind ds DNA (Figure 19). These results suggest that the integrity of region I of UL42 is essential for DNA binding. In experiments performed by Monahan (unpublished data), Region II mutants d202-337, d256-82,d270 and d282,3 also failed to bind ds DNA although mutant d241-61 bound and eluted DNA like wild-type UL42. He found that insertion mutant (i206) bound DNA with a slightly altered profile ( Figure 20) confirms aberrant binding of DNA by this mutant. In this mutant, only 40% of the DNA was bound and most of it eluted with 250-500 mM NaCl. These results suggest that downstream amino acid 261 represent a region of UL42 required for DNA binding that is highly sensitive to mutations. The altered binding profile observed with mutant i206 suggests that a region of UL42 which includes those residues may be involved but not essential for DNA binding. More importantly these results suggest that most of the UL42 mutants tested are essential for UL42 binding to DNA (Table 5)

**Stimulation of pol activity by GST/UL42 fusion proteins**

Since several of the UL42 mutants were found to have a lower affinity for pol than the wild-type protein (Figure 17 and 18), it would be predicted that these mutant forms of UL42 might be required in higher concentrations than wild-type UL42 to achieve maximum stimulation of pol activity. Even at higher concentrations, it is possible that such mutants would not provide the same degree of stimulation as the GST/wild-type UL42 protein. To test the effect that mutations in Region I and Region II of UL42 had on the ability to provide pol accessory function, I tested increasing concentrations of fusions proteins for their ability

to stimulate pol activity. Partially purified GST or GST fusion proteins were prepared by elution from glutathione agarose columns followed by dialysis (as described in Chapter 2). Preparations were shown to be greater than 80% pure by Coomassie blue staining of gels subjected to SDS-PAGE (Figure 21). *In vitro*-expressed pol was incubated with increasing concentrations of GST, GST/UL42, or GST/UL42 mutant protein for 5 minutes at 37 °C to allow for subunit association. Pol activity was measured as the incorporation of [$^3$H] dTTP into trichloroacetic acid insoluble radioactivity in buffer containing 125 mM KCl using activated calf thymus DNA as a template. At this salt concentration, basal pol activity in the absence of UL42 has been shown to be 4-10 fold less than that with stochiometric quantities of UL42. Pol stimulation was saturated at 500 n moles of GST/UL42 fusion protein (Figure 22). All of the mutants that were severely impaired for pol binding, (d129-163, d137-142, d140, i140, R135D, d202-337, d256-282, d282,3, d270, d274-288), failed to stimulate pol activity above basal pol level or the level of GST at concentrations up to 600 nmoles (Table 6). These results suggest that a physical association with pol is required for UL42 to stimulate pol activity. Mutant proteins that maintained some pol accessory function, as assessed by pol stimulation activity, are shown in Figure 23. Mutant d241-261 stimulated pol activity better than wild-type UL42 and activity was saturated at 200 nmoles of GST protein. Variability in the saturation of UL42 pol stimulation activity by UL42 (Figure 22 verses Figure 23) probably reflects the difference in the amount of pol (expressed by IVTT) in each assay. Wild-type and d241-61 stimulated pol activity 9 and 11-fold respectively, compared to basal pol activity in the presence of GST. Another Region II mutant, i206, stimulated pol only two fold and this stimulation was saturable at 200 n moles of protein. These results suggest that

UL42 mutant i206 is severely deficient in its ability to stimulate pol activity on activated calf thymus DNA. The altered DNA binding profile and the wild-type level of i206 pol binding suggest that a simple physical association of pol and UL42 to produce a pol/UL42 complex is not sufficient to provide stimulation of pol activity on activated calf thymus DNA. Region I mutant T142A, stimulated pol at one-half the level of that produced with WT-UL42 at the highest concentration tested (Figure 23). More importantly, 4 times the amount of T142A was required to reach saturation of pol stimulation compared to WT-UL42. The lack of DNA binding of this mutant and its 50% pol binding activity suggests that the inherent ability of UL42 to bind ds DNA is not required for UL42 stimulation of pol activity on activated calf thymus DNA. The interaction of T142A with pol and not with DNA suggests that UL42 may alter the conformation of pol by increasing its affinity for pol and DNA thereby increasing its processivity. The observation that i206 stimulated pol poorly and the fact that the degree of stimulation was not substantially altered by the amount of mutant protein added to pol, suggests that i206 may tether pol to DNA (accounting for the slight enhancement in pol activity) without altering the conformation of pol or rendering it fully processive. It will be important to test purified i206 and T142A in true processitivity assays in order to validate these hypotheses.

**Figure 15. Flow chart of *in vitro* characterization of GST/UL42 mutants.**

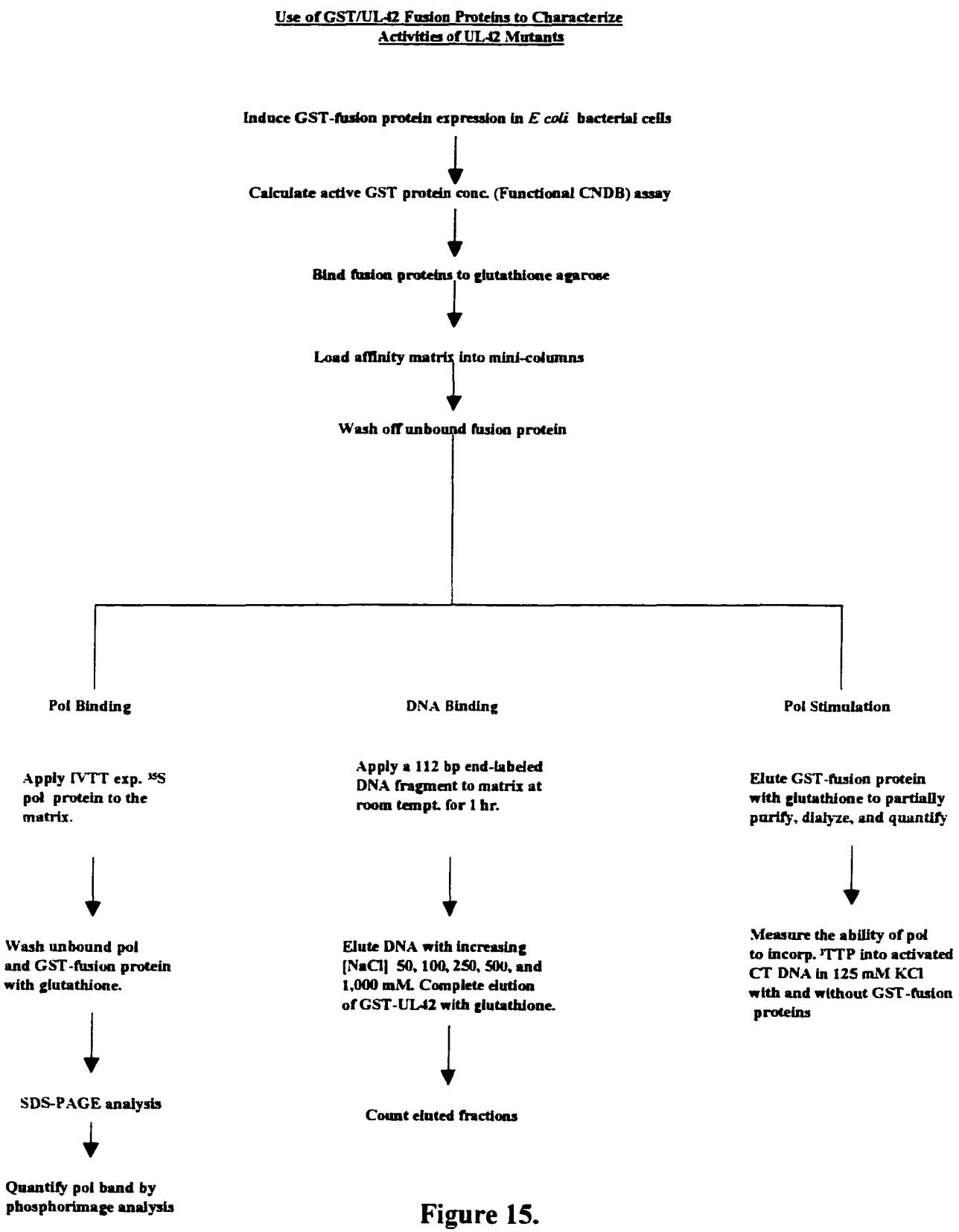

Use of GST/UL-42 Fusion Proteins to Characterize Activities of UL-42 Mutants
Induce GST-fusion protein expression in E coli bacterial cells
Calculate active GST protein conc. (Functional CNDB) assay
Bind fusion proteins to glutathione agarose
Load affinity matrix into mini-columns
Wash off unbound fusion protein
Pol Binding
DNA Binding
Pol Stimulation
Apply IVTT exp. 35S pol protein to the matrix.
Apply a 112 bp end-labeled DNA fragment to matrix at room tempt. for 1 hr.
Elute GST-fusion protein with glutathione to partially purify, dialyze, and quantify
Wash unbound pol and GST-fusion protein with glutathione.
Elute DNA with increasing [NaCl] 50, 100, 250, 500, and 1,000 mM. Complete elution of GST-UL42 with glutathione.
Measure the ability of pol to incorp. 3TTP into activated CT DNA in 125 mM KCl with and without GST-fusion proteins
SDS-PAGE analysis
Count eluted fractions
Quantify pol band by phosphorimage analysis

**Figure 15.**

**Figure 16. Pol binding to GST and GST/UL42.** Pol protein produced by coupled in vitro transcription/translation in the presence of [$^{35}$S] methionine was incubated with GST/UL42 or GST. Matrix was prepared by charging affixed glutathione agarose matrix with 1, 2, or 10 μM of GST:UL42 proteins. Proteins were washed to remove unbound material prior to the addition of [$^{35}$S]-labeled pol. Bound pol was eluted with the GST protein using 5 mM glutathione and the first 3 elutions (E1-E3) were analyzed by SDS-PAGE.

**Lanes:**

1. 1/10 of input pol.
2.-10 pol eluted from GST columns.
11.-19 pol eluted from GST:UL42 columns.

**Figure 17. Binding of pol to UL42 Region I mutants expressed as GST fusion proteins.** TnT expressed [$^{35}$S]-methione labeled pol was incubated with mutant forms of UL42 expressed as GST fusion proteins, previously immobilized to glutathione agarose using three charging concentrations (1, 2, and 10μM). After washing, bound pol was released with 5 mM glutathione, analyzed by SDS-PAGE, and the radioactivities in bands corresponding to pol quantified using a phosphorimager.

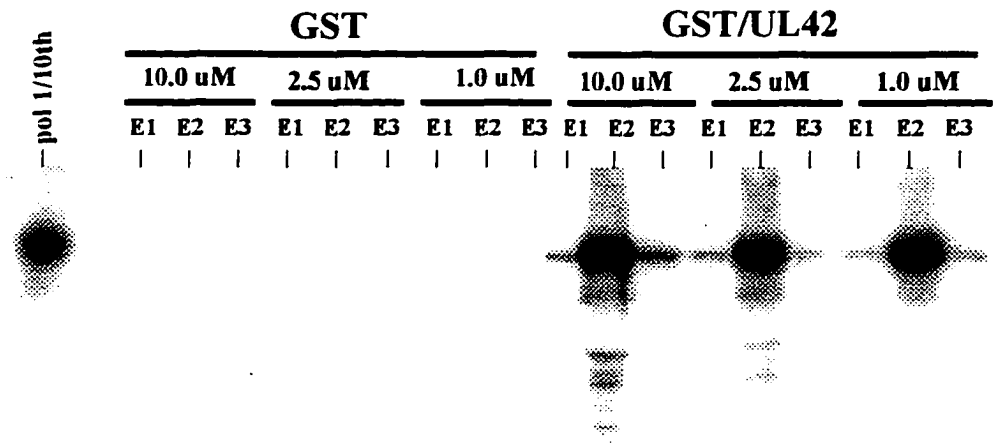

pol 1/10th
GST
GST/UL42
10.0 uM
2.5 uM
1.0 uM
10.0 uM
2.5 uM
1.0 uM
E1 E2 E3 E1 E2 E3 E1 E2 E3 E1 E2 E3 E1 E2 E3 E1 E2 E3

**Figure 16.**

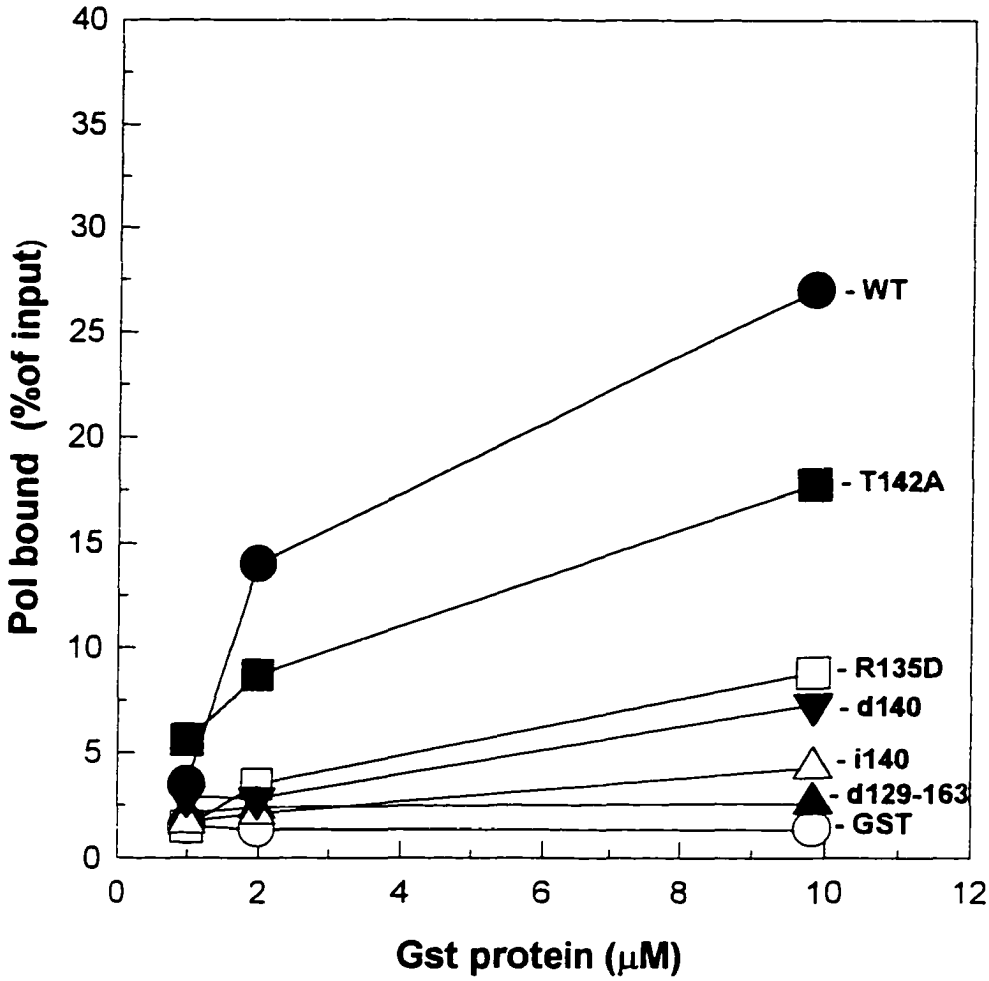

Pol bound (%of input)
Gst protein (μM)
- WT
- T142A
- R135D
- d140
- i140
- d129-163
- GST
0
5
10
15
20
25
30
35
40
0
2
4
6
8
10
12

**Figure 17.**

**Figure 18. Binding of pol to UL42 Region II mutants expressed as GST fusion proteins.** TnT expressed [$^{35}$S]-methione labeled pol was incubated with mutant forms of UL42 expressed as GST fusion proteins, previously immobilized to glutathione agarose using three charging concentrations (1, 2, and 10 μM). After washing, bound pol was released with 5 mM glutathione, analyzed by SDS-PAGE, and the radioactivities in bands corresponding to pol quantified using a phosphorimager.

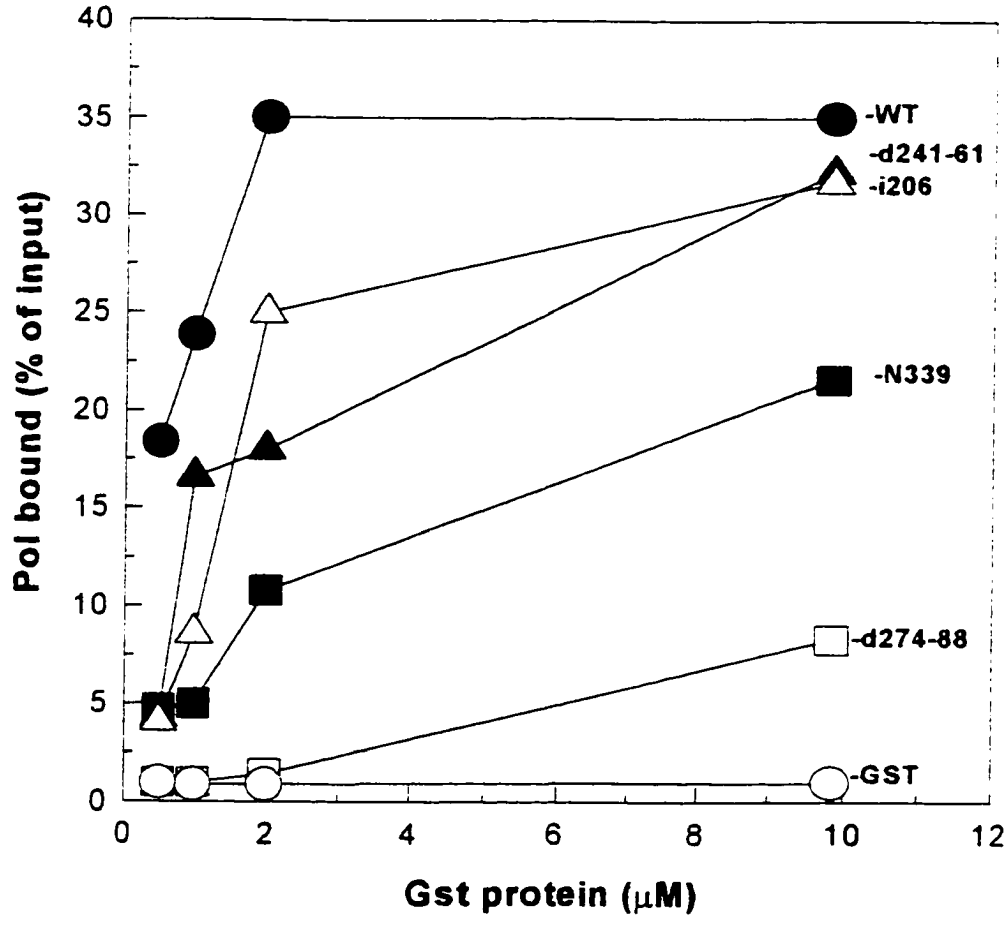
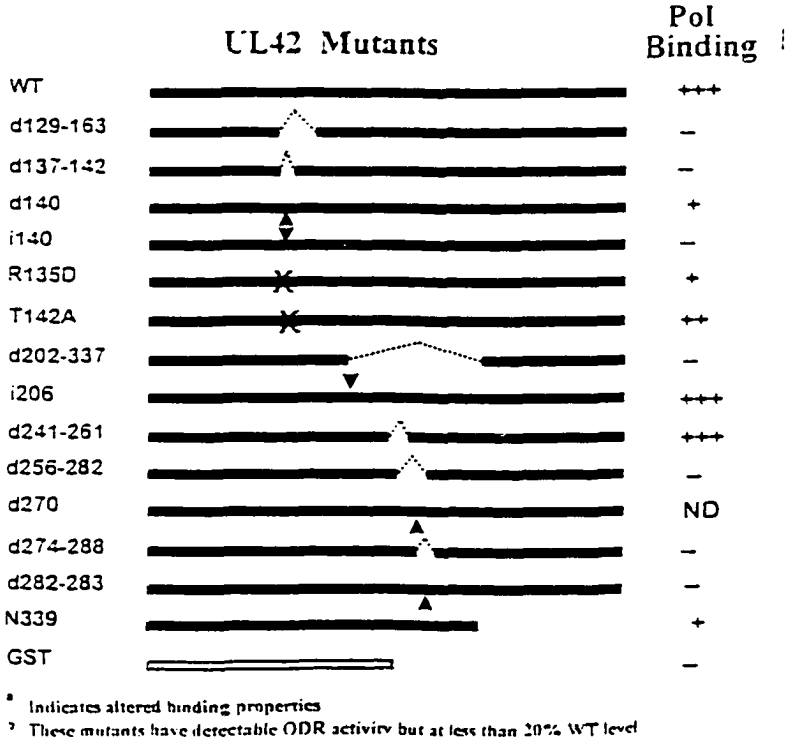

40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
Pol bound (% of Input)
Gst protein (μM)
-WT
-d241-61
-i206
-N339
-d274-88
-GST

**Figure 18**

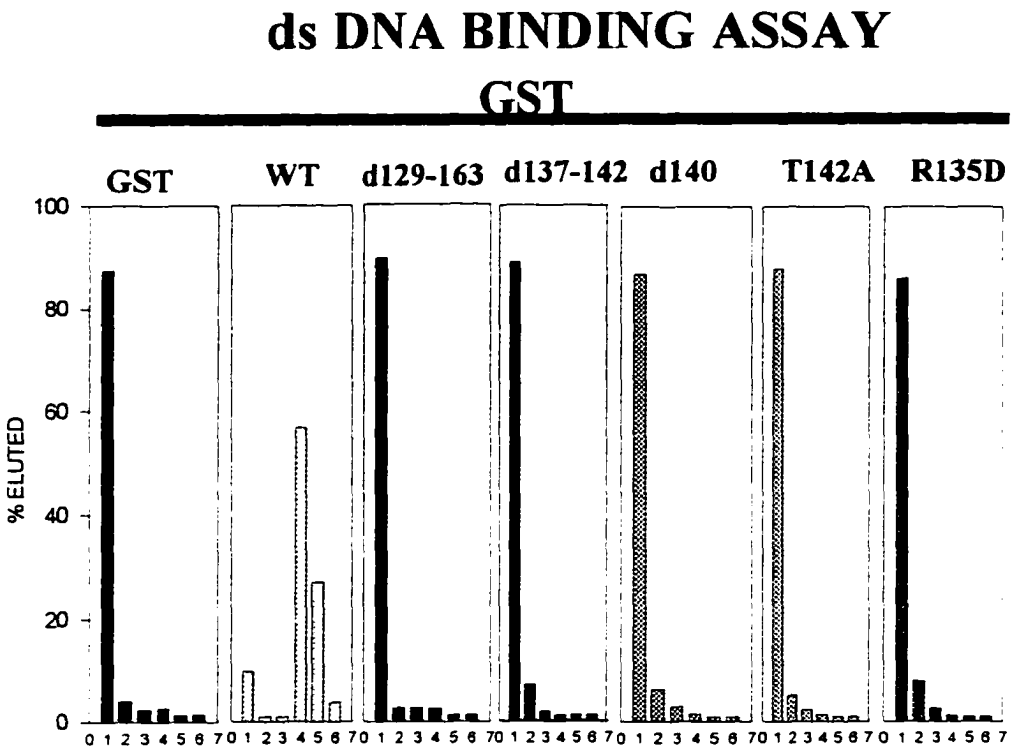



[a] Indicates altered binding properties

[b] These mutants have detectable ODR activity but at less than 20% WT level

**Table 4. Pol binding activity of region I and region II UL42 mutants.**

**Figure 19. Ability of mutant forms of UL42 to bind ds DNA.** UL42 mutant proteins were immobilized to a glutathione agarose matrix. A 112 bp DNA fragment from plasmid pCR II (Invitrogen), labeled at the 3' end with [$^{32}$P]-dATP by end filling, was added to respective columns in a buffer containing 20 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$ (TM Buffer), plus 50 mM NaCl. After 1 hour at room temperature to allow binding, columns were washed sequentially with 500 µl of Tm + 50 mM NaCl, 100 mM, 250 mM, 500 mM, and 1M NaCl. Remaining GST fusion proteins bound to the matrix were eluted by a final wash with 5 mM glutathione. The amount of radioactivity released with each salt elution is expressed as percentage of the total radioactivity loaded per column. Material which eluted with TM containing 50 mM NaCl was considered not bound (NB).




1. 50mM NaCl
2. 100mM NaCl
3. 250mM NaCl
4. 500mM NaCl
5. 1M NaCl
6. Glutathione

**Figure 19.**

**Figure 20. Ability of region II UL42 mutants to bind ds DNA.** UL42 mutant proteins were immobilized to a glutathione agarose matrix. A 112 bp DNA fragment from plasmid pCR II (Invitrogen), labeled at the 3' end with [$^{32}$P]-dATP by end filling, was added to respective columns in a buffer containing 20 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$ (TM Buffer), plus 50 mM NaCl. After 1 hour at room temperature to allow binding, columns were washed sequentially with 500 µl of Tm + 50 mM NaCl, 100 mM, 250 mM, 500 mM, and 1M NaCl. Remaining GST fusion proteins bound to the matrix were eluted by a final wash with 5 mM glutathione. The amount of radioactivity released with each salt elution is expressed as percentage of the total radioactivity loaded per column. Material which eluted with TM containing 50 mM NaCl was considered not bound (NB).

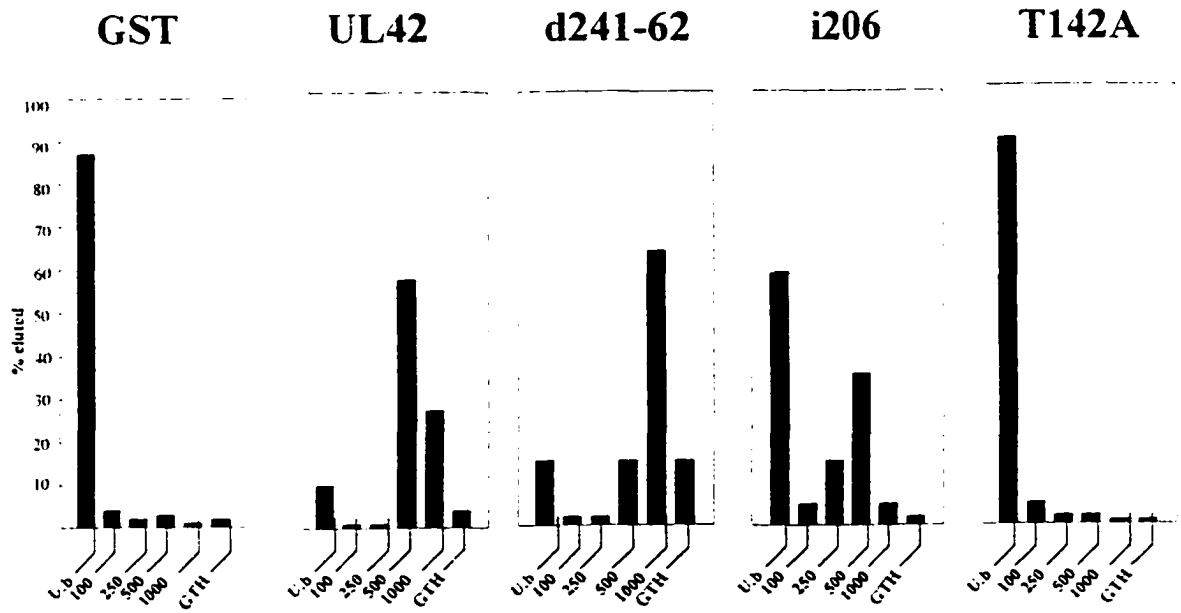
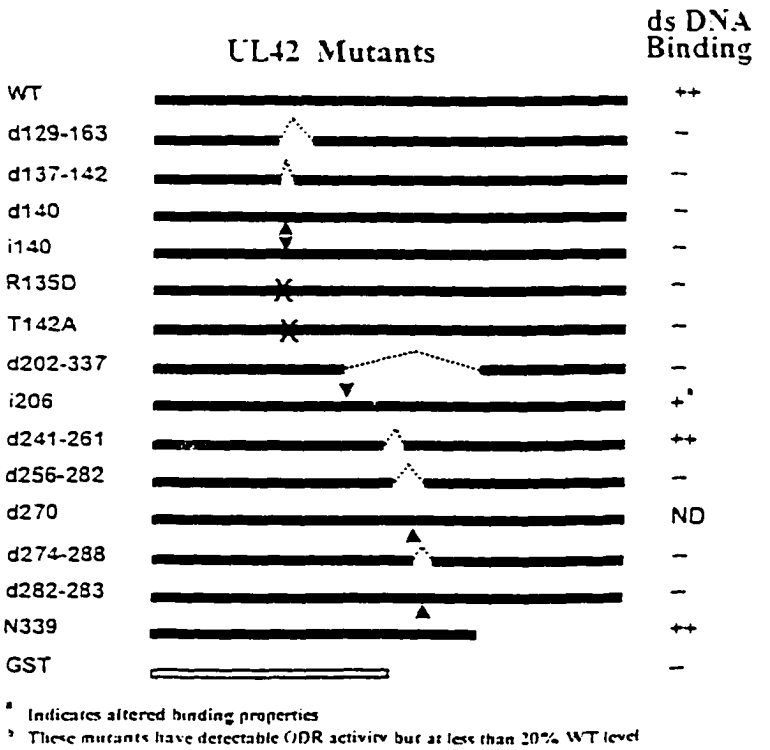



**Figure 20.**

**Figure 20. Affinity of UL42 region II mutants for ds DNA.**




[a] Indicates altered binding properties

[b] These mutants have detectable ODR activity but at less than 20% WT level

**Table 5. DNA binding of region I and region II UL42 mutants..**

**Figure 21. Purification of GST, GST/UL42 or GST/UL42 mutants.** Bacterial cell lysates were mixed with a 50% (v/v) glutathione-S-sepharose beads equilibrated in TED buffer + 0.1 M NaCl + 50 μg/ml BSA. The mixture was incubated overnight at 4°C with gentle shaking. Beads were washed 3 times with TED + NaCl + BSA,collected by centrifugation, and loaded into a 1 ml disposable pipet tip containing glass wool. Bound fusion proteins were eluted in 4 column volumes with 1 column volume of 5 mM glutathione. One-tenth of each elution was subjected to SDS-PAGE analysis, and Coomassie staining was performed.

**Lanes:**

1. Elution 1 GST/UL42
2. Elution 2 GST/UL42
3. Elution 3 GST/UL42
4. Elution 4 GST/UL42

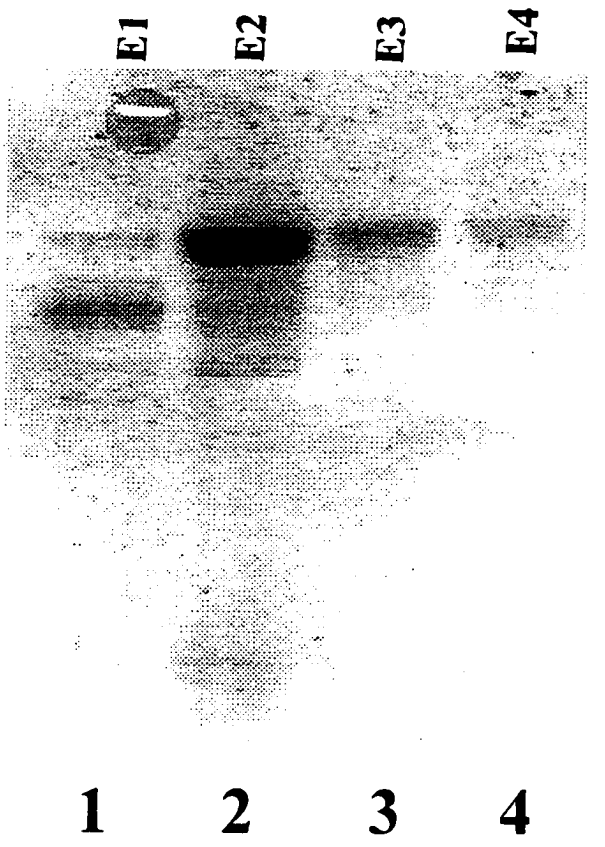



**Figure 21.**

**Figure 22. Titration of pol stimulation by GST/UL42 fusion protein.** UL42 expressed as a GST fusion protein, was partially purified (as in figure 21), dialyzed and measured for its ability to stimulate pol activity in 125 mM KCl. Varying amounts of GST/UL42 fusion protein were incubated with pol (expressed by IVTT) for 5 min. at 37°C and pol activity was measured by the incorporation of [$^3$H]-dTTP in activated calf thymus DNA. A unit of activity was defined as the amount of enzyme required for the incorporation of 1 fmole of dTTP into DNA per hour at 37° C.

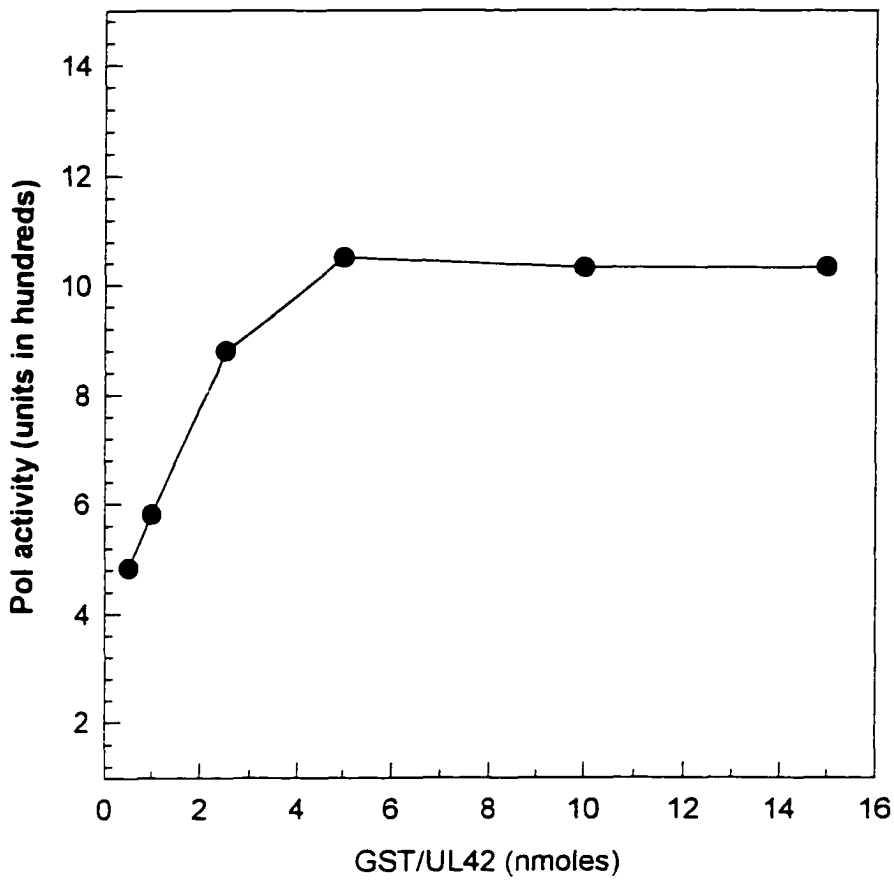

Pol activity (units in hundreds)
14
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
GST/UL42 (nmoles)

**Figure 22.**

**Figure 23. Stimulation of pol activity by GST/UL42 fusion proteins.** Mutant forms of UL42 expressed as GST fusion proteins, were partially purified by binding to and eluting from glutathione agarose. Eluted protein was dialyzed, and measured for its ability to stimulate pol activity in 125 mM KCl. Varying amounts of each fusion protein were incubated with pol (expressed by *in vitro* transcription/translation) for 5 minutes at 37°C and pol activity was measured by the incorporation of [$^3$H]-dTTP into activated calf thymus DNA. A unit of activity was defined as the amount of enzyme required for the incorporation of 1 fmole of dTTP into DNA per hour at 37° C.

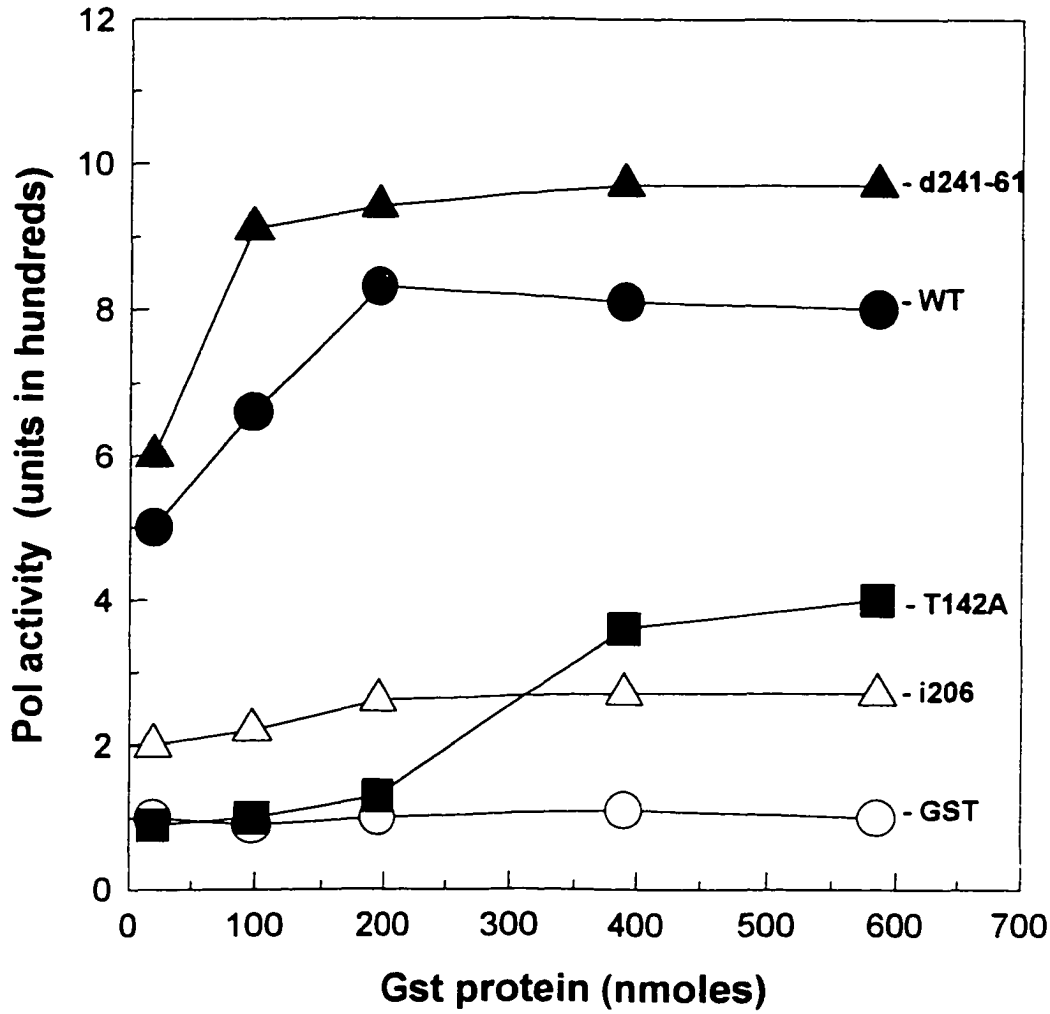
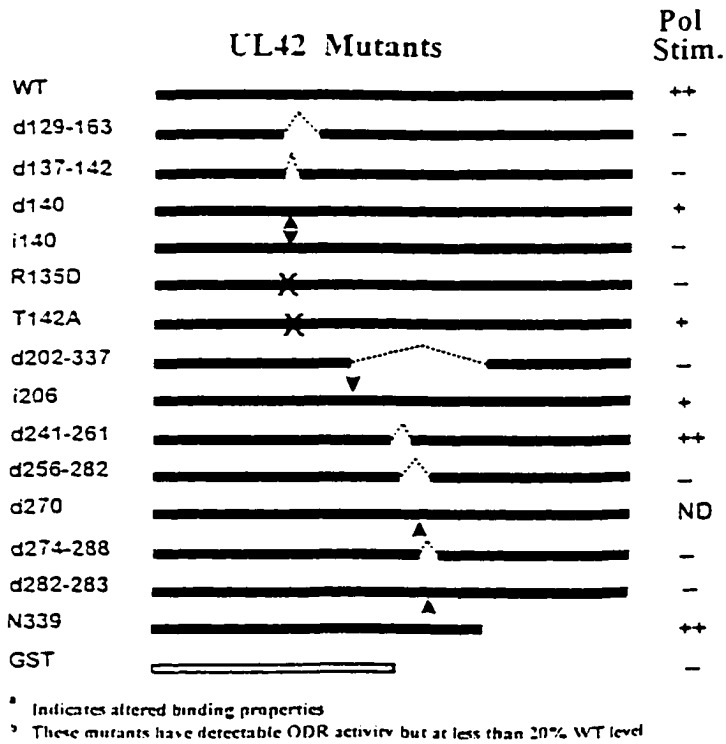

12
10
8
6
4
2
0
Pol activity (units in hundreds)
0
100
200
300
400
500
600
700
Gst protein (nmoles)
- d241-61
- WT
- T142A
- i206
- GST

**Figure 23.**




[a] Indicates altered binding properties

[b] These mutants have detectable ODR activity but at less than 20% WT level

**Table 6. Stimulation of pol activity by region I and region II UL42 mutants.**

# CHAPTER 5

## CHARACTERIZATION OF THE ACTIVITY UL42 MUTANT PROTEIN *IN VIVO*

In order to better characterize the biochemical activities of UL42, it was important to be able to produce large quantities of purified protein and to produce large quantities of UL42 in the absence of other HSV-1 replication proteins. I developed a PCR-based system (Chapter 3) for amplifying and transferring UL42 mutants previously characterized in plasmids, to the baculovirus genome for high level expression. Because future efforts are aimed at utilizing UL42 protein in cell free systems, it was important to determine whether proteins expressed were able to perform all of the replication functions normally carried out during lytic infection of permissive cells in addition to those activities for which convenient *in vitro* assays were available. A transient transfection assay developed to identify a set of 7 HSV-1 genes required for origin dependent replication (ODR) in a mammalian cell line (Wu et al., 1988) and the ability of these same proteins to replicate DNA in Sf9 cells when expressed by recombinant baculovirus (Stow et al.,1992) provide convenient methods in the presence of other DNA replication proteins by which mutated replication proteins can be screened for function. Both assays have laid the foundation for the characterization of UL42 mutants in the context of other cellular or HSV-1 replication proteins.

**Production of recombinant baculovirus expressing UL42 mutants and wild-type proteins.**

Recombinant protein expression systems based on the baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcNPV), have become a highly useful and popular means for expressing eukaryotic proteins. The advantages of the baculovirus system compared to bacterial systems are as follows: a) it is a eukaryotic system that can carry out many of the post-translational modifications found in mammalian proteins, b) large quantities of the biologically active recombinant protein are produced in suspension culture or even in larvae, c) purification of the recombinant protein requires fewer steps compared to purification from mammalian cell culture, tissues or bacterial cultures because of the lower abundance of proteases in insect cells and the high concentration of recombinant vs. cellular protein synthesis (Goswami and Glazer, 1991). Higher levels of expression in insect cells are achieved through recombinant viruses generated by homologous recombination between a contransfected plasmid (harboring the foreign gene) and viral genomic DNA (Gang and Weber, 1995). To accomplish this, a series of plasmids were constructed to introduce wild-type or mutated UL42 into the baculovirus transfer vector, pVL1392 (Figure 24). UL42 was introduced into AcNPV under control of the polyhedrin promoter using the linearized BaculoGold technique (Chapter 2). BaculoGold ® DNA (Serrano et al 1993, Frankel et al 1994) is a modified AcNPV Baculovirus DNA which contains a lethal deletion and is incapable of producing viable virus. Co-transfection of the BaculoGold ® DNA with a complementing baculovirus transfer vector rescues the lethal deletion by complementation and homologous recombination. Since only recombinant BaculoGold ® produces viable virus,

the frequencies for obtaining plaques which contain the desired insert exceed 99%.

In most cases, cells from the original superinfection were sufficient to identify recombinant protein 4 days after the original transfection using Western blot analysis (data not shown). Having demonstrated that UL42 was expressed in Sf9 cells, 3 rounds of plaque purifications were performed. Following addition of a standard agar overlay (Chapter 2), plaques were stained with neutral red and plaque purified. A number of plaques were picked (n=8) and examined for UL42 production by Western blot analysis. The efficiency for the isolation of recombinant wild-type UL42-containing virus approached 100% as demonstrated by the fact that all of the randomly picked plaques screened produced UL42 (Figure 26). For most of the mutants tested, at least 80% of the plaques tested expressed recombinant protein (data not shown). Western blot analysis with a monoclonal antibody 6898 to UL42 indicated the presence of a reactive protein species in Sf9 cells infected with recombinant virus (AcNPV UL42, d129-163, d274-288, d202-337, or AcNPV/β-gal) and harvested at 48 hours p.i. (Figure 25). The 65 kDa or smaller protein present in extracts of UL42-containing recombinants was not present in AcNPV/B-galactosidase infected insect cells (Figure 25, lane 6 ). For the purpose of comparison, protein from cells infected with recombinant virus AcNPV/UL42 (which has the UL42 gene with the 300 bp leader sequence) from Mark Challberg is shown next to recombinants I produced using the PCR approach described in Chapter 2. Recombinants were also created for mutants T142A, R135D and i206 and expression of the mutated UL42 was confirmed by Western blot analysis (results not shown).

The presence of UL42 DNA in the baculovirus genome was confirmed by Southern blot hybridization. UL42-containing recombinant virus (10 PFU/cell) was used to infect Sf9

cells, which were harvested 48 hours post infection. Total cellular DNA was isolated and purified (as described in Chapter 2) . Because the UL42 sequences in the transfer vector are flanked by EcoR1 sites, the DNA was digested with EcoR1 and subjected to agarose gel electrophoresis to demonstrate the 1.5 kbp UL42 DNA fragment, which represents full length UL42 (Figure 27 ). This band was present in the AcNPV/UL42, but not in DNA from virus expressing β-galactosidase (Figure 27, compare lanes 2 and 9). As predicted, bands which hybridized to the probe were detected for wild-type UL42, d129-163, d202-337, T142A, R135D, i206, and d274-288 (Figure 27, lanes 2-8).These results confirmed that the entire ORF of wild-type and mutant UL42 sequences were introduced into the baculovirus genome and that these sequences could be removed as predicted from the recombinant baculovirus.

Metabolic labeling of recombinant protein showed that mutant or wild-type UL42 was the major newly synthesized polypeptide produced between 24 and 36 hours after infection with recombinant baculovirus (Figure 28, lanes 1,3,5,7). When cells were doubly infected with baculovirus recombinants expressing HSV-1 pol and the appropriate UL42 protein, UL42 was expressed at 2 times the level of pol. These results suggest that coinfection of UL42 with pol recombinant baculovirus severely impairs the expression of pol.

Because recombinant proteins expressed in the baculovirus system can range from 0.1% to 50% of the total protein in the insect cells (Baixeras et al., 1990; Caroni et al., 1991; Christensen et al., 1993; Mattion et al., 1991; Hsu et al., 1991) it was important to determine the optimal conditions for expression of UL42 compared to that of other HSV-1 replication proteins (UL9 and pol) in this system. Sf9 cells were adsorbed with recombinant baculoviruses which express UL42, pol or UL9 (20 PFU/cell) for 1 hour at 27°C. Cells were

harvested at 24,36,48, or 60 hours post infection. At 6 hours prior to harvest, infected cells were metabolically labeled with 50 μCi/ml [$^{35}$S] - L-methionine (specific activity, 1000-1200 Ci/mmol, Amersham, Arlington Heights, IL.). Cells were harvested by centrifugation, lysed, and extracts analyzed by SDS-PAGE. The autoradiograph shown in Figure 29 shows that between 24 and 36 hours post infection, UL42, pol, and UL9 were all expressed to an optimum level (Figure 29, lanes 3, 7 and 11). By 48 hours post infection, UL42 expression decreased and little expression was detected between 48 and 60 hours p.i. (Figure 29, lanes 4 and 5). UL9 and pol expression continued through 48 hours p.i. but decreased thereafter. These results suggest that optimum expression of pol, UL42, and UL9 was at 36 hours p.i. and the protein production thereafter varied for each mutant.

In previous UL42 preparations from mammalian cells, UL42 protein was purified using high salt nuclear extract preparations as described by Gallo et al, (1989). In order to determine the location of UL42 in recombinant baculovirus-infected cells, western blot analysis of nuclear and cytoplasmic extracts of viral infected Sf9 cells harvested at 36 hours p.i. was performed. Nuclei were separated from cytoplasmic fractions by dounce homogenization in hypotonic buffer and low speed centrifugation. All of the mutant proteins tested were present in the cytoplasmic fractions and none was detected in the nuclear fractions (Figure 30).Several reasons exist for the inability of UL42 mutants to localize to the nucleus. First, the mutation affected the nuclear localization signal for the mutants. This appears unlikely since sequences common to nuclear localization signals have not been identified in these regions. More importantly, some of these mutants localized to the site of active DNA replication in ODR assays (Figure 43 ).Secondly, these mutants in region I of

UL42 may not be retained in the nucleus due to the loss of DNA binding activity. No data exists that supports or denies a DNA binding requirement for UL42 to be retained in the nucleus of cells infected with recombinant baculovirus expressing UL42 mutant proteins. Although wild-type UL42 protein was present in the cytoplasm, a larger portion of the protein was present in the nuclei of infected cells (Figure 30). Extracts were prepared from cells infected with pol or β-galactosidase recombinant and used as controls for antibody specificy. Results showed that neither of these extracts had reactive protein in either the cytoplasmic or nuclear fractions. These results show that high salt nuclear extract protocol as described by Gallo et al (1989), is not a sufficient method to isolate protein from cells infected with baculovirus representing region I mutants of UL42. However, cytoplasmic extracts provide a better means to isolate protein from recombinant baculovirus expressing UL42 (data not shown).

**Origin-dependent DNA replication**

In order to test the function of baculovirus-expressed mutant UL42 in tissue culture, origin-dependent replication assays were performed. Stow et al, (1992) demonstrated that in insect cells co-infected with baculovirus recombinants expressing all 7 of the DNA replication genes, amplification of an origin-containing plasmid DNA occurred. Total cellular DNA was cleaved with Eco RI and Dpn I. Amplified plasmid could be distinguished from input plasmid by Dpn I digestion. Dpn I cleaves unmethylated input DNA such as that in bacteria but not the amplified DNA. Eco RI cut at a single site within the origin-containing plasmid sequence to provide unit length plasmid sequences. This system (Figure 31) has provided a method to screen for the functions of UL42 mutants in Sf9 insect cells.

Sf9 cells were transfected with a HSV-1 origin-containing plasmid, pT085, and infected at a moi of 5 pfu/cell with 6 recombinant baculovirus representing HSV-1 replication proteins pol, UL9, UL8, UL5, UL52, ICP8 together with wild-type or mutated UL42. Cells were harvested at 64 hours post infection and the DNA extracted. Southern blot analysis showed amplification of origin-containing DNA with PCR-derived UL42 virus (Figure 32, lane 6). Cells infected with 6 of the 7 baculovirus recombinants (lacking UL42) failed to amplify plasmid DNA but did show a DpnI sensitive input DNA band which confirms that the lack of DNA transfected into cells did not account for the negative results obtained. On the other hand, cells infected with AcNPV/UL42 from Mark Challberg had no detectable origin-dependent amplification which probably reflected the lack of origin-containing plasmid transfected into Sf9 cells for that sample. These results showed that origin-dependent DNA replication in insect cells was achieved using a PCR-derived wild-type UL42 recombinant baculovirus.

In cases where baculovirus recombinants expressing mutant proteins from UL42 (d129-163, d202-337, d274-288) were substituted for wild-type UL42, no amplification of the origin-containing plasmid was observed (Figure 33, lanes 6, 17, 18). At the same time, UL42KT and (to a lesser extent) UL42 from Mark Challberg were able to provide origin dependent function (Figure 33, lanes 14, 15). These results suggest that ODR is a sufficient *in vivo* assay to determine the function of UL42 mutants.

**Characterization of HSV-1 UL42 mutant proteins using an interference assay to detect dominant -negative phenotypes**

The interest in regions of UL42 that associate with pol has led to the prediction that

mutant proteins which associate with pol or some other replication protein but lack pol accessory function would have a dominant-negative phenotype by sequestering some or all of the replication complex. A nonfunctional mutant of UL42 capable of binding to pol or another replication protein would be expected to interfere with origin-dependent replication in mixed assays with wild-type UL42 virus (Figure 34). For mutants that lack function, interference could be used to determine the *in vivo* relevance of protein interactions defined using in vitro assays and to directly map domains which physically interact with other proteins in the complex. To quantify the degree of interference provided by the mutants, optimization of the parameters of the origin-dependent replication first was required.

In order to determine the optimal origin-containing template amount required for ODR activity, Sf9 cells were transfected with varying amounts of the HSV-1 origin containing plasmid, pTO85. Cells were infected at a moi of 20 pfu/cell with each of the 7 recombinant baculoviruses indicated above. Amplification was observed for the template amounts (2.5 -10.0 μg) transfected (Figure 35). ODR function increased with increasing plasmid amount to 2.5 μg with no substantial change at higher plasmid amounts. Cells transfected with plasmid and infected with recombinant baculovirus at moi's ranging from 0.5 to 20 PFU/cell provided amplification only at a moi of 2 or 20 (Figure 36). These results demonstrate the variability of results I often observed when using the ODR assay as described above. Reaction conditions such as the transfection efficiency of the origin-containing plasmid or the infection efficiency of individual recombinant baculoviruses expressing the 7 proteins that provide origin-dependent replication activity could have accounted for the variability. In order to determine if the expression of replication proteins was influenced or affected in

the ODR assay, cells were infected with recombinant virus, labeled with methionine (as described in Chapter 2), harvested at 36 hours p.i., and SDS-PAGE analysis was performed.

Metabolic-labeling of cells infected with one of the 7 viruses (Figure 37) demonstrated that all of the proteins were expressed independent of the other replication proteins. However, when all 7 recombinants were coinfected, pol, UL8, UL42, UL5, ICP8 and UL9 were expressed at reduced levels compared to UL52, as observed by western blot analysis (data not shown). The most drastic reductions were in ICP8 and UL42. Since UL52 and ICP8 co-migrated, the presence of ICP8 was confirmed by Western blot analysis. As a result of apparently low expression levels of ICP8, UL52 and UL42, recombinant viruses were titrated in the ODR assay. Cells were transfected with pTO85 and infected with the 7 recombinant viruses (as described above). The input multiplicity of ICP8, UL42, or UL52 was varied from 5 to 30 PFU/cell while holding the moi of the other 6 recombinants to 5 PFU/cell. Template DNA amount (origin-containing plasmid) had a dramatic affect on amplification as shown by the lack of amplification in reactions using 0.4 μg of template. Results show that ODR is highly dependent on the relative moi's of the recombinant baculovirus (Figure 38). An increase in the moi of UL42 increased the amount of full length amplified DNA up to the highest tested (20 PFU/cell) while increasing amounts of AcNPV/ICP8 increased amplification up to a moi of 10 PFU/cell. Results also confirm that UL42, and to a lesser extent ICP8, were limiting in standard co-infection assays when 5 PFU/cell of each of the 7 recombinant baculovirus were tested . These results suggested that the ODR assay was sensitive to multiplicity effects that most likely reflect differences in gene

expression. Thus, I determined that this approach was not a feasible system to quantify interference of ODR.

As described above, a mutant that lacks one of the UL42 pol accessory functions may interfere with the ability of UL42 to stimulate pol activity. As an alternative approach (to the ODR assay) to determine the ability of mutant UL42 protein to interfere with wild-type UL42 in a dominant-negative manner, an *in vitro* based pol stimulation assay was developed, which relied on the quantity of RNA available for in vitro translation. Translated protein from the addition of increasing amounts of T142A, or wild-type UL42 RNA were mixed with IVTT pol (at a constant concentration) and their ability to stimulate pol activity on activated calf thymus DNA was determined (as described in Chapter 2). T142A and WT-UL42 stimulated pol activity (above basal pol activity) 2 and 4 fold respectively, at the highest amount of RNA tested (Figure 39). Increasing amounts of T142A RNA was found to have no effect on the ability of a constant amount wild-type UL42 to stimulate pol activity above basal pol levels when RNA's were mixed, translated and added to pol (Figure 40). These results suggest that T142A does not interfere with pol stimulation by WT- UL42. More importantly, the modified pol stimulation assay described above could be used to detect mutants with a dominant negative phenotype. No other UL42 mutants were tested with this procedure.

**Transient complementation for origin-dependent replication**

In order to investigate if there is a correlation in the *in vitro* activities of UL42 and its activity in HSV-1 viral infected cells, I took advantage of a UL42 null mutant Cgal Δ42 (Johnson et al., 1991). I used a modified transient complementation assay from the procedure described by Reddig et al, (1991), to examine and quantify the ability of UL42 mutant

proteins to provide ODR of a HSV-1 origin-containing plasmid in cells infected with Cgal Δ42 (Figure 41). Because Cgal Δ42 cannot replicate viral DNA or plasmids containing HSV origins, *in vivo* complementation of the Cgal Δ42 virus by wild-type UL42 *in trans* allows the amplification of an HSV-1 origin-containing plasmid. This system is enhanced by the expression of all of the viral proteins necessary for ODR by the parental virus (minus UL42) thereby reducing the variability and multiplicity effects seen in the baculovirus-based ODR system described by Stow et al, (1992). More importantly, specificity of the transient complementation assay provides a method to quantify the degree of amplification of plasmid from cells transfected with wild-type UL42 compared with UL42 mutants.

In order to test for ODR, cells were transfected with pT085 (has 1 copy of $ori_s$) or pON114 (that has 2 copies of $ori_s$) and infected with the KOS wild-type strain of HSV-1 (as described in Chapter 2 ). Total cellular DNA was extracted and hybridized to a plasmid-specific probe (as described in Chapter 2). The results show that both plasmids containing functional origins of replication can be amplified (Figure 42). In additional experiments, I used only pT085 for transient complementation origin-dependent DNA replication. BHK cells were co-transfected with HSV-1 origin containing plasmid (pT085) and a plasmid containing the wild-type or mutated UL42 gene. Sixteen hours later, the cells were infected with Cgal Δ42 at a moi of 10 PFU/cell. As a control, some cells were infected with KOS. Cells were harvested 24 hours later and the DNA extracted. The DNA was subsequently digested with EcoRI and Dpn I and the fragments separated by agarose gel electrophoresis. Southern blot analysis was performed as described in Chapter 2. A band corresponding to full length and amplified plasmid sequences was quantified by phosphorimage analysis. The

band representing WT-UL42 amplification was designated as 100% amplification and all others were normalized to it.

Results show that for UL42 mutants d140 and R135D, ODR was similar to that produced by wild-type UL42 while deletion mutant d270 replicated origin-containing plasmid at 88% of wild-type UL42 (Figure 43 lanes 4,7,12). UL42 mutants T142A, i206, and d282,3 amplified plasmid DNA at 67, 49, and 47% of wild-type levels. All other UL42 mutants (d137-142, i140, 202-337, d241-261, d274-288, and d256-282) failed to provide origin - dependent DNA replication above 20% of wild-type UL42 (Table 7). There is a good correlation between the *in vitro* and *in vivo* activity of UL42 mutants. Most of the mutants that failed to stimulate pol activity *in vitro* (d129-163, d137-142, i140, 202-337,, d274-288, and d256-282) were also unable to provide origin-dependent replication *in vivo* (Table 8). On the other hand, mutants that stimulated pol activity *in vitro* (d140, T142A, i206) provided ODR function. One mutant, d241-61, which was positive in all of the in vitro assays used, failed to provide origin dependent replication. This suggests that the region of UL42 encompassing amino acids 241-61 of UL42 may be involved in the physical or functional interaction with another HSV-1 or cellular protein involved in ODR and that removal of these residues may affect this interaction.

**Figure 24. Production of UL42 Mutant Baculovirus Recombinants.**

# Production of UL42 Mutant Baculovirus Recombinants.

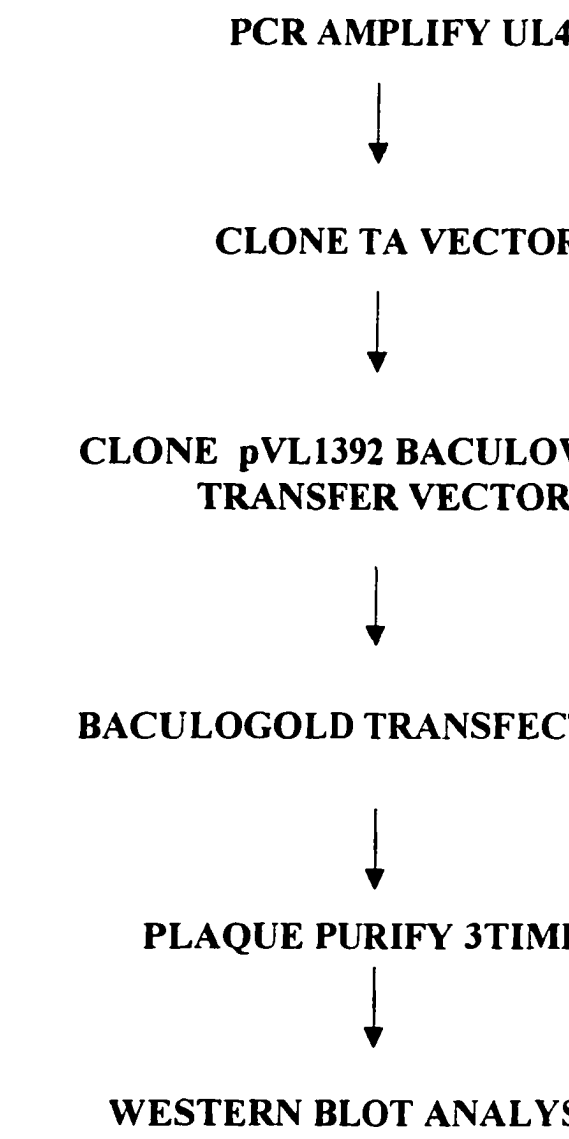



**Figure 24.**

**Figure 25. Western blot analysis of wild-type and mutant UL42 proteins expressed from constructed baculovirus recombinants.** Sf9 cells infected with virus from plaque purified (2 times) recombinants were infected at a moi of 5 pfu/cell. Extracts prepared were subjected to polyacrylamide gel electrophoresis, proteins transferred to nitrocellulose and the filter probed with mAb 6898 to UL42.Arrow indicates full length UL42.

**Lanes:**

1. AcNPV/UL42 (Constructed by Mark Challberg)
2. AcNPV/UL42 (Constructed by Keith Thornton)
3. AcNPV/d129-163
4. AcNPV/d274-288
5. AcNPV/d202-337
6. AcNPV

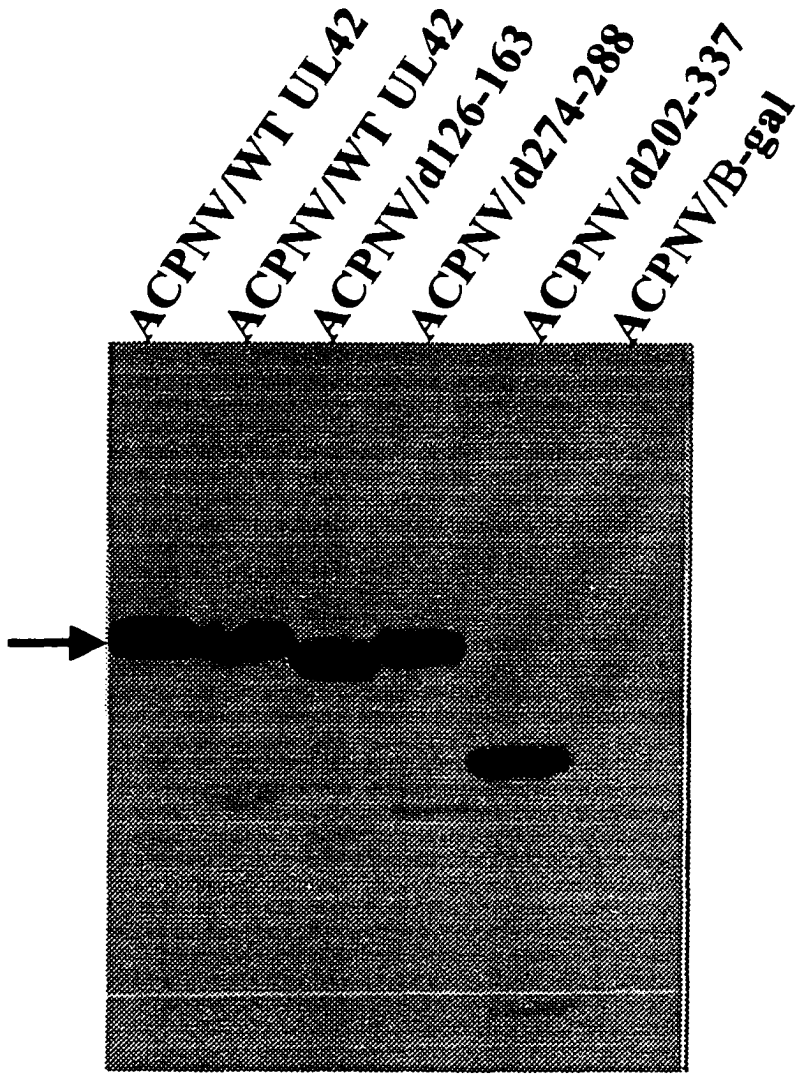

ACPNV/WT UL42
ACPNV/WT UL42
ACPNV/d126-163
ACPNV/d274-288
ACPNV/d202-337
ACPNV/B-gal

**Figure 25.**

**Figure 26. Western blot analysis of recombinant UL42 plaques.** Eight randomly picked plaques were amplified from plates inoculated with AcNPV/UL42 rescued BaculoGold™ virus and Western blot analysis was performed as described in figure 25.Arrow denotes full length UL42.

**Lanes:**

1. UL42 purified
2. AcNPV/ UL42 plaque 1
3. plaque 2
4. plaque 3
5. plaque 4
6. plaque 5
7. plaque 6
8. plaque 7

9 plaque 8

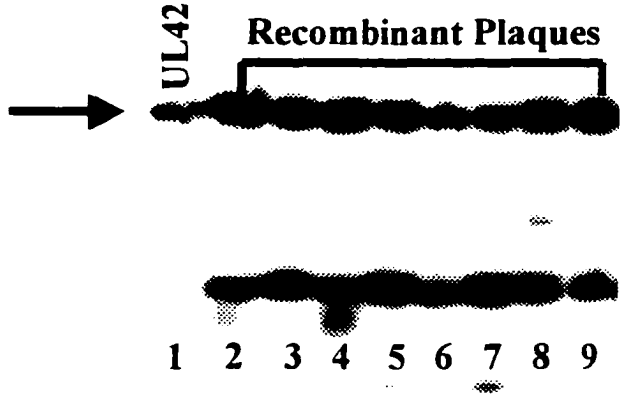



**Figure 26.**

**Figure 27. Southern blot analysis of AcNPV/UL42 and AcNPV/UL42 mutants**. Sf9 cells infected with AcNPV/UL42 or AcNPV/B-gal recombinants. DNA prepared from cells was analyzed following cleavage with EcoR1. The fragments were separated by agarose electrophoresis, transferred to nitrocellulose and hybridized to $^{32}$P-labeled UL42 sequence (700 bp Pst I fragment). An autoradiograph of the washed membrane is shown. The arrow indicates the EcoR1 fragment containing the UL42 ORF. Bands above the arrow are partial digestion products.

**Lanes:**

1. pVL1392/ UL42 vector (10.6kb)
2. AcNPV/UL42
3. AcNPV/T142A
4. AcNPV/d129-163
5. AcNPV/d202-337
6. AcNPV/R135D
7. AcNPV/d274-288
8. AcNPV/i206
9. AcNPV/B-gal

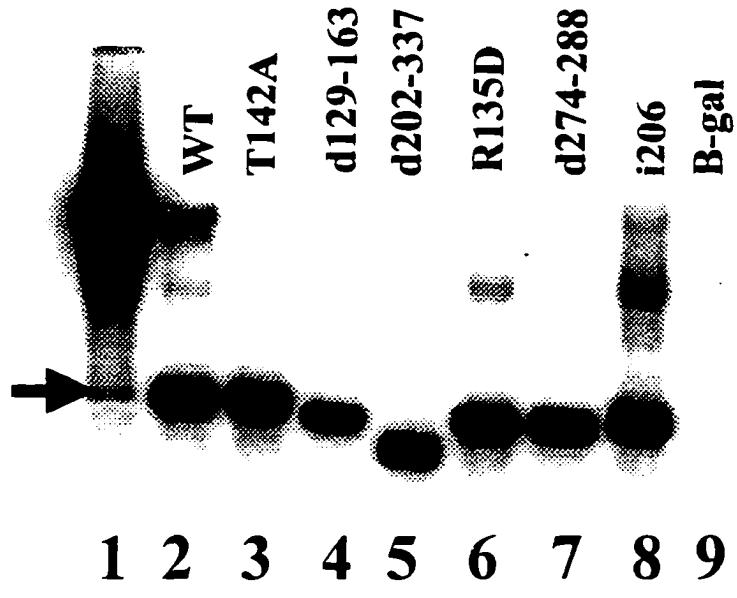

WT
T142A
d129-163
d202-337
R135D
d274-288
i206
B-gal
1 2 3 4 5 6 7 8 9

**Figure 27.**

**Figure 28. [$^{35}$ S]- methionine labeling of AcNPV/ wild-type or mutant UL42 recombinants.** Sf9 cells were infected with wild-type or mutant UL42 recombinant baculovirus the pol recombinant or both. Cells were labeled with [$^{35}$ S]- methionine (50 μCi/ml), harvested at 36 hours post -infection,and high salt nuclear extracts were prepared. Samples were electrophoresed by SDS-PAGE.

**Lanes:**

1. Wild-type UL42
2. Wild-type Ul42 +pol
3. d129-163
4. d129-163 +pol
5. d202-337
6. d202-337 +pol
7. d274-288
8. d274-288 +pol
9. pol
10. Sf9

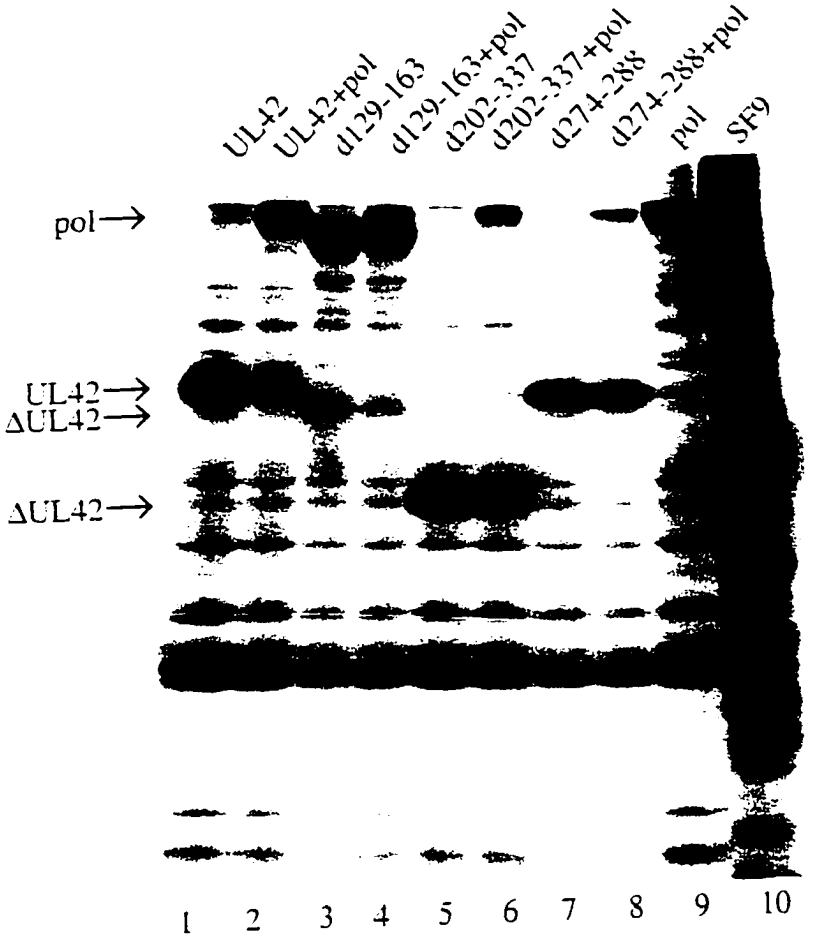

UL42
UL42+pol
d129-163
d129-163+pol
d202-337
d202-337+pol
d274-288
d274-288+pol
pol
SF9
pol→
UL42→
ΔUL42→
ΔUL42→
1 2 3 4 5 6 7 8 9 10

**Figure 28.**

**Figure 29. Time course of Baculovirus expression of HSV-1 replication proteins UL42, pol, and UL9.** Sf9 cells were infected with AcNVP/UL42, pol, or UL9 (20 PFU/cell). Cells were harvested at 24, 36, 48, and 60 hours post infection and labeled with[$^{35}$S]-methionine for 6 hours prior to harvest. Proteins from cell lysates were separated by electrophoresis through a 10-20% acrylamide gel, embedded with En$^{3}$Hance and exposed to X-ray film. The products of the inserted genes are indicated by open circles.

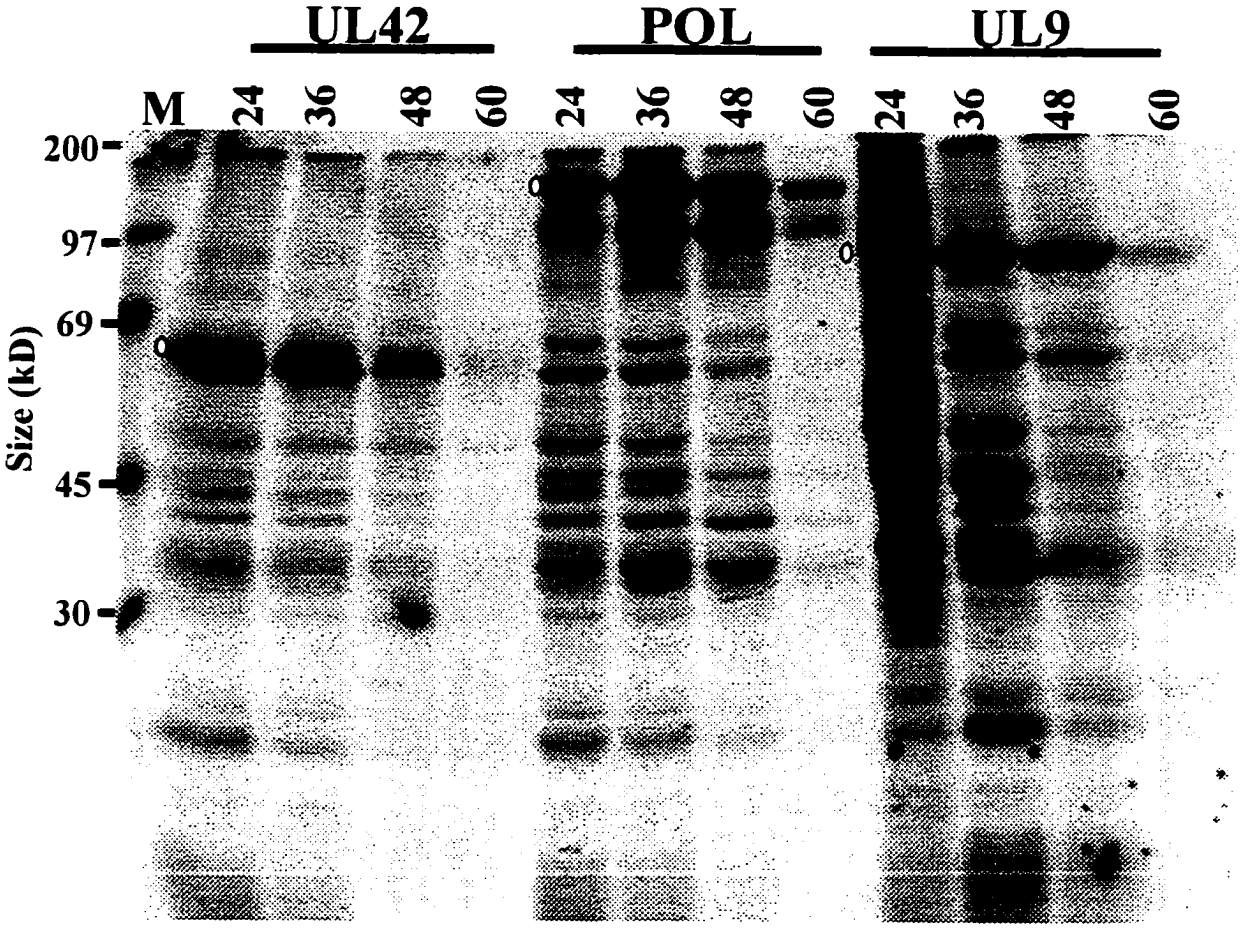

UL42
POL
UL9
M
24
36
48
60
24
36
48
60
24
36
48
60
200
97
69
45
30
Size (kD)

**Figure 29.**

**Figure 30. Cellular localization of Region I AcNPV/UL42 recombinant proteins.** Sf9 cells infected with recombinant virus were harvested and nuclei were separated from cytoplasmic extracts by dounce homogenization in hypotonic buffer. Lanes 1-5 represent cytoplasmic extracts while lanes 6-10 represent nuclear extracts.

**Lanes:**

1. AcNPV/ UL42
2. AcNPV/d129-163
3. AcNPV/T142A
4. AcNPV/R135D
5. AcNPV/B-gal
6. AcNPV/UL42
7. AcNPV/d129-163
8. AcNPV/T142A
9. AcNPV/R135D
10. AcNPV/B-gal

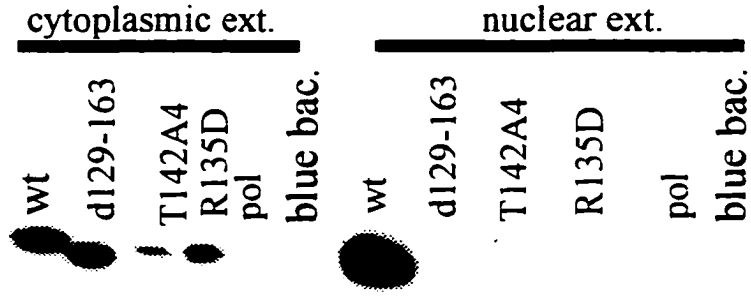

cytoplasmic ext.
nuclear ext.
wt
d129-163
T142A4
R135D
pol
blue bac.
wt
d129-163
T142A4
R135D
pol
blue bac.

**Figure 30.**

**Figure 31. Flowchart of the origin-dependent replication assay.**

# Origin-Dependent Replication Assay

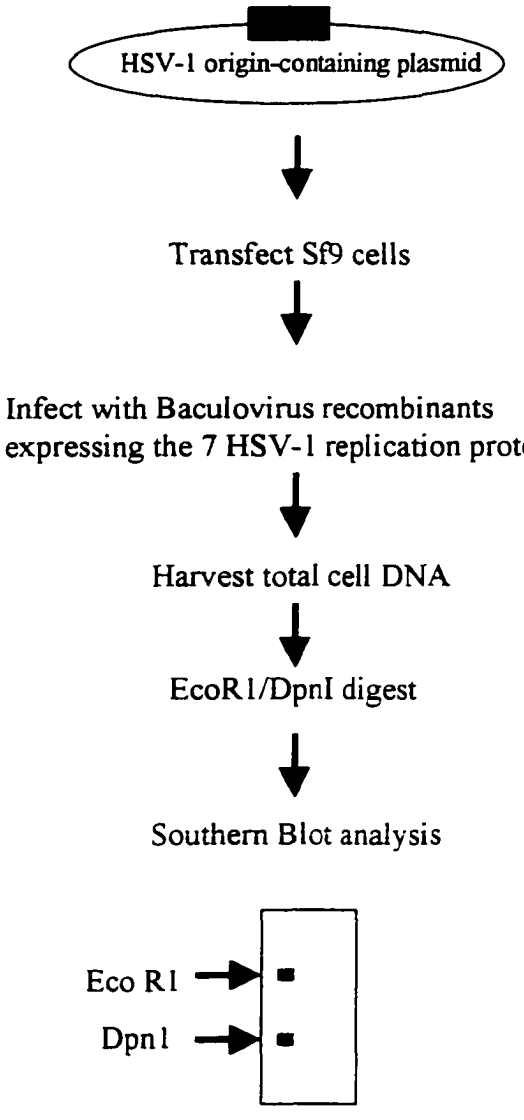



**Figure 31.**

**Figure 32. Ori-dependent replication assay (ODR) of UL42 baculovirus in Sf9 cells.** Sf9 cells were transfected with a HSV-1 origin containing plasmid pT085 and infected at a moi of 5 PFU/cell with 6 recombinant baculovirus encoding HSV-1 DNA replication proteins pol, UL9, UL8, UL5, UL52, ICP8,without UL42 or with 2 different wild-type UL42 constructs Cells harvested at 64 hours pi were phenol/chloroform extracted and RNase treated. DNA was EcoRI digested (lanes 1-3) to identify linearized unit-length pTO85 and EcoRI/DpnI digested (lanes 4-7) to identify DpnI-resistant origin DNA (upper band) from input DpnI sensitive DNA (lower band).

EcoRI
EcoRI/DpnI
-UL42
+UL42MC
+UL42KT
-UL42
+UL42MC
+UL42KT
-UL42
←DpnI
input
1 2 3 4 5 6 7

**Figure 32.**

**Figure 33. ODR of UL42 mutant baculovirus in Sf9 cells.** Sf9 cells were transfected and infected recombinant baculovirus (as described in figure 32) except for the substitution of mutant UL42 recombinant virus, as indicated.

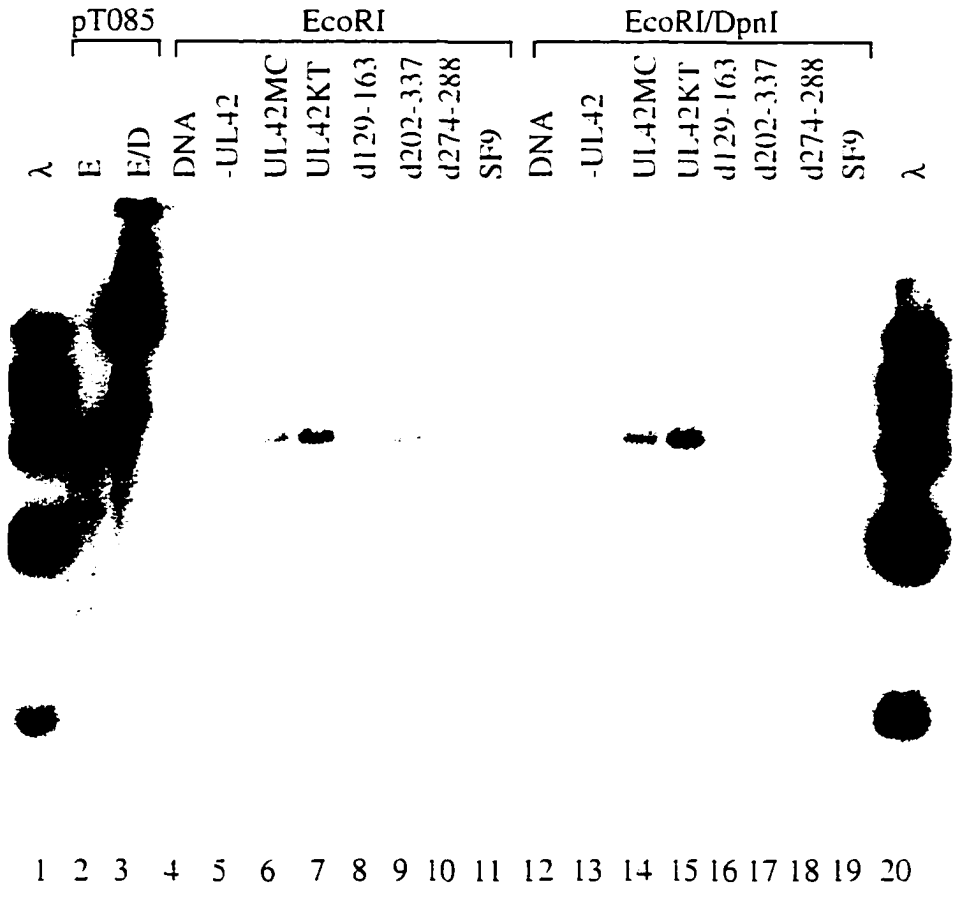

pT085
EcoRI
EcoRI/DpnI
λ
E
E/D
DNA
-UL42
UL42MC
UL42KT
d129-163
d202-337
d274-288
SF9
DNA
-UL42
UL42MC
UL42KT
d129-163
d202-337
d274-288
SF9
λ
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

**Figure 33.**

**Figure 34. Model for the Dominant-negative effect in the ODR assay.**

MODEL 1. Dominant -Negative Effect in ODR Assay.

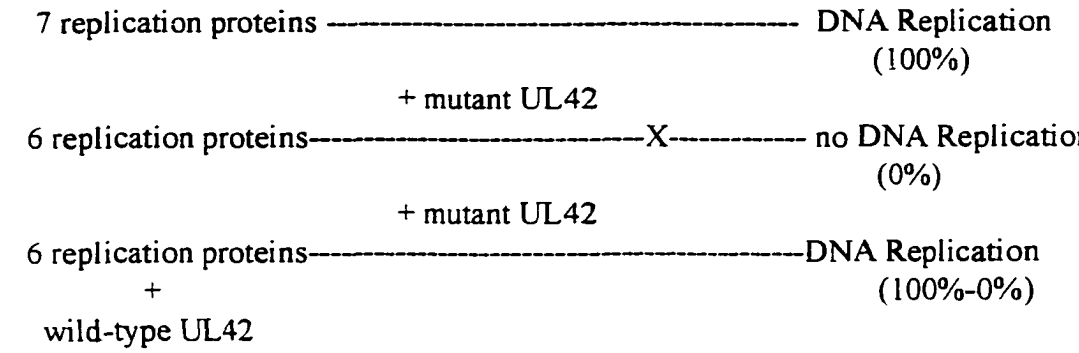



**Figure 34.**

**Figure 35. Origin-dependent replication *in vivo* with varying template concentrations.** Sf9 cells were transfected with a HSV-1 containing plasmid (pTO85) at varying amounts (0.5 μg, 1 μg, 2.5 μg. 5 μg. 10 μg) and infected at a moi of 10 or 20 PFU/cell with 7 recombinant baculovirus representing HSV-1 DNA replication proteins. Cells were harvested at 60 hours p.i., DNA extracted, digested with EcoR1 and Dpn1, and analyzed by southern blot as indicated in Chapter 2.

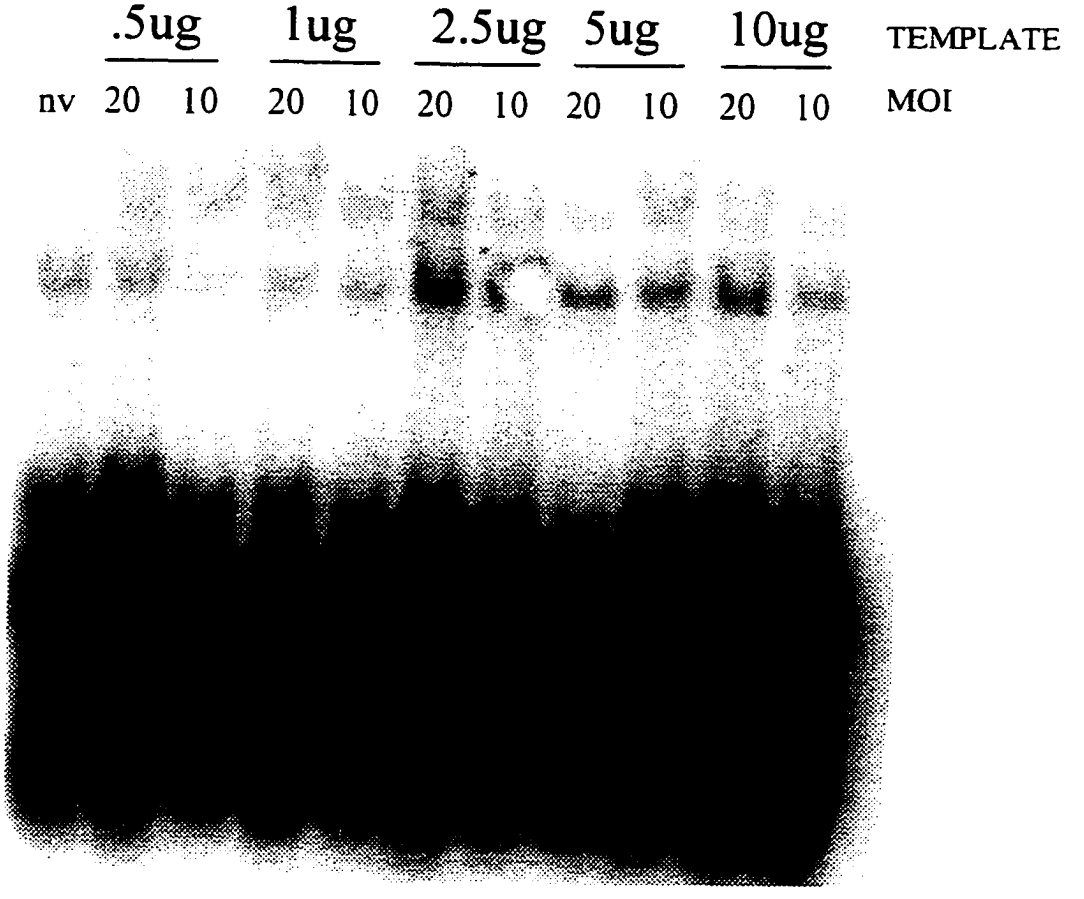

.5ug
1ug
2.5ug
5ug
10ug
TEMPLATE
nv 20 10 20 10 20 10 20 10 20 10
MOI

**Figure 35.**

**Figure 36. Origin-dependent Replication assay with varying moi.** Sf9 cells were transfected with plasmid pTO85 and infected with 7 recombinant baculoviruses representing HSV-1 replication proteins at a moi ranging from 0.5 to 20 PFU of each cell. DNA was analyzed as indicated in Figure 35.

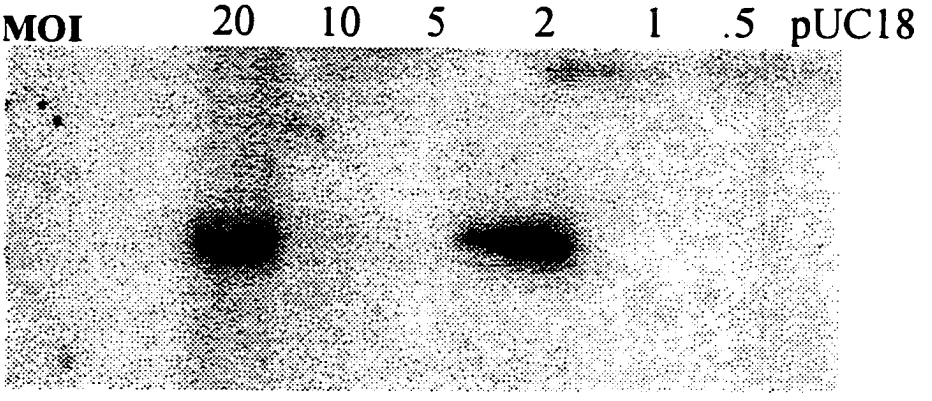



**Figure 36.**

**Figure 37. $^{35}S$ labeling of Recombinant proteins.** Sf9 cells were infected singly with recombinant baculoviruses (moi 10 PFU/ml) expressing each of 7 HSV-1 replication proteins or by co-infection with all 7 baculoviruses recombinants. Cells were labeled with [ $^{35}S$] methionine (50 μCi/mi),were harvested and were sonicated. Samples were electrophoresed by SDS-PAGE followed by fluorography. Lanes nv, and all 7 represent no virus and all 7 virus co-infected, respectively.

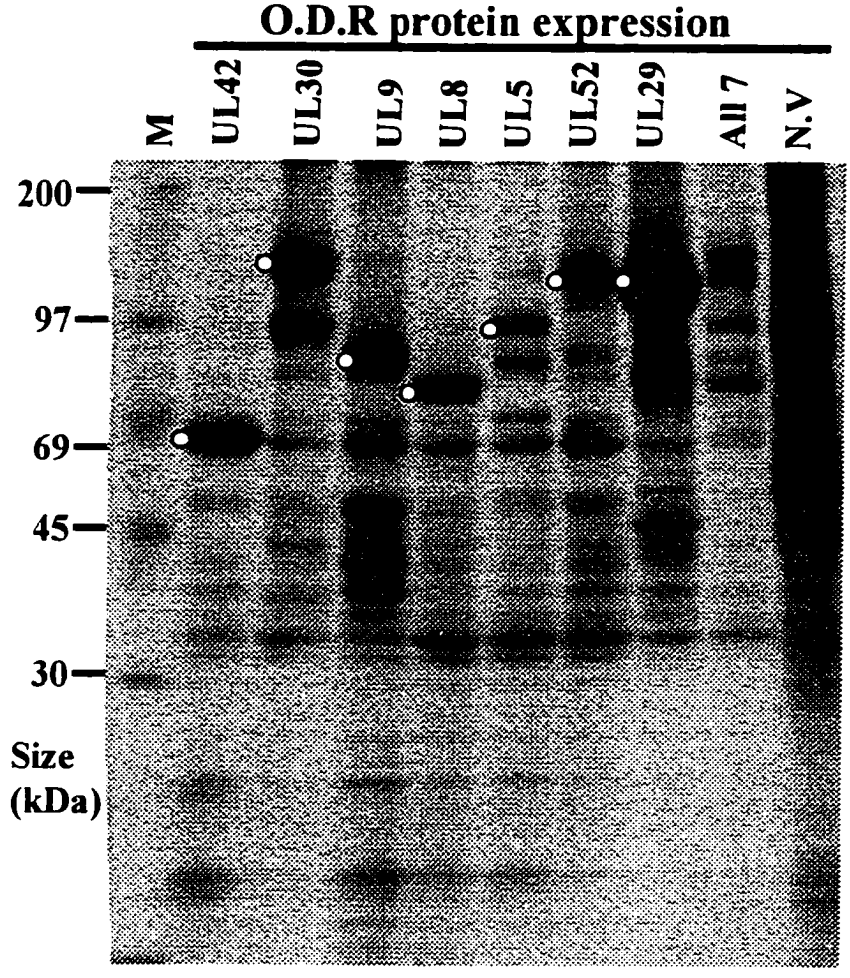

O.D.R protein expression
M
UL42
UL30
UL9
UL8
UL5
UL52
UL29
All 7
N.V
200
97
69
45
30
Size
(kDa)

**Figure 37.**

**Figure 38. Origin-dependent replication assay with varying ICP8, UL52 and UL42 moi.** Sf9 cells were transfected with pTO85 and infected with 7 recombinant baculoviruses at a moi of 5 PFU/cell for all but the indicated viruses. The input multiplicity of ICP8, UL42, and UL52 recombinant varied for 5 PFU/cell to 30 PFU/cell. Cells harvested at 64 hr pi were phenol/chloroform extracted and RNase treated, the DNA cleaved with Eco R1 and Dpn1, subjected to electrophoresis, analyzed by hybridization to a pT085 specific DNA probe.

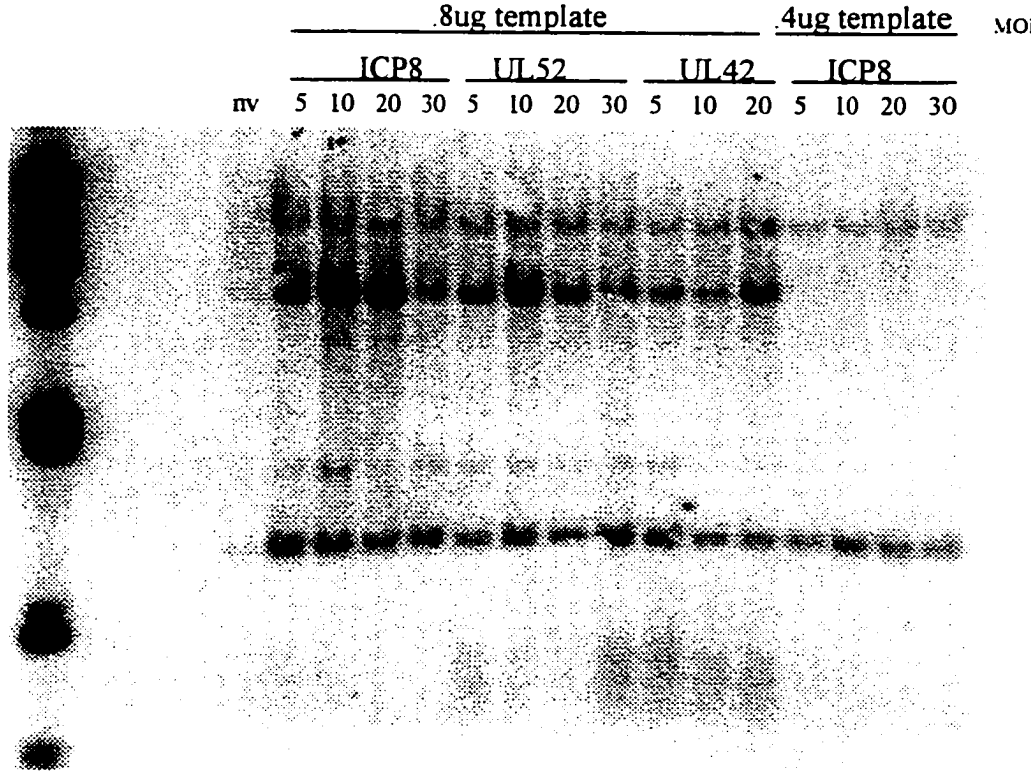

.8ug template
.4ug template
MOI
ICP8
UL52
UL42
ICP8
mv 5 10 20 30 5 10 20 30 5 10 20 5 10 20 30

**Figure 38.**

**Figure 39. Titration of T142A and WT UL42 RNA in pol stimulation assays.** Increasing amounts of T142A or UL42 RNA were translated (as described in chapter 2) and mixed with a constant amount of pol RNA. Pol assays were assayed for the incorporation of $^{3}$TTP into activated calf-thymus DNA. Results are shown as the fold stimulation of pol activity above basal pol (pol translated and assayed alone).

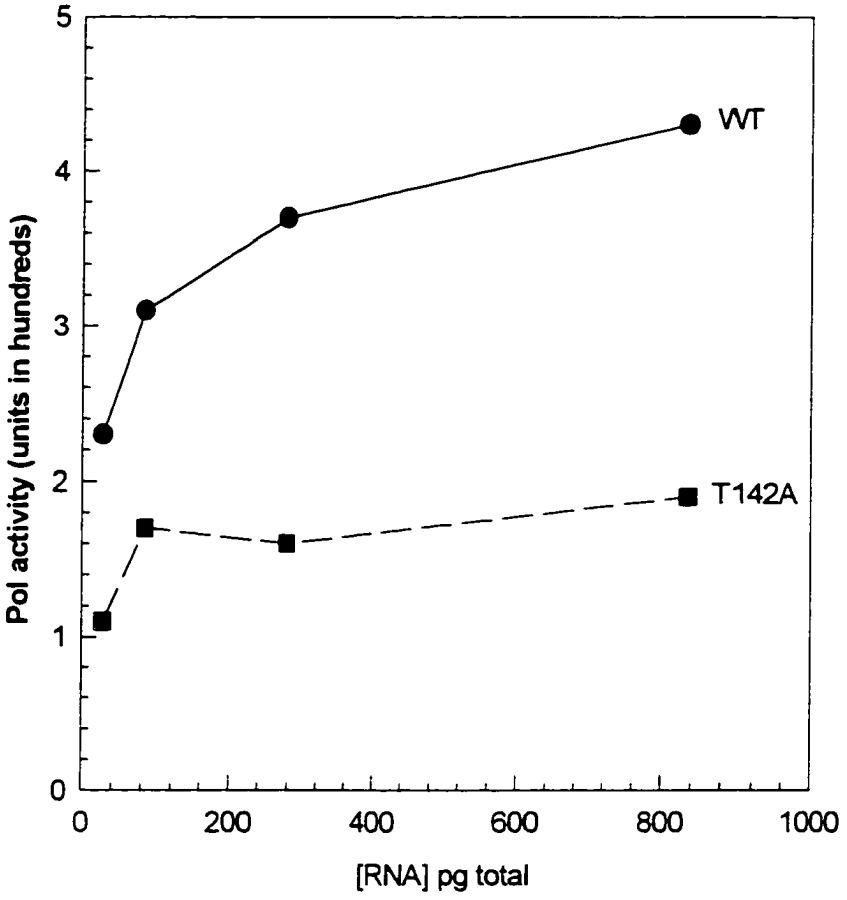

5
4
3
2
1
0
0
200
400
600
800
1000
Pol activity (units in hundreds)
[RNA] pg total
WT
T142A

**Figure 39.**

**Figure 40. IVTT polymerase stimulation interference assay.** Increasing amounts of UL42 and T142A RNA's were translated (as described in chapter 2). Increasing amounts of translated T142A was mixed with 30 ng of translated UL42. Constant amounts of translated pol was added to each mixture to initiate pol stimulation. Results are shown as $^{3}$TTP incorporated (in thousands).

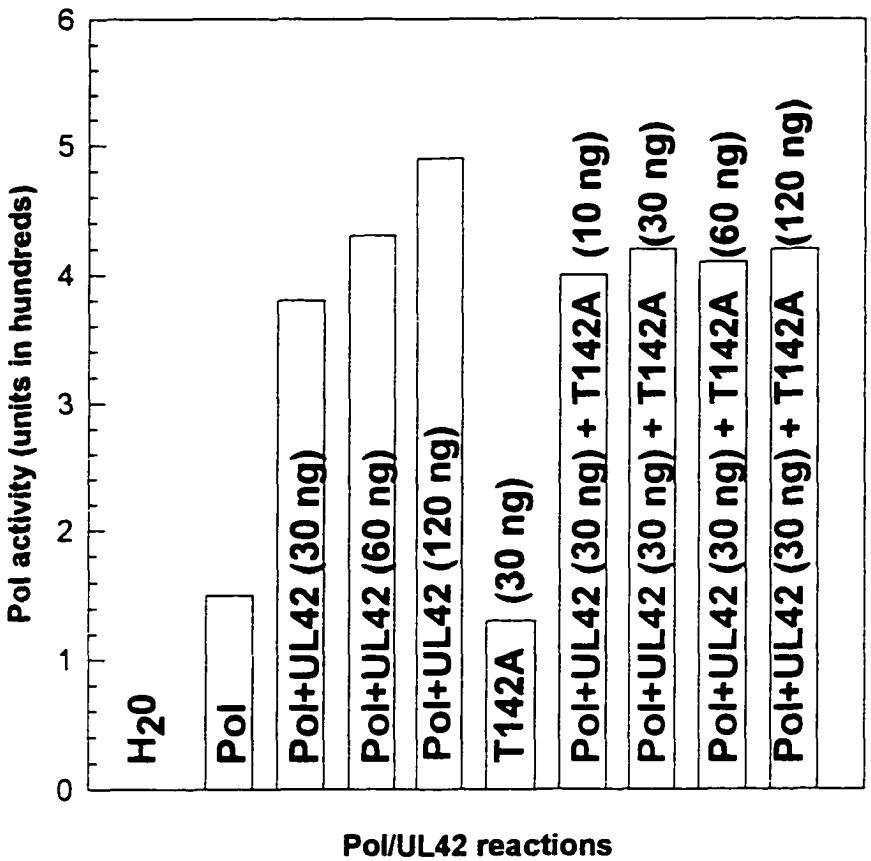

Pol activity (units in hundreds)
0
1
2
3
4
5
6
H2O
Pol
Pol+UL42 (30 ng)
Pol+UL42 (60 ng)
Pol+UL42 (120 ng)
T142A (30 ng)
Pol+UL42 (30 ng) + T142A (10 ng)
Pol+UL42 (30 ng) + T142A (30 ng)
Pol+UL42 (30 ng) + T142A (60 ng)
Pol+UL42 (30 ng) + T142A (120 ng)
Pol/UL42 reactions

**Figure 40.**

**Figure 41. Transient ODR Replication Assay.**

# Transient TransfectionOrigin-Dependent Replication Assay

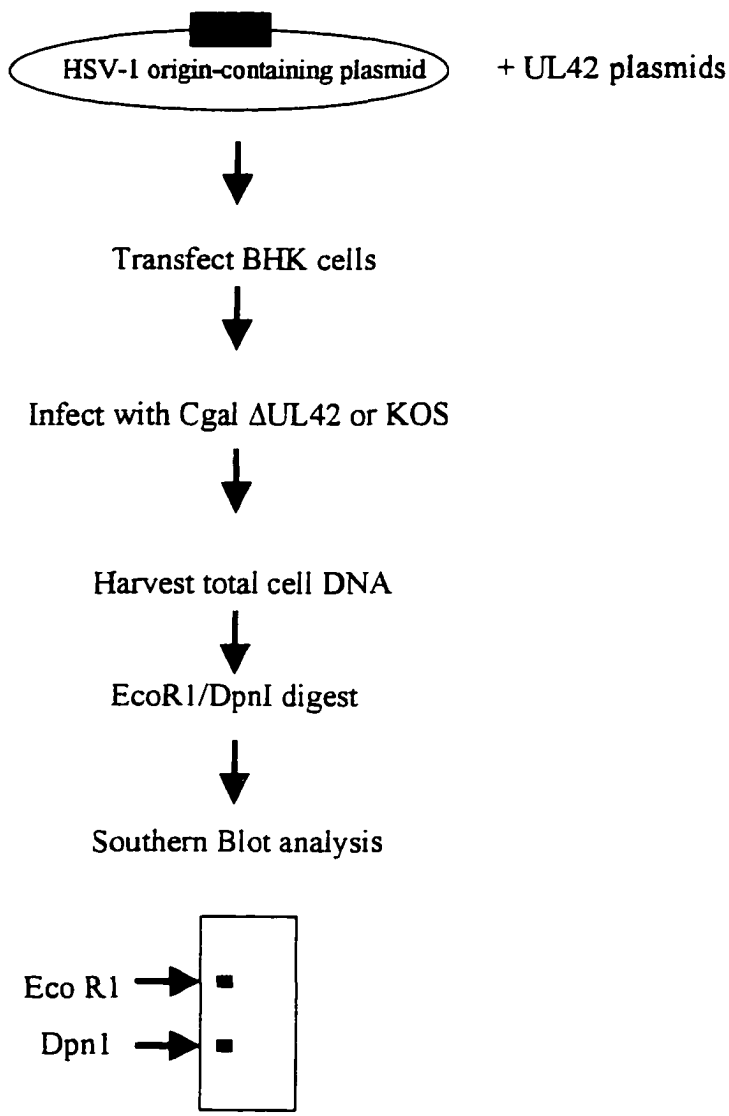



**Figure 41.**

**Figure 42. Replication of HSV-1 origin-containing plasmids pTO85 (1 copy of $Ori_s$) and pol14 (2 copies of $Ori_s$).** BHK cells transfected with HSV-1 origin-containing plasmids were infected with KOS for 24 hours. DNA prepared from cells were digested with EcoRI and DpnI. Fragments were subjected to Southern blot analysis with a plasmid sequence as described in Chapter 2. Arrows show amplified ori-containing plasmids.

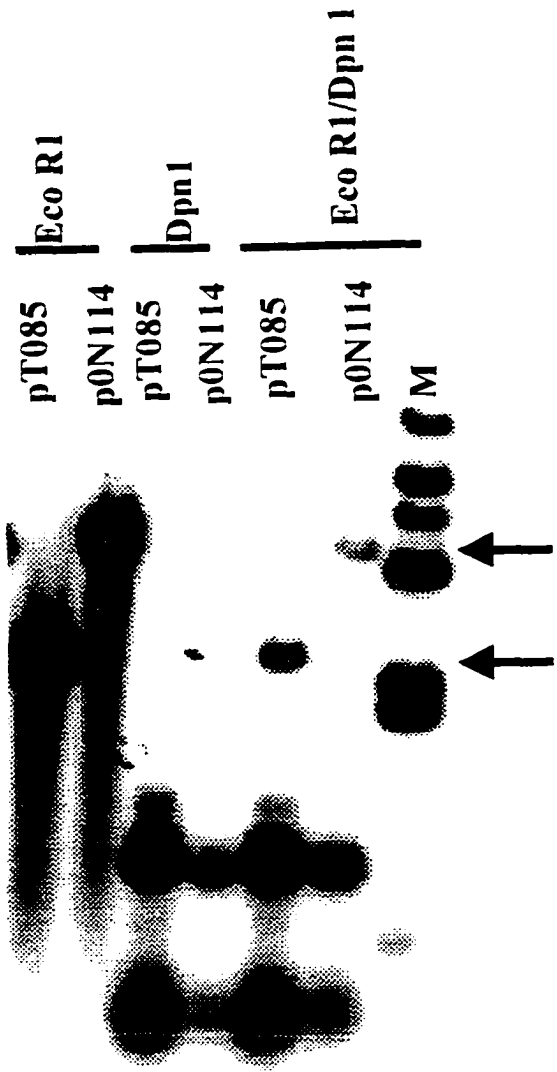
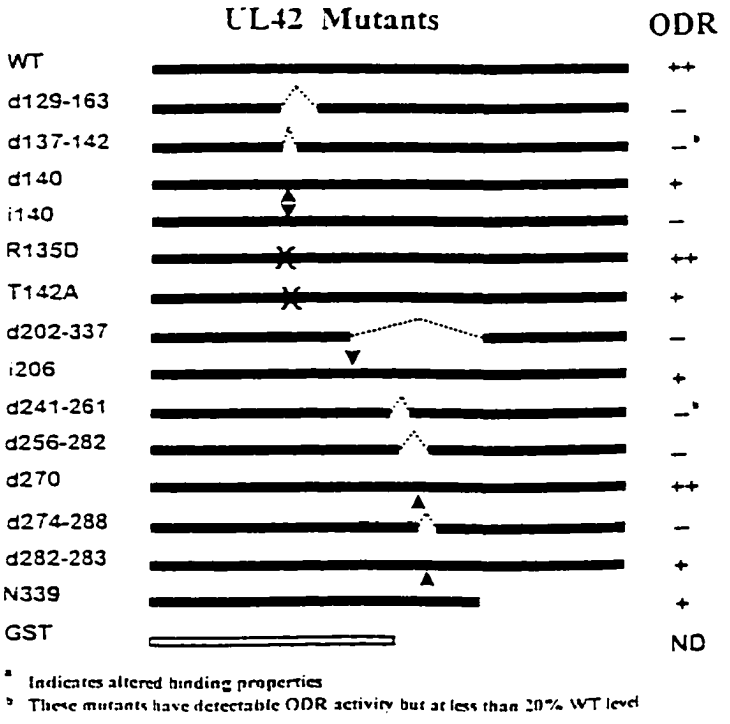

Eco R1
Dpn1
Eco R1/Dpn 1
pT085
p0N114
pT085
p0N114
pT085
p0N114
M

**Figure 42.**

**Figure 43. HSV-1 Origin-dependent DNA replication (ODR) assay.** BHK cells were co-transfected with an HSV-1 origin-containing plasmid (pTO85) and a plasmid containing a wild-type or mutant UL42 gene. These cells were infected later with CgalΔ42 at a moi of 10 PFU/cell. Cells were harvested at 24 hrs post infection and the DNA was extracted and RNase treated. The DNA was subsequently digested with EcoRI and DpnI. Digestions were electrophoresed on a 1% agarose gel, transferred to nitrocellulose and probed with vector sequence. The band corresponding to full length plasmid (→) was quantified by the phosphorimage analysis and the level of ODR adjusted to 100% for the wild-type UL42 plasmid.

100
17
102
10
67
101
0
49
17
0
88
0
47
VECTOR
VECTOR
WT UL42
WT UL42
d137-142
d140
i140
T142A
R135D
d202-337
i206
d241-261
d256-282
d270
d274-288
d282,3
E
D
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

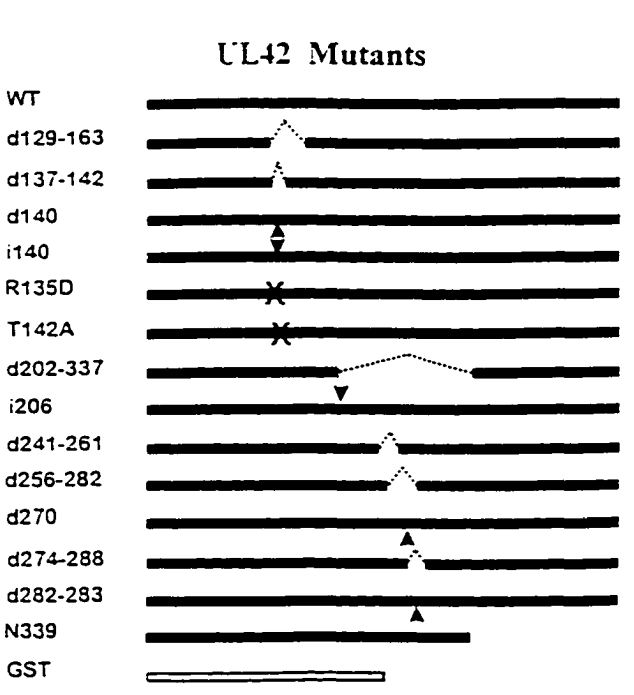
Figure 43




[a] Indicates altered binding properties
[b] These mutants have detectable ODR activity but at less than 20% WT level

**Table 7. ODR or region I and region II UL42 mutants.**

**Table 8.Summary of *in vivo* and *in vitro* functions of mutant UL42 polypeptides.** Dark areas indicate portions of the UL42 open reading frame expressed by mutant plasmids while the (▾) indicates the site of in-frame insertion mutations. Some deletion mutants are indicated by (▴) while site-directed mutations are shown by (x). The *in vitro* function of each mutant was determined by the ability of GST fusion proteins expressing the mutant protein to bind ds DNA, pol, or stimulated pol activity on activated calf-thymus DNA. The ability of each of the mutants to provide origin-dependent replication was determined by its ability to complement the replication of an origin-dependent plasmid when infected with CgalΔ42 as indicated in figure 44. The ability of each UL42 gene to function *in vivo* was determined by its ability to complement the replication of CgalΔ42.




| UL42 Mutants | ds DNA Binding | Pol Binding | Pol Stim. | ODR | C' Δ42 |
|---|---|---|---|---|---|
| WT | ++ | +++ | ++ | ++ | ++ |
| d129-163 | – | – | – | – | – |
| d137-142 | – | – | – | –[b] | – |
| d140 | – | + | + | + | ++ |
| i140 | – | – | – | – | + |
| R135D | – | + | – | ++ | ++ |
| T142A | – | ++ | + | + | ++ |
| d202-337 | – | – | – | – | – |
| i206 | +[a] | +++ | + | + | + |
| d241-261 | ++ | +++ | ++ | –[b] | – |
| d256-282 | – | – | – | – | – |
| d270 | ND | ND | ND | ++ | ++ |
| d274-288 | – | – | – | – | – |
| d282-283 | – | – | – | + | + |
| N339 | ++ | + | ++ | + | ND |
| GST | – | – | – | ND | ND |

[a] Indicates altered binding properties
[b] These mutants have detectable ODR activity but at less than 20% WT level

**Table 8. Summary of *in vivo* and *in vitro* functions of mutant UL42 polypeptides.**

# CHAPTER 6

## DISCUSSION

The HSV-1 UL42 protein is essential for the replication of HSV-1 DNA (Marchetti et al., 1988; Wu et al., 1988; Johnson et al., 1991). UL42 increases the activity and processivity of pol *in vitro* (Gallo et al., 1989; Gottlieb et al., 1990; Hernandez and Lehman, 1990; Monahan et al., 1993; Reddig et al., 1994). In this study, I have used three separate systems (glutathione-S-transferase affinity chromatography, recombinant baculoviruses, and origin-dependent replication) to examine indicators of various UL42 mutations on HSV-1 pol accessory function. The goal of this research was to define the minimal domains of UL42 that are required for UL42's pol accessory function *in vitro* and *in vivo*. This research was also extended to determine whether the UL42 activities which include pol binding, ds DNA binding and pol stimulation, are located within the same minimal domains of UL42.

### *In vitro* activity of UL42 mutants expressed as GST fusion proteins

The association of DNA polymerase with factors that increase the processivities of pol is a common occurrence among replication polymerases. These associations include the eukaryotic DNA pol δ + PCNA (Prelich et al., 1997), the E. coli Pol III + β subunit (Fay et al., 1982), and the bacteriophage T7 pol + E coli. thioredoxin (Tabor et al., 1987).

There is substantial evidence for the role of UL42 in HSV-1 replication (Marchetti et al., 1988; Wu et al., 1988; Johnson et al., 1991; Gallo et al., 1989; Gottlieb et al., 1990;

Hernandez and Lehman, 1990; Monahan et al., 1993; Reddig et al., 1991; Powell and Purifoy, 1977; Crute and Lehman, 1989; Hernandez and Lehman, 1990; Hamatake et al., 1993; Stow et al., 1993; Digard and Coen, 1990; Digard et al., 1993; Marsden et al., 1994; Digard ,1995; Gao et al., 1993; Gibbs et al., 1991; Tenney et al., 1993; Owisanka et al., 1993; Chow and Coen, 1995). For most of these studies, IVTT- expressed UL42 proteins were used to determine UL42 function. The variability of expression and the limited amount of protein expressed by *in vitro* translation limited the type of assays which could be used to characterize UL42 and compromised quantitation of the results of such assays. These limitations have led to conflicting results obtained with similar mutants of UL42 (Chow and Coen, 1995; Monahan et al., 1993). As a way to circumvent this problem and to more accurately quantify the activities of UL42 mutant proteins, I employed affinity chromatography with GST fusion proteins to characterize the ability of UL42 mutants to determine various aspects of pol accessory functions *in vitro*.

The existence of pol/UL42 complex within infected cells and the tight association of pol:UL42 ($1.2 \times 10^8$ $M^{-1}$, Hamatake et al., 1993) suggest that the ability of UL42 to complex with pol is critical during HSV-1 infection (Digard et al., 1990; Hernandez and Lehman, 1990; Gottlieb et al., 1990). The ability of UL42 mutant proteins to bind pol correlated with their abilities to complement UL42 null mutants (Digard et al., 1993) and the observation that pol mutants which retain catalytic activity but are unable to complex with UL42 are incapable of supporting viral replication (Digard et al., 1993) or origin-dependent DNA synthesis (Stow et al.,1993) further supports the importance of UL42 in HSV-1 DNA replication. I have demonstrated that UL42 is sensitive to mutations in that most of the

mutations made throughout the N-terminal 2/3 of UL42 (d137-142, d129-163, d202-337, d256-282) of the protein eliminated pol accessory function and completely abolished the ability of the protein to function *in vitro* and *in vivo* (Table 8). Pol binding is a excellent indicator of the ability of UL42 mutants to function as pol accessory proteins in that all of the mutants that failed to bind pol failed to stimulate pol activity *in vitro*. On the other hand, UL42 mutants that bound pol even partially (above 10%) maintained the ability to stimulate pol activity *in vitro* and provide origin-dependent replication (Table 8). These results were predicted since several labs have demonstrated the importance of the pol/UL42 interaction for processive DNA replication. (Marchetti et al., 1988; Wu et al., 1988; Johnson et al., 1991; Gallo et al., 1989; Gottlieb et al., 1990; Hernandez and Lehman, 1990; Monahan et al., 1993; Reddig et al., 1991; Powell and Purifoy, 1977; Crute and Lehman, 1989; Hernandez and Lehman, 1990; Hamatake et al., 1993; Stow et al., 1993; Digard and Coen, 1990; Digard et al., 1993; Marsden et al., 1994; Digard ,1995; Gao et al., 1993; Gibbs et al., 1991; Tenney et al., 1993; Owisanka et al., 1993; Chow and Coen, 1995).

My results are consistent with the hypothesis that there are multiple distinct regions of UL42 required for pol binding. One region is upstream of amino acid residue 241 and includes a previously described region I of UL42 (Monahan et al., 1993). Mutations in this region have a less profound effect on pol binding than mutations made in other regions of UL42 in that point mutants (T142A, R135D) retained some pol binding activity, while the larger deletions failed to bind pol at any significant level above that of GST. A second region is downstream residues 261 of UL42 and includes previously described region II mutants (Monahan et al., 1993). This region was also equally sensitive to mutations in that all of the

mutants C-terminal of amino acid deletion 261 failed to bind pol. These observations are consistent with those found by Digard et al., (1993), where internal deletions spanning the entire UL42 open reading frame failed to bind pol . However, two of their deletion mutants, deletions of amino acids 9-20 and 242-250, bound pol and maintained the ability to stimulate pol activity *in vitro* (Digard et al., 1993) while a single insertion at residue 160 (i160) from a group of sensitive insertion mutations spanning UL42 maintained pol binding activity . The loss of both pol binding and DNA binding of all of their deletion mutants and the inability of insertion mutants to lose those activities support the hypothesis that loss of activity may be due to protein misfolding. My studies were able to address this hypothesis by creating and testing more subtle site-directed mutations. Mutants T142A, R135D, and d140 used in my study within region I (d129-163) of UL42, which also includes residue 160 (previously described by Digard et al., (1993), were reduced in pol binding by at least 50% and retained partial pol stimulation activity *in vitro*. The fact that these small insertion, deletions and site-directed mutations of UL42 retain only partial pol binding and pol stimulation strongly suggests that the effect of the mutations in this region of UL42 may not a result of the global misfolding of the protein and that this region is involved in the ability of UL42 to bind pol. However , I cannot totally rule out this possibility. Defining regions that affected the ability of UL42 to bind pol did not determine if these regions are involved in the direct contact with pol or if they comprise part of the protein that stabilizes another region of UL42 that binds pol or provide a conformation that assists in the interaction with pol. The limitations of my and other experiments do not allow determination between the ways that mutations in these regions of UL42 affect pol binding. Although I did not identify a single defined region of

UL42 that affected pol binding, my results did show that there is a severe structural dependence on UL42 required for its physical interaction with pol since mutations spanning most of UL42 are defective in pol binding. Although no direct evidence exists on the effects of these mutations on pol binding, Chou-Fasman (Monahan, unpublished) and Robson-Garner (Chow and Coen, 1995) secondary structure predictions have identified important motifs that may be involved in protein:protein or protein:DNA interaction. Conclusive evidence of the effects of these mutations on UL42 structures can be elucidated through X-ray crystallography of the mutants alone and in the presence of pol.

Previous data showed that UL42 bound to ds DNA (Gallo et al., 1988, Vaughan et al., 1985). There are no known structural models for ds DNA binding domains, which makes it difficult to determine the mode of the UL42:DNA interaction and the domains of UL42 involved in this interaction. Although there is no direct evidence that the ds DNA binding activity of UL42 is directly responsible for the the ability of UL42 to increase the processivity of pol, the findings that pol's affinity for a primer:template junction increased by 10 fold with the addition of UL42 (Gottleib et al, 1994) supports the notion that UL42 may act as a clamp for pol. At the same time, the finding that UL42 binds DNA in a complex with pol and increases the nuclease protection of ds DNA by pol with no effect on the protection of ss DNA demonstrated that UL42 does not function like the class of pol accessory proteins which include β, PCNA, gene 45 or thioredoxin that do not bind to DNA (Kong et al., 1995, Collman et al., 1995, Younger et al., 1992).

As an alternative method to identify the ability of UL42 mutants to associate with DNA, I used a GST-affinity method which was a reciprocal approach compared to the

previous used techniques that eluted IVTT expressed UL42 mutants from ds DNA cellulose (Chow and Coen, 1995; Digard et al., 1993). A problem with the latter method was that it allowed leakiness and spreading of the elution profiles over several NaCl elutions and could not be readily quantified. By contrast, my GST-based assay was sufficiently informative in identifying ds DNA binding and non-binding behavior and was reproducible. I found that DNA binding is not a good indicator of UL42 function in that mutations spanning UL42's open reading frame abolished DNA binding, yet retained the ability to bind pol, stimulate pol activity, and provide origin-dependent replication in tissue culture (Table 5). Results demonstrated that UL42 is sensitive to mutations that affect its ability to bind ds DNA (Table 5 ) in that most of the mutants failed to bind ds DNA. The loss of DNA binding activity of UL42 mutations was consistent with results of Digard et al 1993, where all of the internal mutants tested failed to bind DNA cellulose. In their studies, the fact that insertion mutants created within the UL42 had no affect on DNA binding again did not rule out the possibility of misfolding of the protein. In my studies, the inability of point mutations T142A and R135D to bind ds DNA suggests that global mis-folding of the protein *per se* is not the sole reason for the loss of DNA binding activity of UL42. These results also suggest that residues in and around this region of UL42 are essential for DNA binding. The loss of activity of mutants in this region is supported by the predictions of Monahan (unpublished) who used the Chou-Fasman and Robson-Garnier algorithms to predict that residues 130-162 of UL42 form a helix-loop-helix motif similar to DNA binding regulatory proteins and eukaryotic transcription factors (Pabo et al., 1992). More importantly, the ability of these mutants to stimulate pol activity in vitro suggests that DNA binding is not required for partial stimulation

of pol activity by UL42 even *in vitro*.

I observed a second region which altered DNA binding which includes residue 206 and areas downstream of residues 261 of UL42. The behavior of mutants which failed to bind DNA in low salt (50 mM NaCl) was more easily distinguishable than those which behaved like wild-type and mainly eluted in high salt (250 mM NaCl). On the other hand, insertion mutant at residue 206 (i206) displayed an elution profile different than wild-type UL42 in subtle ways. Only 40% of this mutant bound DNA and bound material was eluted in 250 mM NaCl. These results are consistent with those mutants i203 and i206 produced by Coen and Chow, 1995, which had similar aberrant binding profiles to ds DNA cellulose. There could be several reasons for the elution profile of protein in both high and low salt. First, the impaired ds DNA binding ability of the mutant could account for an altered elution profile. However, this would not adequately explain why some mutants elute in high salt. Secondly, the altered profile could mean that the GST/i206 does not bind to the glutathione agarose like wild-type and thus less protein might have been available to bind DNA. I excluded this possibility since GST/ i206 and GST-wild-type UL42 remained bound to glutathione agarose at 1000 mM NaCl as demonstrated by coomassie blue staining of SDS polyacrylamide gels (data not shown). Thirdly, partial misfolding of protein could make DNA binding less stable than for wild-type UL42 with only a small fraction of the proteins, properly folded, bound DNA. These mutations could inhibit UL42 from interacting with other domains important for DNA binding or partially destabilize the protein without completely destroying the conformation of the DNA binding region.

Using multiple alignment of UL42 homologs VZV, PRV, and EHV Chow and Coen,

1995 identified a phenylalanine at residue 203 to be absolutely conserved with high conservation upstream of these residues being noted among the alphaherpesvirus. More importantly, Robson-Garnier secondary structure predictions by these investigators suggested that the region around 203 and 206 might be flexible and they hypothesized that alteration in the flexibility of this region could alter DNA binding (Chow and Coen, 1995).

Owsianka et al 1993, reported that 15 peptides between a larger region of UL42 which included amino acids 269-303 could bind DNA. These results are consistent with the hypothesis that non-contigious regions of UL42 are required for DNA binding (Digard et al, 1993; Chow and Coen 1995). One region could stabilize another with only one interacting directly with DNA. Taken together, these results suggest that the DNA binding activity of UL42 is located in multiple regions of UL42 that contain structures that are common to DNA binding proteins. As seen with pol binding, the true indicator of the roles of the mutations in DNA binding would be through X-ray crystallographic determination of the structure of UL42 (mutants and wild-type) with DNA and pol.

UL42 stimulation of pol activity is indicative of its ability to provide pol accessory function *in vitro*. I have demonstrated that the ability of UL42 mutants to stimulate pol activity in vitro was dependent on the ability of the mutants to retain one or more UL42 functions (DNA binding or pol binding) (Table 3). My results are similar to those in other studies of UL42 where pol stimulation was linked to DNA and pol binding ( Chow and Coen 1995; Monahan et al., 1993 Redding et al., 1994; Tenney et al., 1993; Digard et al., 1993). These assays differ from my studies in that they used *in vitro* transcribed/translated proteins to stimulate pol activity or to produce full length M13 DNA while my studies involved the

use of partially purified fusion proteins to stimulate pol activity. While my pol stimulation assay does not directly measure processive DNA synthesis, there is a good correlation between the ability of an active pol/UL42 complex to stimulate pol activity on calf thymus DNA and to generate long products and increased pol processivity ( Hamatake et al., 1993). Whereas pol stimulation identifies an increase in the ability of pol to fill small gaps in DNA, a processivity assay measures the ability of pol to remain bound to DNA primer template through many successive additions of nucleotides. For example, the inability of Chow and Coen to observe processivity with UL42 mutant i206 while our i206 mutant stimulated pol activity by 2-fold by my assay, reflects the limited specificity of the proccessivity assays which record the production of full length DNA products only. Some mutants tested by both assays yielded the same results (i.e. UL42 mutant d129-163 which failed to stimulate pol activity Monahan et al 1993, also failed to provide processive DNA synthesis in proccessivity assays (Chow and Coen, 1995) suggesting that this specific mutation was absolutely involved in pol binding and may abolish all of the possible activities involved in UL42 pol accessory activity. Inconsistencies in their assays may result from an inability to differentiate mutants that bind pol and change its conformation of pol to a processive enzyme from those that bind pol and merely tether it to DNA without a conformational change. It will be important to examine the exact mechanism by which each of these mutants affects pol activity to gain a better understanding of the entire spectrum of interactions possible between UL42 and pol.

**Mechanism of UL42 as a Processivity Factor**

The mechanism by which UL42 increases the processivity is slowly becoming unraveled. Because UL42 does not fit into the standard class of polymerase accessory factors known to

date, it may represent a novel class of replication proteins (specific to the herpesvirus family) that binds ds DNA independently to increase the ability of UL42 to stimulate pol activity. The ability of UL42 to bind DNA is difficult to reconcile with known mechanisms of pol processivity factors ( eukaryotic DNA pol δ + PCNA (Prelich et al., 1997), the E. coli Pol III + β subunit (Fay et al., 1982),gp43 of T4 bacteriophage + gp45 and the bacteriophage T7 pol + thioredoxin (Tabor et al., 1987).

UL42 is similar to other pol accessory proteins in that it is required for DNA replication and increases the processivity of the pol catalytic subunit. This is where the similarities between UL42 and the β subunit of E coli pol III end. β subunits of E. coli pol, PCNA of eukaryotes and gene 45 of T4 all require ATP for loading and cofactors for activity. At the same time, none of these proteins binds DNA by standard assays. Evidence does suggest that these proteins form a torus around DNA and that this activity allows them (β, PCNA, and T4) to tether pol to DNA to increase the processivity of pol (Naktining et al 1996, Yao et al 1996, Younger et al 1992). *E coli* thioredoxin is the accessory protein of T7 pol and may be more closely related to UL42 because ATP and cofactors are not required for loading (Tabor et al, 1993).

The results obtained with the partially purified GST fusion proteins provide a more accurate method to characterize the effect of UL42 on pol stimulation than previous studies, which were limited by ability of IVTT-expressed proteins to inhibit pol stimulation at high concentrations, (Digard et al 1993, Tenney et al 1993, Monahan et al 1993, Reddig et al 1993). In my studies, the titration of mutant proteins over several concentrations allowed me to detect measurable changes in relative affinities of mutant proteins in pol stimulation and

suggests 2 mechanisms by which UL42 may independently affect the ability of UL42 to stimulate pol activity.

I proved that intrinsic DNA binding was not necessary for UL42 to provide pol accessory function though pol binding appears to be important. Regions of UL42 including residues 129-163 and 202-337 are involved in pol binding, DNA binding , and pol stimulation (Monahan et al, 1993). Reduction of UL42's ability to bind ds DNA or pol diminishes pol accessory function *in vitro* and *in vivo*. I was unable to identify a mutant form of UL42 which abolished binding to either pol or DNA but not both. The closest case was mutant T142A, which bound pol at 50% of wild-type UL42, did not bind ds DNA, and stimulated pol at only 50% the level of wild-type UL42. Four times the amount of T142A was required to reach saturation of pol stimulation as required for wild-type UL42. These results suggest that the inherent DNA binding of UL42 is not absolutely required for pol stimulation. More importantly, it suggests that simply tethering pol to DNA does not account for all of the UL42-induced pol stimulation. Pol activity could also be enhanced independently through the physical association of UL42 to pol that alters the conformation of pol to enhance the affinity for template. Another UL42 mutant, i206, which bound pol like wild-type and bound DNA with an altered profile stimulated pol only 2 fold (even at the highest concentrations tested), suggesting that i206 may aberrantly bind to DNA and pol and tether pol to DNA without altering the conformation of pol and rendering it fully proccessive. Alternately i206 could provide a conformational change in pol that is different from the wild-type UL42/pol complex which makes pol less active. This would account for the low stimulation of pol activity provided by i206 and the lack of pol activity observed by Chow and Coen et al (1993).

Although the afore mentioned mutants retain some activity, results show that UL42 is highly sensitive to mutations. Taken together, these results suggest that a physical association of pol and UL42 to produce a heterodimeric complex is not sufficient to provide full pol stimulation. Results suggest that fully functional UL42 is dependent upon a conformation which requires most of the N-terminal 2/3 of the protein. Results showed that there are no defined short domains which can independently bind to pol or DNA, suggesting that UL42 is a protein in which several regions act together to provide a conformation that is sufficient for UL42's pol stimulation activity .

One possible mechanism of UL42 pol accessory function could include the tethering of pol to a DNA template. A second mechanism could involve a conformational change in pol that increases its processivity. My results suggest that both DNA binding and pol binding play a role in increasing the activity of pol. In my model, UL42's physical association with pol alters the conformation of pol to independently allow enhancement of pol activity (this would explain the results of mutant T142). A conformational change in pol produced by UL42 binding could allow pol to slide along template and increase its affinity for the template. At the same time, the DNA binding ability of UL42 tethers pol to DNA to allow an increased affinity to DNA which would result in an increase in processivity (this would explain the low level enhancement of pol activity by mutant i206). The partial activity of the two mutants (T142A and i206) suggests that an overall enhancement of pol activity could be achieved independently through UL42's ability to alter the conformation of pol and provide an enhanced affinity for the template. Interestingly, evidence in support of this idea include the results that demonstrated that a pol/UL42 complex has a 1000-fold higher affinity for

primer/template than pol alone in high salt conditions (Sun, Chaudhuri, and Parris, unpublished). This is far beyond the predicted binding of protein that simply tethers pol to DNA. Gottlieb et al., (1993) determined the binding dissociation constants ($K_D$) of $7.1x10^{-9}$ for pol, $7.8x10^{-10}$ for pol:UL42, and $1.1x10^{-9}$ for free UL42 by Scatchard analysis of the gel shift and filter binding data. If this model is correct, it suggests a different mode of action for pol accessory proteins (PAP's) and raises the question of the mechanism by which a pol/UL42 complex could slide along and elongate primer without acting as a brake on pol. Although the limitations of my experiments do not show which occurs first, I would predict that UL42 bound to pol would bind DNA, which would in turn cause another conformational change that would allow Pol to move along the DNA template . A third possibility is that one or both mechanisms play a role or that they are required for the same protein structure needed for activity. Again, my assays have not proved or disproved this theory. There could also be a situation where 2 domains of UL42 separated by a linker region that is essential for DNA binding such that the interaction with pol occurs at one or both regions allowing formation of a complex which partially encircles duplex DNA templates. The definitive way to determine this model would be to observe the X-ray crystal structure of pol, pol:UL42, and both bound to DNA.

**Correlation of *In vitro* and *In vivo* Activities of Mutant UL42 Proteins**

*In vitro* assays demonstrate that UL42 is specifically required for maximum pol activity in high salt conditions thought to occur in infected cell nuclei in the absence of other viral proteins. If UL42 is required *in vivo* for its pol accessory function, it is predicted that there would be a correlation between the ability of mutant forms of UL42 to stimulate pol

activity *in vitro* and provide *in vivo* functions in the context of other HSV-1 or cellular proteins. My results demonstrated that all of the mutants that failed to stimulate pol activity *in vitro*, also failed to provide function for replication of an HSV-1 origin containing plasmid (Table 3). The results suggest that the inability of the mutants to provide pol accessory function was not complemented or overcome by cellular or HSV-1 proteins. As expected, mutants that stimulated pol activity were able to provide origin-dependent replication *in vitro*. However, several of the mutants that retained some pol binding activity ( d140, d282 3, R135D, d241-126, i206) *in vitro* retained some *in vivo* activity. What this suggests is that these mutants were able to overcome their lack of DNA binding by possibly interacting with other HSV-1 replication proteins when bound at the origin to allow replication .Alternately, the level of synthesis of the mutant proteins in the context of virus replication is sufficient to compensate for low affinity binding to DNA. The fact that mutant i206 provided minimal pol stimulation in vitro, failed to provide partial origin-dependent replication, and only partially complemented a null UL42 virus suggests the limited DNA binding provided by i206 may be sufficient to provide limited *in vivo* function. These results can be reconciled if the level of UL42 produced by the mutants is sufficient to complex with pol partially when aided by complex association with some of the other HSV-1 DNA replication proteins. In experiments performed by Lynn Grinstead (unpublished), many of the UL42 mutants that failed to provide ODR were unable to fully complement CgalΔ42 *in vivo*.

One notable mutant, d241-261, bound pol, bound DNA and stimulated pol activity *in vitro*. However, it was incapable of providing replication function in the presence of the other DNA replication proteins by *in vivo* complementation of a null CgalΔ42 virus or by

origin-dependent replication. This is consistent with results by Digard et al., (1993), who found that Δ242-250 was not affected in DNA binding and pol binding but did not complement a null UL42 mutant. Hamatake et al (1993), showed that this region was not associated with DNA binding but was flanked by 2 or more protease sensitive regions that are required for the association with DNA.

There are several reasons that a mutant protein that functions *in vitro* is inactive *in vivo*. First, there could exist a different conformation *in vivo* vs. *in vitro*. This appears unlikely since wild-type UL42 protein expressed by IVTT and the GST-fusion systems both stimulated pol. However conformation cannot be totally ruled out. Second, there could be different protein amounts in each assay used. Third, inaccurate localization of the mutant could inhibit *in vivo* activity. Identification of the ability of d241-26 to localize to the nucleus is currently being performed in our lab. Fourth, the mutant protein expressed *in vivo* could influence the ability of UL42 to interact with other HSV-1 replication proteins. Support for this idea is based on the observation that UL42 has been shown to bind to UL9 (Monahan et al.,1998). Preliminary experiments by Lynn Grinstead with GST/UL42 mutants showed that d241-261 maintained the ability to bind UL9. It will be interesting to determine if these UL42 mutants interact with other HSV-1 proteins. This could possibly provide a third mechanism by which UL42 could enhance pol activity *in vivo* in the background of other cellular and viral proteins.

Evidence for the association of UL42 with other replication proteins is supported by Robson-Garnier 2° structure prediction algorithm (Garnier et al., 1978) that showed that the final 35 amino acids of pol has 3 -4 hydrophobic repeats. That raised the possibility that pol and UL42 heterodimerize by hydrophobic interaction, similar to proteins of the leucine zipper

class of transcription factors. Interestingly, a match for an amphipathic and helical region of UL42 lies between residues 242-253 (Digard et al., 1993). These observations suggest that this region of UL42 could bind to another replication protein and the absence of this region could inhibit UL42's ability to function *in vivo*.

Despite the structural and functional similarities of pol accessory proteins, they show no obvious sequence homology (O'Donnell et al 1993). Nevertheless, it is clear from my studies and the studies of others that apparently minor differences in the amino acid sequence can affect the ability of the accessory proteins to interact with pol. The specificity of the interaction of UL42 with pol is necessary for *in vivo* function (Digard et al 1993, Stow et al 1993) and indicates a possible avenue for the development of antiviral compounds which target the interaction of pol and UL42 and is highly specific for blocking viral DNA replication.

The knowledge that the assembly of various complexes including protein:protein and protein:DNA interactions are critical for HSV-1 DNA replication, suggests that interference of any of these interactions may inhibit DNA replication. The targeting of protein:protein interactions may be a useful strategy since more than one protein is required for replication so it would be hard for single resistant strains to develop. The interest in protein:protein interactions has grown from observations that HSV ribonucleotide reductase is specifically inhibited by a nonapeptide corresponding to the small subunit of the enzyme (Coen et al., 1986; Dutia et al., 1989). The peptide acted by competing for a site on the large subunit to which the smaller one binds and so inhibits normal association of the 2 subunits (Coen et al., 1986, Dutia et al., 1989, Darling et al., 1990, McClements et al., 1988, Luzzi et al., 1994).

These results suggested that in principle, any biological process that depends on protein:protein interaction between 2 subunits could be blocked by compounds that interfere with that interaction.The interaction between pol and UL42 with an association constant of $1 \times 10^8 M^{-1}$ (Hamatake et al., 1993) and the requirement of this interaction for viral replication has led to growing interest in UL42 as a potential novel antiviral drug target using compounds that can interfere with heterodimerization. It is also possible that antiviral agents that mimic the DNA binding region of UL42 may also interfere with viral DNA replication. This would make a UL42 DNA binding site a good target. Such an antiviral agent could be HSV specific and not affect cellular functions.

DNA replication is a complex phenomenon that has been studied in a variety of prokaryotic, eukaryotic, and viral systems. It is becoming increasingly apparent that the fundamental mechanisms of DNA replication have been highly conserved during evolution. Often, many of the replication proteins found in eukaryotes have analogs in prokaryotic and viral systems. For example, the 3'-5' exonuclease proofreading activity used to maintain the fidelity of DNA replication appears to be a common activity for many of the systems studied. Analogous in many of these systems is the fact that at some point the replication fork develops and is asymmetrical whereby the leading strand is continuous, while lagging strand is discontinuous. At the same time, accessory proteins may modify the properties of DNA pol.

However, while the mechanisms involved in DNA replication may be conserved, the actions of the proteins involved may not. In many instances, little homology exists between functionally similar proteins from different replication systems. In some cases, the similar proteins within the same species may have conservation of function and have some (minor)

homology. The regions of highest homology are generally important for substrate binding and the catalysis of pol or for various protein:protein or protein:DNA interactions. In any case, UL42 reacts like pol accessory proteins but differs in its mode of action from that on *E coli*, prokaryotic, eukaryotic and other viral systems. However, the activity of UL42 has been found to be consistent with that of BMF1 of EBV (Tsurumi et al., 1993) and UL44 of CMV (Ertl and Powell, 1992). The fact that there are functional homologs of UL42 from viruses closely related to HSV-1 suggests that we are dealing with a new mechanism by which pol accessory proteins may affect DNA replication and the mechanism may be specific for herpesviruses. The mutations that I have made and characterized are invaluable in the elucidation of important functional domains of UL42 that are involved in pol accessory function. More importantly, my work has laid the foundation for a means to accurately quantify the activities (pol binding, DNA binding, and pol stimulation) of this unique class of pol accessory proteins.

The interactions of all of the replication proteins that coordinate to assemble multiprotein complexes to promote efficient leading and lagging strand DNA replication has not been fully exposed. My studies have developed a very good model using GST-fusion proteins to examine these interactions and the regions throughout the proteins that are involved in these associations. The advantage of this system is that it would allow identification of protein:protein or protein:DNA interactions and in my opinion can be extended to determine if various protein:protein interactions are DNA dependent or independent. For example, it would interesting to determine if the addition of DNA to GST:UL42 or GST:UL9 enhances the binding of the two proteins. At the same time, the

assay may be extended to determine the order of assembly of the replication proteins at the origin by defining interactions required between early proteins like UL9, UL42, and ICP8 for the assembly of pol and the helicase-primase complex. These experiments will be dependent on the ability of the other replication proteins to complex within this system. The development of more sensitive technologies like surface plasmon resonance to detect biomecular interactions have provided a way to more carefully examine the effects of mutations on the DNA binding and pol binding activities of UL42 and may explain more fully the mechanism by which UL42 stimulates pol activity.

I have also developed a system to study the *in vivo* functions of mutant UL42 proteins to replicate DNA that is efficient and can be quantitated. It would be interesting to determine if mutants that lack function *in vivo* but maintain *in vitro* activity, like d241-261 of UL42, can interfere with other HSV-1 replication proteins. A dominant negative effect would be expected for a protein that retains the ability to bind pol or DNA while losing the ability to functionally interact with other proteins of the replication complex. A dominant negative phenotype will result if increasing amounts of plasmid expressing mutant UL42 protein reduces the amount of origin-dependent replication from cells infected with KOS wild-type virus and co-transfected with an origin-containing plasmid. One potential problem with this technique, would be with obtaining a fitting transfection efficiency to allow an increased expression of the recombinant protein. I believe that this problem is minor.

Clearly, UL42 plays an important role in the complex HSV-1 DNA replication system in a way that is different from many of the other pol accessory proteins in its interaction with pol and DNA. Interestingly, upon elucidation of its interaction with pol and DNA, it is

becoming increasingly obvious that UL42's role in DNA replication may extend and be influenced by the addition of viral or cellular proteins. The experiments described in this work identify some of the possible ways by which UL42 may influence HSV-1 DNA replication and provide a basis to greatly facilitate the dissection and understanding of the mechanisms and interactions required for the development of high processive, high fidelity DNA replication for not only HSV-1 but also for those of related systems.

## REFERENCES


Arbuckle, M.I., and N.D. Stow. (1993). A mutational analysis of the DNA-binding domain of the herpes simplex virus type 1 UL9 protein. J. Gen. Virol. 74:1349-1355.

Baixeras, E., S. Roman-Roman, S. Jitsukawa, C. Genevee, S. Mechiche, E. Viegas-Pequignot, T. Hercend, and F. Triebel. (1990). Cloning and expression of a lymphocyte activation gene (Lag-1). Mol. Immunol. 27: 1091-11102.

Becker, Y., H. Dym , I. Sarov . Herpes simplex virus DNA.( 1968)Virology : 184-192.

Berthomme, H., S.J. Monahan, D.S. Parris, B. Jacquemont, and A. Epstein. (1995). Cloning, sequencing, and functional characterization of the two subunits of the pseudorabies virus DNA polymerase holoenzyme: evidence for specificity of interaction. J. Virol. 69:2811-2818.

Blanco, l., A. Benad, M.A. Blasco, and M. Salas. (1991). A general structure for DNA-dependent DNA polymerase. Gene 100:27-38.

Boehmer, P.E., M.S. Dodson, and I.R. Lehman. (1993). The herpes simplex virus type-1 origin binding protein: DNA helicase activity. J. Biol. Chem. 268:1220-1225.

Boehmer, P.E., and I.R. Lehman. (1993). Physical interaction between the herpes simplex virus 1 origin-binding protein and single-stranded DNA-binding protein ICP8. Proc. Natl. Acad. Sci. USA. 90:8444-8448.

Boehmer, P.E., M.C. Craigie, N.D. Stow, and I.R. Lehman. (1994). Association of origin binding protein and single strand DNA-binding protein, ICP8, during herpes simplex virus type 1 DNA replication in vivo. J. Biol. Chem. 269:29329-29334.

Bruckner, R.C., J.J. Crute, M.S. Dodson, and I.R. Lehman. (1991). The herpes simplex virus 1 origin binding protein: a DNA helicase. J. Biol. Chem. 266:2669-2674.

Bush, M., D.R. Yager, M. Gao, K. Weisshart, A.I. Marcy, D.M. Coen, and D.M. Knipe, (1991). Correct intranuclear localization of herpes simplex virus DNA polymerase requires the viral ICP8 DNA-binding protein. J. Virol. 65: 1082-1089.

Calder, J.M., E.C. Stow, and N.D. Stow. 1992. On the cellular localization of the components of the herpes simplex virus type 1 helicase-primase complex and the viral origin-binding protein. J. Gen. Virol. 73: 531-538.

Chadler, J.M., and N.D. Stow. (1990). Herpes simplex virus helicase-primase: the UL8 protein is not required for DNA dependent ATPase and DNA helicase activities. Nucleic Acids Res. 18:3573-3578.

Challberg MD. A method for identifying the viral genes required for herpesvirus DNA replication. Proc. Natl. Acad. Sci. 1986 83: 9094-9098.

Christensen, J., T. Storgaard, B. Bloch, S. Alexandersen, and B. Aasted. (1993). Expression of aleutian mink disease parvovirus proteins in a baculovirus vector system. J. Virol. 67: 229-238.

Chu C.T., D.S. Parris, R.A.F. Dixon, F.E. Farber, and P.A. Schaffer. (1979). Hydroxylamine mutagenesis of HSV DNA and DNA fragments: introduction of mutations into selected regions of the viral genome. Virology 98:168-181.

Conley, A. J., D.M. Knipe, P.C. Jones, and B. Roizman. (1981). Molecular genetics of herpes simplex virus. VII. Characterization of a temperature-sensitive mutant produced by in vitro mutagenesis and defective in DNA synthesis and accumulation of g polypeptides. J. Virol. 37: 191-206.

Crute, J.J., and I.R. Lehman. (1989). Herpes simplex-1 DNA polymerase. Identification of an intrinsic 5'-3' exonuclease with ribonuclease H activity. J. Boil. Chem. 264:19266-19270.

Crute, J.J., T. Tsurumi, L. Zhu, S.K. Weller, P.D. Olivo, M.D. Challberg, E.S. Mocarski, and I.R. Lehman. (1989). Herpes simplex virus 1 helicase-primase: a complex of three herpes-encoded gene products. Proc. Natl. Acad. Sci. USA. 86:2186-2189.

de Bruyn Kops, A., and D.M. Knipe. (1988). Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. Cell 55: 857-868.

Derbyshire, V., N.D.F. Grindley, and C.M. Joyce. (1991). The 3'-5' exonuclease of DNA polymerase I of Escherichia coli: contribution of each amino acid at the site to the reaction. EMBO J. 10: 17-24.

Digard. P., and D.M. Coen. (1990). A novel functional domain of an α-like DNA polymerase. The DNA binding site on the herpes simplex virus polymerase for the viral UL42 protein. J. Biol. Chem. 265: 17393-17396.

Digard. P.,C.S. Chow, L. Pirrit, and D.M. Coen. (1993). Functional analysis of the herpes simplex virus UL42 protein. J. Biol. Chem. 67: 1159-1168.

Digard. P., W.R. Berin, K. Weisshart, and D.M. Coen. (1990). The extreme C terminus of herpes simplex virus DNA polymerase is crucial for functional interaction with processivity factor UL42 and for viral replication. J. Virol. 67: 398-406.

Dodson, M.S., and I.R. Lehman. (1991). Association of DNA helicase and primase activities with a subassembly of the herpes simplex virus 1 helicase-primase composed of the UL5 and UL52 gene products. Proc. Natl. Acad. Sci. USA 88:1105-1109.

Dodson, M.S., and Lehman, I. R. (1993). The herpes simplex virus type 1 origin binding protein. J. Biol. Chem. 268: 1213-1219.

Dodson, M. S., J.J. Crute, R.C. Bruckner, and I.R. Lehman. (1989). Overexpression and assembly of the herpes simplex virus type 1 helicase-primase in insect cells. J. Biol. Chem. 264: 20835-20838.

Dorsky, D. I., and C.S. Crumpacker. (1988). Expression of herpes simplex virus type 1 DNA polymerase gene by in vitro translation and effects of gene deletions on activity. J. Virol. 62: 3224-3232.

Downey, K.M., C.-K Tan, and A.G. So. (1990). DNA polymerase delta: A second eukaryotic DNA replicase. BioEssays 12: 231-236.

Elias, P., C.M. Gustafasson, O. Gammarsten, and N.D. Stow. (1992). Structural elements required for the cooperative binding of the herpes simplex virus origin binding protein to $ori_s$ reside in the N-terminal part of the protein. J. Biol. Chem. 267:17424-17429.

Elias, P., C.M. Gustafsson, and O. Hammarsten. (1990). The origin binding protein of herpes simplex virus 1 binds cooperatively to the viral origin of replication $ori_s$. J. Biol. Chem. 265: 17167-17173.

Elias, P., C.M. Gustafsson, O. Hammarsten, and N.D. Stow.(1992). Structural elements required for the cooperative binding of the herpes simplex virus origin binding protein to oriS reside in the N-terminal part of the protein. J. Biol. Chem. 267: 17424-17429.

Ertl, P.F., and K.L. Powell. (1992). Physical and functional interaction of human cytomegalovirus DNA polymerase and its accessory protein (ICP36) expressed in insect cells. J. Virol. 66:4126-4133.

Roizman, B.M. and A.E. Spears. (1996). Herpes simplex viruses and their replication.3rd edition, edited by B.N. Fields, D.M. Knipe, P.M. Howley, et al., Lippincott-Raven Publishers, Philadelphia, PA 401-424.

Fierer, D.S., and M.D. Challberg. (1992). Purification and characterization of UL9, the herpes simplex virus type 1 origin-binding protein. J. Virol. 66:3986-3995.

Frankel, A., H. Roberts, L. Afrin, J. Vesely, and M. Willingham. (1994). Expression of ricin B chain in Spodoptera frugiperda. Biochem. J. 303: 787-794.

Gac, N.T.L., G. Villani, J-S Hoffman, and P.E. Boehmer. (1996). The UL8 subunit of the herpes simplex virus type-1 DNA helicase-primase optimizes utilization of DNA templates covered by the homologous single-strand DNA binding protein ICP8. J. Biol. Chem. 271: 21645-21651.

Gallo, M.L., D.H. Jackwood, M. Murphy, H.S. Marsden, and D.S. Parris. (1988). Purification of the herpes simplex virus type 1 65-kilodalton DNA binding protein: properties of the protein and evidence of its association with the virus encoded DNA polymerase. J. Virol. 62:2874-2883.

Gallo, M.L., D.I. Dorsky, C.S. Crumpacker, and D.S. Parris. (1989). The essential 65-kilodalton DNA-binding protein of herpes simplex virus stimulates the virus-encoded DNA polymerase. J. Virol. 63:5023-5029.

Gang, D.R. and D.J. Weber. (1995). Preparation of genomic DNA for RAPD analysis from thick-walled dormant teliospores of Tilletia species. Biotechniques 1: 92,94,96-97.

Gao, M., and D.M Knipe. (1989). Genetic evidence for multiple nuclear functions of the herpes simplex virus ICP8 DNA-binding protein. J. Virol. 63:5258-5267.

Gao, M., S.F. DiTusa, and M.G. Cordingley. (1993). The C-terminal third of UL42, a HSV-1 DNA replication protein, is dispensable for viral growth. Virology. 194:647-653.

Gao, M., and D.M. Knipe. (1991). Potential role for herpes simplex virus ICP8 DNA replication proteins in stimulation of late gene expression. J. Virol. 65: 2666-2675.

Garber, D. A., S. M. Beverly, and D.M. Coen. 1993. Demonstration of circulation of herpes simplex virus DNA following infection using pulsed field gel electrophoresis. Virology 179: 459-462.

Gibbs, J.S., K. Weisshart, P. Digard, A. deBruyn Kops, M.J. Retondo, S.K. Weller, and D.M. Coen.(1985).Sequence and mapping analysis of the herpes simplex virus DNA polymerase gene predict a C-terminal substrate binding domain. Proc. Natl. Acad. Sci. 23: 7969-7973.

Gibbs, J.S., H.C. Chow, P. Digard, J.D. Hall, D.W. Mount, D.M. Knipe, and D.M. Coen. 1991. Polymerization activity of an alpha-like DNA polymerase requires a conserved 3'-5' exonuclease active site. Mol. Cell. Biol. 11: 4786-4795.

Goodrich, L. D., P.A. Schaffer, D.I. Dorsky, C.S. Crumpacker, and D.S. Parris. (1990). Localization of the herpes simplex virus type 1 65-kilodalton DNA-binding protein and DNA polymerase in the presence and absence of viral DNA synthesis. J. Virol. 64: 5738-5749.

Goswami, B.B and R.I. Glazer. (1991). A simplified method for the production of recombinant baculovirus. Biotechniques. 5: 626-630.

Gottlieb, J., and M.D. Challberg. (1994). Interaction of herpes simplex virus type 1 DNA polymerase and the UL42 accessory protein with a model primer template. J. Virol. 68: 4937-4945.

Gottlieb, J., A.I. Marcy, D.M. Coen, and M.D. Challberg. (1990). The herpes simplex virus type 1 UL42 gene product: a subunit of DNA polymerase that functions to increase processivity. J. Virol. 64: 5976-5987.

Gustafsson, C. M., M. Falkenberg, S. Simonsson, H. Valdi, and P. Elias. (1995). The DNA ligands influence the interactions between the herpes simplex virus 1 origin binding protein and the single strand DNA-binding protein, ICP8. J Biol. Chem. 270: 19028-19034.

Hall, J.D., K.L Orth, K.L Ser, B.M. Swihart, and R.A. Senese. (1995). Mutations within the conserved motifs in the 3'-5' exonuclease domain of herpes simplex virus DNA polymerase. J Gen. Virol. 76: 2999-3008.

Hamatake, R.K., M. Bilfano, D.J. Tenney, W.W. Hurlburt, and M.G. Cordingley. The herpes simplex virus type 1 DNA polymerase accessory protein, UL42, contains a functional protease-resistant domain. J. Gen. Virol. 74: 2181-2189.

Hayward, G.S., R.J. Jacob, S.C. Wadsworth, B. Roizman. (1975). Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short segments. Proc. Natl. Acad. Sci. 72:4243-4247.

Hazuda, D.J., J.C. Perry, A.M. Naylor, and W.L. McClements. (1991). Characterization of the herpes simplex virus origin binding protein interaction with $Ori_s$. J. Biol. Chem. 266:24621-24626.

Hernandez, T. R., and I.R. Lehman. (1990). Functional interaction between the herpes simplex-1 DNA polymerase and UL42 protein. J. Biol. Chem. 265: 11227-11232.

Himawan, J.S., and C.C Richardson. (1992). Genetic analysis of the interactions between bacteriophage T7 DNA polymerase and Escherichia coli thioredoxin. Proc. Natl Acad. Sci. 89: 9774-9778.

Hori, K., D.F. Mark, and C.C. Richardson. (1979). Deoxyribonucleic acid polymerase of bacteriophage T7. Characterization of the exonuclease activities of the gene 5 protein and the reconstituted polymerase. J. Biol. Chem. 254: 11598-11604.

Huber, H., S. Tabor, and C.C. Richardson. 1987. Escherichia coli thioredoxin stabilizes complexes of bacteriophage T7 DNA polymerase and primed templates. J. Biol. Chem. 262: 16224-16232.

Jacob, R.J., L.S. Morse, B. Roizman.(1979). Anatomy of herpes simplex virus DNA. XIII. Accumulation of head to tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. J. Virol. 9: 448-457.

Jacob, R.J., L.S. Morse, B. Roizman.(1977). Anatomy of herpes simplex virus DNA. VIII. Properties of replicating DNA. J. Virol. 23: 394-411.

Jacob R.J., L.S. Morse, and B. Roizman. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatomers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. J. Virol.29: 448-457.

Jarvis, T.C., J.W. Newport, and P. H. von Hippel. (1991). Stimulation of the processivity of the DNA polymerase of bacteriophage T4 by the polymerase accessory proteins. J. Biol. Chem. 266: 1830-1840.

Jofre, J.T., P.A. Schaffer, and D.S. Parris. (1977). Genetics of resistance to phosphonoacetic acid in strain KOS of herpes simplex virus type 1. J. Virol. 23:833-836.

Johnson, P.A., M.G. Best, T. Friedman, and D.S. Parris. Isolation of a Herpes Simplex Virus Type 1 Mutant Deleted for the Essential UL42 gene and Characterization of its Null Phenotype. J. Virol. 65:700-710.

Kieff, E.D., S.L. Bachenheimer, B. Roizman.(1971). Size, composition and structure of the DNA of subtypes 1 and 2 herpes simplex virus. J. Virol. 8: 125-129.

Kiehl, A., and D. I. Dorsky. (1991). Cooperation of EBV DNA polymerase and EA-D (BMRF1) in vitro and co-localization in nuclei of infected cells. Virology 184: 330-340.

Kiehl, A., and D. I. Dorsky. 1995. Bipartite DNA-Binding Region of the Epstein-Barr Virus BMRF1 Product essential for DNA polymerase accessory function. J. Virol. 69:1669-1677.

Klinedinst, D. K., and M.D. Challberg. (1994).Helicase-primase complex of herpes simplex virus type 1: a mutation in the UL52 subunit abolishes primase activity. J. Virol. 68: 3693-3701.

Koff, A., J.F. Schwedes, and P. Tegtmeyer. (1991). Herpes simplex virus origin-binding protein (UL9) loops and distorts the viral replication origin. J. Virol. 65: 3284-3292.

Koff, A., and P. Tegtmeyer.(1988).Characterization of major recognition sequences for herpes simplex virus type 1 origin-binding protein. J. Virol. 62: 4096-4103.

Kong, X-P, R. Onrust, M. O' Donnell, and J. Kuriyan. (1992). Three dimensional structure of the β subunit of E. coli DNA polymerase III holoenzyme: A sliding clamp. Cell 69: 425-435.

Kornberg, A., and T.A. Baker. (1992). DNA replication, 2nd ed. W. H. Freeman & Co., San Francisco.

Kroutil, L.C., K. Register, K. Bebenek, and T.A. Kunkel. (1996). Exonuclease proofreading during replication of repetitive DNA. Biochemistry. 35: 1046-1053.

Kunkel, T.A., S.S. Patel, and K.A. Johnson. (1994). Error-prone replication of repeated DNA sequences by T7 DNA polymerase in the absence of its processivity subunit.

Lai, J.-S., and W. Herr. (1992). Ethidium bromide provides a simple test for identifying genuine DNA-independent protein associations. Proc. Natl. Acad. Sci. USA 89: 6958-6962.

Lee, C.K., and D.M. Knipe. (1985). An immunoassay for the study of DNA-binding activities of herpes simplex virus protein ICP8. J. Virol. 54:731-738.

Lee, S.S.-K., Q. Dong, T. S.-F. Wang, and I.R. Lehman. (1995). Interaction of herpes simplex virus 1 origin-binding protein with DNA polymerase a. Proc. Natl. Acad. Sci. USA. 92:7882-7886.

Liptak, L.M., S.L. Uprichard, and D.M. Knipe. (1996). Functional order assembly of herpes simplex DNA replication proteins into prereplicative site structures. J. Virol. 70: 1759-1767.

Lukonis, C. J., Burkham, J., and Weller, S. K. (1997). Herpes simplex virus type 1 prereplicative sites are a heterogenous population: only a subset are likely to be precursors to replication compartments. J. Virol. 71, 4771-4781.

Lukonis, C. J., and S.K. Weller. (1996). Characterization of nuclear structures in cells infected with herpes simplex virus type 1 in the absence of viral DNA replication. J. Virol. 70: 1751-1758.

Lukonis, C.J., and S.K. Weller. (1997). Formation of herpes simplex virus type 1 replication compartments by transfection: requirements and localization to nuclear domain 10. J Virol. 71: 2390-2399.

Makhov A.M., P.E. Boehmer, I.R. Lehman, and J.D. Griffith. (1996). The herpes simplex virus type 1 origin-binding protein carries out origin specific DNA unwinding and forms stem-loop structures. EMBO J. 15: 1742-1750.

Maniatis, T., E.F. Fritch, and J. Sambrook. (1992). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Marians, K.J., Prokaryotic DNA replication . (1992). Annu Rev. Biochem. (1992) 61: 673-713.

Matz B., J.H. Subak-Sharpe, and V.G. Preston. (1983). Physical mapping of temperature-sensitive mutations of herpes simplex virus type 1 using cloned restriction endonuclease fragments. J. Gen. Virol. 64: 2261-2270.

McGeoch, D. J., V.G. Preston, S.K. Weller, and P.A. Shaffer. (1994). Herpes simplex virus, In S. J. O'Brein (ed.), Genetic Maps. p. 1.115-1.120.

Marchetti, M.E., C.A. Smith, and P.A. Schaffer (1988). A temperature-sensitive mutation in a herpes simplex virus type 1 gene required for viral DNA synthesis maps to coordinates 0.609 through 0.614 in $U_L$. J. Virol. 62:715-721.

Mark, D.F., and C.C Richardson. (1976). Escherichia coli thioredoxin: a subunit of bacteriophage T7 DNA polymerase. Proc. Natl Acad. Sci. 73: 780-784.

Marsden, H.S., M.E.M. Campbell, L. Harr, M.C. Frame, D.S. Parris, M. Murphy, R.G. Hope, M.T. Muller, and C.M. Preston. (1987). The 65,000-Mr DNA-binding and virion trans-inducing proteins of herpes simplex virus type 1. J. Virol. 61:2428-2437.

McGeoch, D.J., M.A. Dalrymple, A.J. Davison, A. Dolan, M.C. Frame, D. McNav, L.J. Perry, J.E. Scott, and P. Taylor. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. 69: 1531-1574.

Modrich, P., and C.C Richardson. (1975). Bacteriophage T7 deoxyribonucleic acid replication in vitro. A protein of Escherichia coli required for bacteriophage T7 polymerase activity. J. Biol. Chem. 250: 5508-5514.

Modrich, P., and C.C Richardson. (1975). Bacteriophage T7 deoxyribonucleic acid replication in vitro. Bacteriophage T7 DNA polymerase : an enzyme composed of phage and host-specific enzymes. J. Biol. Chem. 250: 5515-5522.

Monahan, S. J., T.F. Barlam, C.S. Crumpacker, and D.S. Parris. (1993). Two regions of the herpes simplex virus type 1 UL42 protein are required for its functional interaction with the viral DNA polymerase. J. Virol. 65: 5922-5931.

McLean, G.W., A.P. Abbotts, M.E. Parry, H.S. Marsden, and N.D. Stow. (1994). The herpes simplex virus type 1 origin-binding protein interacts specifically with the viral UL8 protein. J. Gen. Virol. 75:2699-2706.

Naktinis, V., J.Turner, and M. O'Donnell. (1996). A molecular switch in a replication machine defined by an internal competition for protein rings. Cell 84: 137-145.

O'Donnell, M.E., P. Elias, and I.R. Lehman. (1987). Processive replication of single-stranded DNA templates by the herpes simplex virus-induced DNA polymerase. J. Biol. Chem. 262:4252-4259.

O'Donell, M., R. Onrust, R.B. Dean, M. Chen, and J. Hurwitz. (1993). Homology in accessory proteins for replicative polymerase E coli to humans. Nucleic Acids Res. 21: 1-3.

Olivo, P.D., N.J. Nelson, and M.D. Challberg. (1988). Herpes simplex virus DNA replication: the UL9 gene encodes an origin binding protein. Proc. Natl. Acad. Sci. USA 85:5414-5418.

Olivo, P.D., N.J. Nelson, and M.D. Challberg. (1989). Herpes simplex type 1 gene products required for DNA replication: identification and overexpression . J. Virol. 63: 196-204.

Orberg, P., P.A. Schaffer. (1987). Expression of herpes simplex virus type 1 major DNA-binding protein, ICP8, in transformed cell lines: complementation of deletion mutants and inhibition of wild-type virus. J. Virol. 61: 1136-1146.

Owsianka, A.M., G. Hart, M. Murphy, J. Gottlieb, and R. Boehme, M. Challberg, and H.S. Marsden. (1993). Inhibition of herpes simplex virus type 1 DNA polymerase activity by peptides from the UL42 accessory protein is largely nonspecific. J. Virol.67: 258-264.

Parris, D.S., R.J. Courtney, and P.A. Schaffer. (1978).Temperature sensitive mutants of herpes simplex virus type 1 defective in transcriptional and post transcriptional functions required for viral DNA synthesis. Virology. 90:177-186.

Parris, D.S., A. Cross, L. Haarr, A. Orr, M.C. Frame, M. Murphy, D.J. McGeoch, and H.S. Marsden. (1988). Identification of the gene encoding the 65-kilodalton DNA-binding protein of herpes simplex virus type 1. J. Virol. 62:818-825.

Poffenberger, K.L., and B. Roizman. (1985). A noninverting genome of a viable herpes simplex virus 1: presence of head-to-tail linkages in packaged genomes and requirements for circularization after infection. J. Virol. 53: 587-595.

Powell, K.L., and D.J.M. Purifoy. (1976). DNA-binding proteins of cells infected by herpes simplex virus type 1 and type 2. Intervirology 7:225-239.

Powell, K.L., and D.J.M. Purifoy. (1977). Nonstructrual proteins of herpes simplex virus. I. Purification of the induced DNA polymerase. J. Virol. 24:618-626.

Powell, K.L., E. Littler, and D.J.M. Purifoy. (1981). Nonstructrual proteins of herpes simplex virus. II. Major virus-specific DNA-binding protein. J. Virol. 39:894-902.

Purifoy, D.J.M., R.B. Lewis, and K.L. Powell. (1977). Identification of the herpes simplex virus DNA polymerase gene. Nature (London) 269:621-623.

Rabkin, S.D., and Hanlon, B. (1991). Nucleoprotein complex formed between herpes simplex virus UL9 protein and the origin of DNA replication: inter- and intramolecular interaction. Proc. Natl. Acad. Sci. USA 88: 10946-10950.

Reddig, P.J., L.A. Grinstead, S.J. Monahan, P.A. Johnson, and D.S. Parris. (1994). The essential in vivo function of the herpes simplex virus UL42 protein correlates with its ability to stimulate the viral DNA polymerase in vitro. Virology. 200:447-456.

Ruyechan, W. T. and A.C. Weir. (1984). Interaction with nucleic acids and stimulation of the viral DNA polymerase by the herpes simplex virus type 1 major DNA-binding protein. J. Virol. 52: 727-733.

Saiki, R.K., S. Scharf, K.B. Faloona, G.T. Mullis, H.A. Horn, H.A. Erlich and N. Arnheim. (1985). Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1354.

Sarkar, G., S. Kapelner and S.S. Sommer. (1990). Formamide can dramatically improve the specificity of PCR. Nucleic Acids Res. 18: 7465.

Serrano, M., C. Gutierrez, and J.M. Hermoso. (1993). Superhelical path of the DNA in the nucleoprotein complex that activates the initiation of phage phi 29 DNA replication. J. Mol. Biol. 230: 248-259.

Serrano, M., G.J. Hannon, and D. Beach. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDCK4. Nature. 366: 704-707.

Severini, A., A.R. Morgan, D.R. Tovell, and D.L.J. Tyrrell. (1994). Study of the structure of replication intermediates of HSV-1 DNA by pulsed-field gel electrophoresis. Virology 200: 428-435.

Severini, A., D.G. Scraba, and D.L. Tyrrell. (1996). Branched structures in the intracellular DNA of herpes simplex virus type 1. J. Virol. 70: 3169-3175.

Sherman, G., J. Gottlieb, and M.D. Challberg. (1992). The UL8 subunit of the herpes simplex virus helicase-primase complex is required for efficient primer utilization. J. Virol. 66:4884-4892.

Skaliter, R., and I.R. Lehman. (1994). Rolling circle DNA replication in vitro by a complex of herpes simplex virus type 1-encoded enzymes. Proc. Natl. Acad. Sci. USA 91: 10665-10669.

Skaliter, R., A.M. Makhov, J.D. Griffith, and I.R. Lehman, (1996). Rolling circle DNA replication by extracts of herpes simplex virus type 1-infected human cells. J. Virol. 70: 1132-1136.

Smith, D.B., and K.S. Johnson. (1988). Single step purification of polypeptides expressed in E. coli as fusions with GST. Gene 67:31-40.

Smith, K.O. (1964). Relationship between the envelope and the infectivity of herpes simplex virus. Pro. Soc. Exp. Biol. Med. 115: 814-816.

Southern, E.M., (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503-517.

Stabell, E.C., and P.D. Olivo. (1993). A truncated herpes simplex virus origin binding protein which contains the carboxyl terminal origin binding domain binds to the origin of replication but does not alter its confirmation. Nucleic Acids Res. 21:5203-5211.

Stow, N. D., J.H. Subak-Sharpe, and N.M. Wilkie. (1978). An improved technique for obtaining enhanced infectivity with herpes simplex virus type 1 DNA. J. Virol. 28: 182-192.

Stow, N. D. (1982). Localization of an origin of DNA replication within the TRS/IRS repeated region of the herpes simplex virus type 1 genome. EMBO J 1: 863-867.

Stow, N. D., and E.C. McMonagle.(1983). Characterization of the $TR_S/IR_S$ origin of DNA replication of herpes simplex virus type 1. Virology 130: 427-438.

Stow, N. D. (1992). Herpes simplex virus type 1 origin-dependent DNA replication in insect cells using recombinant baculoviruses. J. Gen. Virol. 73: 313-321.

Stow, N. D., O. Hammarsten, M.I. Arbuckle, and P. Elias. (1993). Inhibition of herpes simplex virus type 1 DNA replication by mutant forms of the origin-binding protein. Virology 196: 413-418.

Stukenberg, P.T., P.S. Studwell-Vaughan, and M. O'Donnell. (1991). Mechanism of the sliding beta-clamp of DNA polymerase III holoenzyme. J. Biol. Chem. 266: 11328-11334.

Tabor, S., H. Huber, and C.C. Richardson. (1987). Escherichia coli thioredoxin confers processivity on the DNA polymerase activity of the gene 5 protein of bacteriophage T7. J. Biol. Chem. 262: 16212-16223.

Tenney, D. J., W.W. Hurlburt, P.A. Micheletti, M. Bifano, and R.K. Hamatake.(1994). The UL8 component of the herpes simplex virus helicase-primase complex stimulates primer synthesis by a subassembly of the UL5 and UL52 components. J. Biol. Chem. 269: 5030-5035.

Tenney, D. J., W.W. Hurlburt, P.A. Micheletti, M. Bifano, and R.K. Hamatake, M.G. Cordingley. (1993). Deletions of the carboxyl terminus of herpes simplex virus type 1 UL42 define a conserved amino-terminal functional domain. J. Virol. 67: 1959-1966.

Tenney, D. J., P. A. Micheletti, Jr. Stevens, R.K. Hamatake, J.T. Matthews, A.R. Sanchex, W.W. Hurlbert, M Bilfano, and M.G. Cordingley. (1993). Mutations in the C terminus of herpes simplex virus type 1 DNA polymerase can affect binding and stimulation by its accessory protein UL42 without affecting basal polymerase activity. J. Virol. 67: 543-547.

Towbin, H., T. Staehelin, and J. Gordon. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76: 4350-4354.

Tsurumi, T. (1993). Purification and characterization of the DNA-binding activity of the Epstein-Barr virus DNA polymerase accessory protein BMRF1 gene products, as expressed in insect cells by using the baculovirus system. J. Virol. 67: 1681-1687.

Vaughn, P.J., K.J.M. Purifoy, and K.L. Powell. (1985). DNA-binding protein associated with herpes simplex virus DNA polymerase. J. Virol. 53:501-508.

Weiland, K.L., N.L. Oien, F. Homa, and M.W. Wathen. (1994). Functional analysis of human cytomegalovirus polymerase accessory protein. Virus Res. 34: 191-206.

Weller, S.K., K.J. Lee, D.J. Sabourin, and P.A. Schaffer. (1983). Genetic analysis of temperature-sensitive mutants which define the gene for the major herpes simplex virus type 1 DNA-binding protein. J. Virol. 45:354-366.

Wu, C.A., N.J. Nelson, D.J. McGeoch, and MD. Challberg. (1988). Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. J. Virol. 62:435-443.

Yao, N., J. Turner, Z. Kelman, P.T. Stukenburg, F. Dean, D. Shechter, Z-Q Pan, J. Hourwitz, and M. O'Donnell. (1996). Clamp loading, unloading and intrinsic stability of the PCNA, beta, and gp45 sliding clamps to human, E. coli, and T4 replicases. Genes to Cell 1: 101-113.

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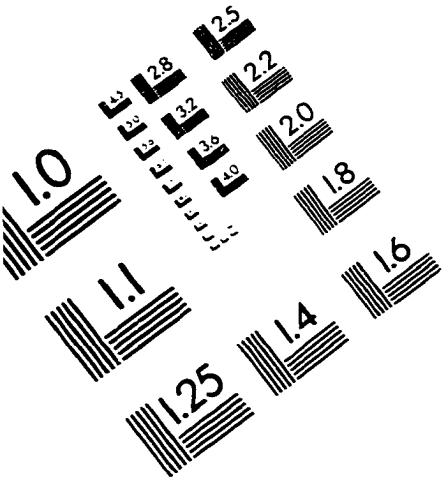


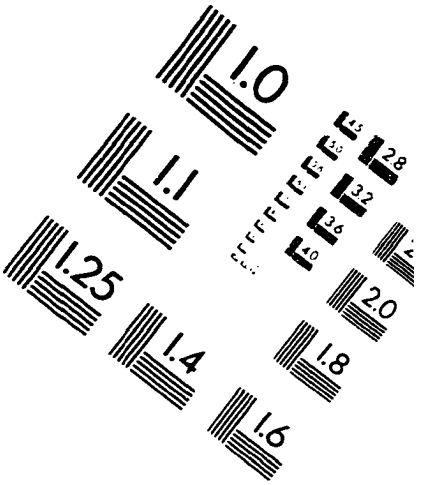


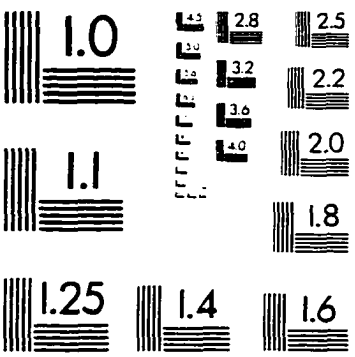


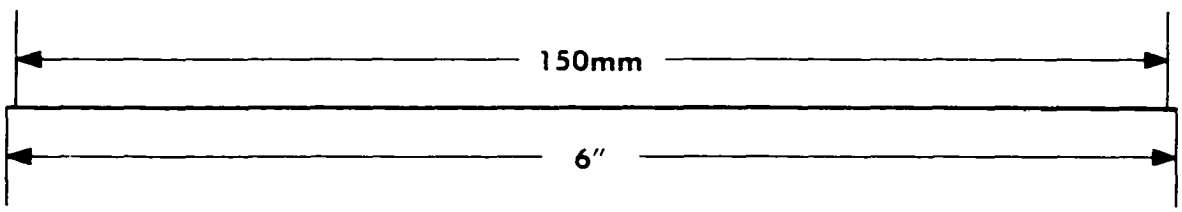


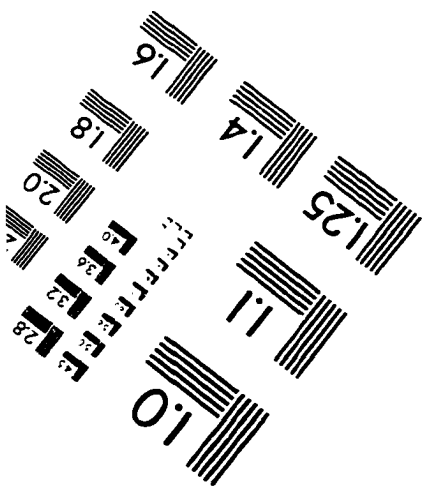


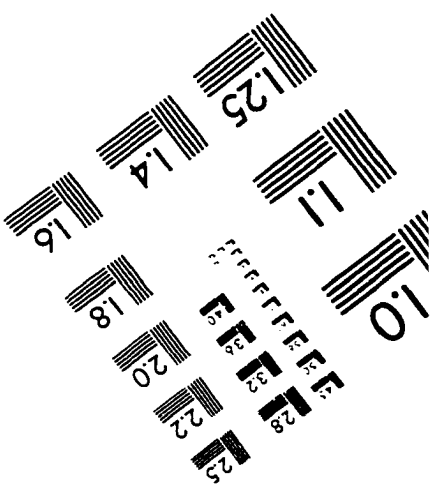


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