Virology 275, 373–390 (2000)
doi:10.1006/viro.2000.0506, available online at http://www.idealibrary.com on IDEAL®

Analysis of *in Vitro* Activities of Herpes Simplex Virus Type 1 UL42 Mutant Proteins: Correlation with *in Vivo* Function

Keith E. Thornton,* Murari Chaudhuri,† Steven J. Monahan,† Lynn A. Grinstead,* and Deborah S. Parris*,†,[1]

*Program in Molecular, Cellular, and Developmental Biology and †Department of Molecular Virology, Immunology, and Medical Genetics, Comprehensive Cancer Center, 333 West Tenth Avenue, Ohio State University, Columbus, Ohio 43210

Received September 24, 1999; returned to author for revision January 4, 2000; accepted July 7, 2000

The DNA polymerase (pol) catalytic subunit of herpes simplex virus type 1, encoded by UL30, and its accessory factor, UL42 protein, are both essential for the replication of the virus. Because the stable interaction between UL42 and pol renders the pol fully processive for replicative DNA synthesis, disruption of this interaction represents a potential goal in the development of novel antiviral compounds. To better compare the effects of mutations in UL42 protein on its known *in vitro* functions, mutations were expressed as glutathione-S-transferase (GST)-fusions and the fusion proteins used in affinity chromatography. In this report, we demonstrate the relationship between the abilities of mutant UL42 fusion proteins to bind pol and to stimulate pol activity *in vitro,* and the abilities of nonfusion mutant proteins to function in viral replication. The pol stimulation assay using GST fusion proteins was found to be a more accurate and sensitive measure of the ability of the UL42 protein to function *in vitro* than the pol binding assay using the fusion proteins linked to a solid matrix. We also found an excellent correlation between the ability of purified GST fusion proteins to stimulate pol activity *in vitro* and the ability of full-length nonfusion UL42 mutant genes to support DNA replication in infected cells. Our results demonstrate that two noncontiguous stretches of amino acids, from 137 to 142 and from 274 to 282, are essential for UL42 function *in vivo* and *in vitro.* Although mutant d241–261 exhibited close to wild-type abilities to stimulate pol activity *in vitro,* it was not capable of complementing the replication of a UL42 null mutant virus. The region of UL42 protein within or close to 241–261 may serve to hinge the essential regions within the N- and C-terminal portions of the protein which are thought to interdigitate. It is hypothesized that reduction in the length of the hinge region could alter the ability of UL42, and/or its complex with pol, to function with one or more of the other proteins present in the DNA replisome within infected cells.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) encodes at least seven of the proteins which are directly involved in the initiation and synthesis of viral DNA in infected cells (reviewed in Challberg, 1991; Weller, 1991). The HSV-1 DNA-dependent DNA polymerase activity, responsible for the addition of deoxyribonucleotides, is a heterodimer composed of a 140-kDa catalytic subunit (pol), encoded by UL30, and an accessory protein, encoded by UL42 (Parris *et al.,* 1988; Gallo *et al.,* 1989; Crute and Lehman, 1989; Gottlieb *et al.,* 1990). Although pol possesses intrinsic activity *in vitro* in the absence of other viral proteins (Dorsky and Crumpacker, 1988; Haffey *et al.,* 1988; Marcy *et al.,* 1990), the UL42 protein stimulates the activity of pol in the presence of high salt and increases its processivity on artificial primer/templates (Gallo *et al.,* 1989; Hernandez and Lehman, 1990; Gottlieb *et al.,* 1990). The requirement of UL42 for processive DNA synthesis in HSV-infected cells is suggested by the fact that the UL30 and UL42 genes are both essential for virus replication (Purifoy *et al.,* 1977; Jofre *et al.,* 1977; Marchetti *et al.,* 1988; Johnson *et al.,* 1991).

The mechanism by which UL42 protein increases the processivity of pol is not known. However, the apparent lack of a requirement for ATP for assembly of UL42 protein and pol onto primed templates and the 1:1 stoichiometry of pol and UL42 within the processive holoenzyme suggest that the HSV-1 enzyme is most similar to the T7 pol:thioredoxin holoenzyme (Tabor *et al.,* 1987; Huber *et al.,* 1987; Gottlieb *et al.,* 1990). However, unlike the T7 pol processivity factor, thioredoxin, which does not bind to DNA alone, UL42 protein has been shown to bind to double-stranded DNA (Marsden *et al.,* 1987; Gallo *et al.,* 1988; Gottlieb and Challberg, 1994). The latter property has led to the hypothesis that UL42 protein may increase the processivity of pol by tethering it to the DNA template (Gottlieb *et al.,* 1990). The hypothesis further predicts that the ability of UL42 protein to bind to both DNA and pol is required for a fully processive pol holoenzyme. Nevertheless, the intrinsic ability of UL42 to bind to DNA presents a potential paradox in this proposed mechanism because such binding could be envi-

[1] To whom correspondence and reprint requests should be addressed at Department of Molecular Virology, Immunology, and Medical Genetics, 2198 Graves Hall, 333 West Tenth Avenue, Columbus, OH 43210. Fax: (614) 292-9805. E-mail: dparris@postbox.service.ohio-state.edu.

0042-6822/00 $35.00

sioned to prevent the pol from moving readily along the template (Tsurumi *et al.,* 1993; Chow and Coen, 1995).

We and others have shown that the N-terminal two-thirds of the UL42 protein is sufficient to provide pol accessory function *in vitro* (Digard *et al.,* 1993; Tenney *et al.,* 1993; Monahan *et al.,* 1993) and in virus-infected cells (Digard *et al.,* 1993; Gao *et al.,* 1993; Reddig *et al.,* 1994). Furthermore, noncontiguous domains within this portion of UL42 have been reported to be required for both DNA binding and pol binding (Digard *et al.,* 1993; Monahan *et al.,* 1993; Chow and Coen, 1995). Indeed, most mutations which abrogate DNA binding activity also destroy the ability to bind to pol. In two notable exceptions, Chow and Coen (1995) reported that two insertion mutations in UL42 destroy the abilities of the encoded proteins to bind to DNA, without affecting their abilities to bind to the pol catalytic subunit. These authors proposed a model for processivity which requires interaction of UL42 with DNA based on their results which demonstrated that these mutant proteins failed to stimulate long-chain DNA synthesis and to complement the replication of a UL42 null mutant.

To further address the question of requirements for pol accessory function, we have constructed a variety of deletion, insertion, and point mutations within the two distinct regions of UL42 we previously demonstrated to be required for the activity of UL42 in stimulating *in vitro* pol activity (Monahan *et al.,* 1993). These mutations were expressed as glutathione-S-transferase (GST) fusion proteins and affinity chromatography was used to assess the ability of the mutant GST fusion proteins to bind to pol and to obtain relatively large amounts of substantially purified protein. In this study, we demonstrate a strong correlation between the ability of mutant UL42 fusion proteins to bind to pol and their abilities to stimulate pol activity *in vitro* and to function in viral replication. However, the ability of a mutant UL42 protein to stimulate pol activity *in vitro* proved a more accurate and sensitive means to predict the ability of a mutant gene to function in the context of the infected cell.

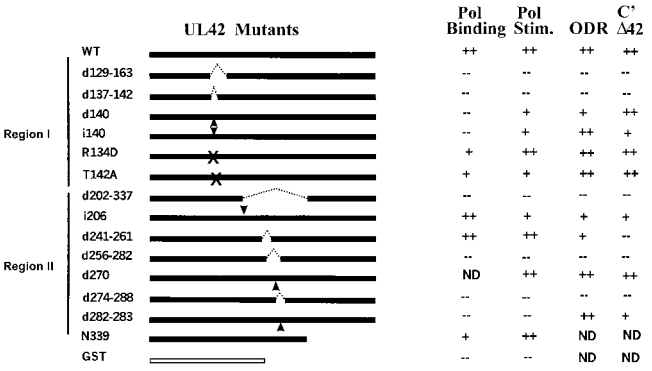

FIG. 1. Structure and activities of UL42 mutant proteins. UL42 mutations were constructed as detailed under Materials and Methods. Thick solid lines indicate residues present within each mutant protein. Deletions of one or two amino acids residues are noted with (▲), while longer deletions are indicated by the dotted lines. Locations of insertion mutations are noted with (▼) and point mutations by (x). All activities were measured relative to those of the appropriate wild-type protein with the use of + or ++ to indicate relative intensity. Activities not above background or negative control levels are indicated by (- -). The pol binding and pol stimulation (stim.) activities were measured using GST fusions of the indicated mutant proteins. All such proteins were derived from a GST fusion with residues 20–456 of the wild-type (WT) UL42 protein. The ability to support ori-dependent DNA replication (ODR) and production of infectious progeny virus by complementation (C′) of the HSV-1 null virus CgalΔ42 (Δ42) utilized plasmids derived from pLBN19A which expressed the indicated UL42 genes from a viral promoter. The GST protein is denoted by the open bar. ND, not determined.

RESULTS

A number of studies have investigated the functions of UL42 mutant proteins. Although there is a consensus that the C-terminal third of the protein is not required for activity *in vitro* or *in vivo,* most deletions in the remainder of the protein result in the simultaneous loss of the ability of UL42 to bind to ds DNA and to pol, as well as the loss of ability to increase pol activity and processivity (Digard *et al.,* 1993; Gao *et al.,* 1993; Tenney *et al.,* 1993; Monahan *et al.,* 1993). There is less agreement on the regions within the N-terminal two-thirds of the protein which are required for these activities. Previously, we demonstrated that two distinct regions within this portion of UL42 were absolutely essential for UL42 function (Monahan *et al.,* 1993; Reddig *et al.,* 1994). Region I (AA 129–163) was extremely sensitive to mutation, as indicated by the loss of function of proteins encoded by d137–142 and altered function of those encoded by d140 and i140. Moreover, computer-based secondary structure analysis predicted this region to form a helix–coil–helix structure (Monahan *et al.,* 1993); thus, we created several base substitution mutations within Region I in an attempt to disrupt the putative domain. Due to the relatively large size of Region II, defined by the deletion of AA 202–337, and to further characterize the function of this region, we also created nested, smaller deletion mutations within this portion of the UL42 gene (Fig. 1). Inasmuch as binding analysis in previous studies utilized small amounts of protein synthesized by *in vitro* transcription/translation, and coimmunoprecipitation analysis used antibodies of differing avidities and specificities, it would have been difficult to distinguish low-level binding from negative or nonspecific binding. In an effort to obtain larger amounts of mutant UL42 proteins for *in vitro* analysis of their activities, we chose to express the wild-type and mutant versions of the gene as fusions with the gene encoding glutathione-S-transferase. Affinity chromatography of the GST–UL42 fusion proteins provided us with a facile means to score for pol-binding activity using standard pull-down experiments and to obtain substan-

tially purified preparations of fusion proteins for more quantitative analysis of the activity in enzyme assays.

Binding of HSV-1 pol to GST fusion proteins

We previously demonstrated that a GST fusion protein containing UL42 amino acid residues 20–456 was functional, as demonstrated by its ability to stimulate, under high-salt reaction conditions, the activity of HSV-1 pol translated *in vitro* (Monahan *et al.,* 1998). We expressed a variety of UL42 mutations as GST fusion proteins in *Escherichia coli.* Analysis of extracts by SDS–PAGE revealed large differences in the amounts of some fusion proteins detected by Coomassie blue staining, when comparable amounts of protein present in extracts were loaded. To attempt to account for differences in expression and/or accumulation, we determined the amount of GST activity in each extract spectrophotometrically using the transferase-mediated reaction between 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione. Such a method provides only an indirect means for quantifying GST–UL42 fusion proteins in crude extracts and does not differentiate full-length from C-terminally truncated products which retain a functional GST moiety. Nevertheless, we found it superior to estimating the relative level of fusion protein expression rather than total protein content, since the poorest expression was observed among those with the lowest level of GST activity while the strongest expression was observed for those with the greatest level of GST activity by this assay (results not shown). Thus, affinity matrices were prepared by adjusting the amount of extract added according to GST activity, rather than absolute protein amount.

We previously demonstrated that the amount of another HSV-1 protein, UL9, which bound to GST–UL42 affinity columns, varied as a function of concentration of fusion protein used to charge the matrix (Monahan *et al.,* 1998). Therefore, we reasoned that optimum sensitivity for detecting binding of pol to various GST–UL42 fusion proteins would be obtained using affinity matrices to which was bound saturating, or close to saturating, amounts of fusion protein. In preliminary experiments, we bound increasing concentrations of GST–UL42 to glutathione agarose, eluted bound proteins with 5 mM glutathione, and examined eluates by SDS–PAGE. Based upon visual examination of Coomassie-blue-stained gels, we determined that binding of GST–UL42 to the gluathione agarose approached saturation at charging concentrations of 9.8 μM (not shown). That maximum sensitivity for detecting pol binding was obtained using affinity matrices containing saturating amounts of fusion protein was confirmed by testing matrices charged with increasing concentrations of GST or GST–UL42 (wild-type) for their abilities to bind small amounts of pol prepared by *in vitro* transcription/translation (Fig. 2). Two independent experiments are shown for which efficiencies of the translation of pol differed slightly. Despite differences in the amount of pol added to the individual matrices, there was a clear dose-response in the amount of pol bound compared to the concentration of GST–UL42 protein when low (subsaturating) charging concentrations were used. However, binding of pol approached or reached maximum levels when columns were charged with saturating amounts (9.8 μM) of GST–UL42. No binding of pol was detected for affinity matrices charged with GST, regardless of charging concentration (Fig. 2).

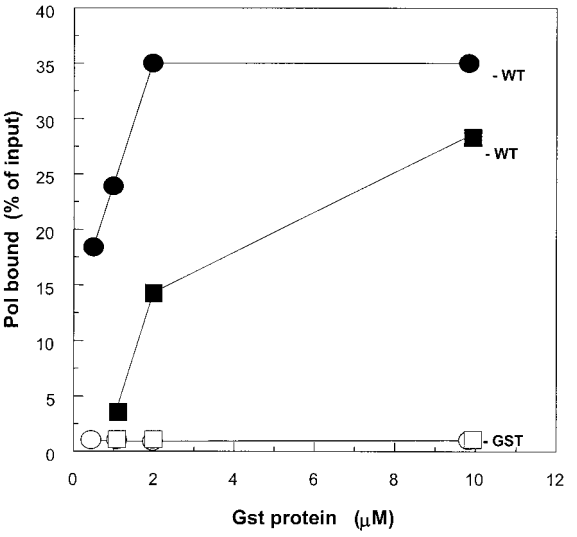

FIG. 2. Binding of pol to GST–UL42 as a function of charging concentration. Glutathione agarose was charged with the indicated concentrations of GST (open symbols) or GST–UL42 (closed symbols) and incubated with HSV-1 pol prepared by *in vitro* transcription/translation of 100 ng of pT7-7.1 in the presence of [^{35}S]methionine. The material which bound and eluted in 5 mM glutathione was subjected to SDS–PAGE and the radioactivity in the band corresponding to the pol in each sample was quantified by phosphorimage analysis. The results of two independent binding experiments (circles or squares) are shown.

To determine the validity of the approach for screening various mutant forms of UL42 expressed as fusions with GST, we prepared glutathione agarose matrix charged with 9.8 μM of GST or GST fusions with wild-type or mutated UL42. Nuclear extracts of insect cells, which were infected with AcNPV-pol and metabolically labeled with [^{35}S]methionine, were incubated with the affinity matrices, and columns were eluted with 5 mM glutathione. Figure 3 demonstrates that little or no binding of proteins in the extract was detected for matrix charged with GST only. However, matrix charged with the same concentration of GST–UL42 (WT) bound the HSV-1 pol in the extracts (lanes 6–9). The specificity of binding of the pol to the GST–UL42 (WT) matrix is further evident by the fact that no other protein from this complex extract bound to and specifically eluted from the GST–UL42 (WT) column (compare lanes 6–9 with lane 1). The large Region II deletion mutant protein (GST–d202–337) failed to bind any pol, consistent with our previous results which

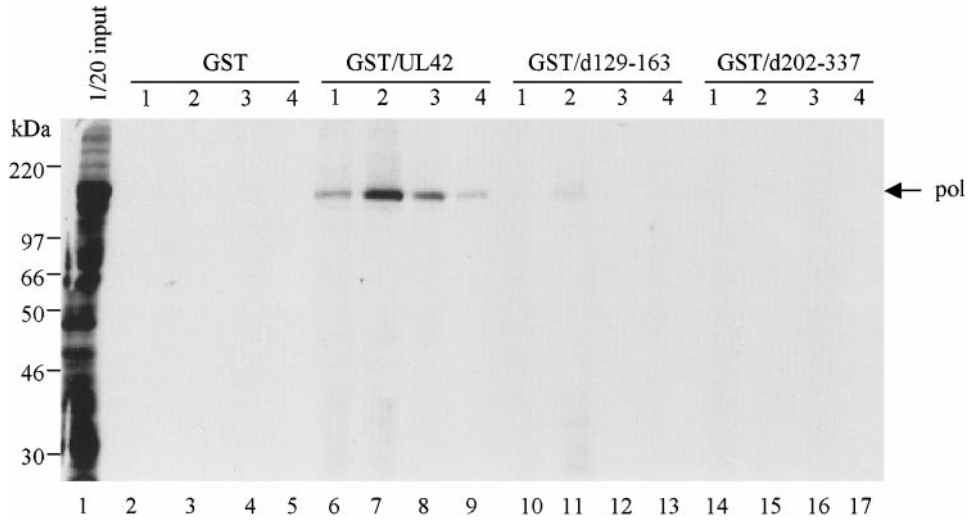

FIG. 3. Binding of pol to GST affinity matrices. GST and GST fusion proteins (9.8 μM) were bound to glutathine agarose, as indicated under Materials and Methods, and incubated with nuclear extracts of insect cells which had been infected with a baculovirus recombinant which expresses the HSV-1 pol and metabolically labeled with [^{35}S]methionine from 12 to 36 h p.i. Unbound protein was washed from the matrix and bound protein was removed with four sequential one-column volumes of 5 mM glutathione (numbered 1–4 above lanes for each matrix). Eluted fractions were analyzed by SDS–PAGE, fixed, soaked with EN^{3}HANCE, and exposed to X-ray film at −80°C. An autoradiograph of the gel is shown. The left-most lane (lane 1) represents 1/20 of the input applied to each matrix. The migration positions of molecular weight markers are shown to the left.

showed a lack of coimmunoprecipitation of a d202–337 UL42-protein and pol with UL42-specific antibody (Monahan *et al.,* 1993). However, we did observe that a small amount of pol could bind to columns charged with the Region I deletion mutant protein, GST–d129–163 (lane 11). Nevertheless, the amount of pol which could bind to the GST–d129–163 matrix in this experiment was less than 5% of that bound to the GST–UL42 (WT) matrix and was, therefore, considered negligible. To exclude the possibility that failure of the pol to bind to the mutant UL42 affinity columns was due to poor binding of the mutant forms of the fusion protein to the affinity matrix, we stained parallel gels with Coomassie blue and found that the amount of GST–d129–163 or GST–d202–337 which eluted from the respective columns was within a twofold range of that eluting from the GST–UL42 (wild-type) columns (results not shown). Taken together, these results confirm both the utility and the sensitivity of GST pull-down experiments for determining the ability of mutant UL42 proteins to physically associate with pol.

We determined the ability of various mutant forms of UL42, expressed as GST fusion proteins, to bind pol using similar GST pull-down experiments, except that pol was expressed by *in vitro* transcription/translation to achieve high specific activity radiolabeling. Table 1 shows the results of triplicate assays in which affinity matrices were prepared by binding 9.8 μM of the indicated fusion proteins to glutathione agarose and the binding of pol determined by SDS–PAGE of column eluates. Bands corresponding to full-length pol were quantified by phosphorimage analysis and binding was expressed as the percentage of the input material applied to the respective columns which were eluted with glutathione. The negative control for the binding of Region II mutants (experiment 1) was GST. However, the negative control selected for the binding of Region I mutants (experiment 2) was GST–d137–142, due to the low level of nonspecific binding we consistently observed for the Region I mutants (see for example, Fig. 3). The results were evaluated by analysis of variance to determine whether the experimental results differed significantly from those of the negative ($P \leq 0.001$) or wild-type ($P < 0.05$) controls. Binding of pol to test matrices which did not vary significantly from that bound to matrices charged with GST–UL42 wild-type protein was scored as ++, while pol binding which did not vary significantly from that bound to GST matrices was scored as negative. The results (Table 1 and Fig. 1) demonstrate that only two of the mutant UL42–GST fusion proteins, i206 and d241–261, could associate with pol as well as the GST–UL42 wild-type protein. Binding was scored as + if it was significantly different (greater) than that to the negative control, but also different (less) than that to the wild-type matrix. Thus, an intermediate level of pol binding was observed for some of the test matrices (R134D, T142A, and N339). Control experiments (not shown), based on visual inspection of Coomassie-stained gels, indicated that the amounts of the UL42 mutant fusion proteins which eluted from columns charged with 9.8 μM protein (calculated by the CDNB assay) could vary slightly from experiment to experiment, but were generally within a twofold range of the amount of GST–UL42 fusion protein bound. Since significant pol binding could be demonstrated when 10 times less wild-type fusion protein was used to charge columns (Fig. 2), the results demonstrate that the mutant proteins that scored negative for pol

TABLE 1

Ability of pol to Bind to Affinity Columns of GST Fusion Proteins[a]

Mutant	pol bound (%) ± SD[b] Experiment 1	Experiment 2	Binding of pol[c]
GST	1.3 ± 1.1	n.d.[d]	−−
GST–UL42 (WT)	31.2 ± 1.7	62.0 ± 6.9	++
GST–N339	12.8 ± 4.5	20.7 ± 3.8	+
GST–d241–261	11.0 ± 2.7	47.5 ± 5.5	++
GST–d256–282	2.5 ± 0.7	n.d.	−−
GST–d274–288	1.2 ± 0.5	n.d.	−−
GST–d282–283	3.8 ± 1.1	n.d.	−−
GST–d137–142	n.d.	9.9 ± 2.8	−−
GST–d140	n.d.	14.9 ± 2.1	−−
GST–i140	n.d.	10.0 ± 1.8	−−
GST–T142A	n.d.	37.7 ± 2.2	+
GST–R134D	n.d.	43.6 ± 15	+
GST–i206	11.6 ± 5.8	55.2 ± 11	++

[a] Glutathione agarose columns were charged with 9.8 μM GST or GST fusion protein.

[b] [^{35}S]Methionine HSV-1 pol was prepared by coupled *in vitro* transcription/translation and bound to columns in triplicate for 3 h at 4°C. Results represent the mean of the percentage of applied pol ± standard deviation (SD) which was recovered following elution with 5 mM glutathione.

[c] Determined by analysis of variance with Dunnett's post hoc comparisons with a control. Binding was scored as ++ if the value differed significantly from the negative control ($P \leq 0.001$) but not from the positive control ($P < 0.05$), + if it differed from both, and −− if it did not differ from the negative control. The positive control for both experiments was the amount of pol which bound the GST–UL42 (WT) columns. The negative control for experiment 1 was the amount of pol which bound to the GST column. In experiment 2, the negative control was the amount of pol which bound to the Region I deletion mutant protein, GST–d137–142. The GST–d241–261 and GST–i206 scored + for pol binding in experiment 1 and ++ in experiment 2.

[d] Not determined.

binding (d137–142, d140, i140, d202–337, d256–282, d274–288, and d282–283) by our GST pull-down assay (Table 1) are at least substantially compromised (compared to the wild-type protein) for their abilities to physically associate with pol.

Ability of GST–UL42 proteins to stimulate pol activity

As a more quantitative measure of function, the ability of UL42 mutant proteins to provide pol accessory function was determined using partially purified GST fusion proteins. Pol accessory function was determined by the ability to stimulate the activity of pol in high-salt buffer containing 125 mM KCl using activated calf thymus DNA as a template. We have used this assay extensively to demonstrate that UL42 provides pol accessory function by stimulating basal pol activity from 4- to 10-fold under these reactions conditions (Gallo *et al.,* 1989; Monahan *et al.,* 1993, 1998). Because pol and UL42 form a stable heterodimer which is more active in high salt than pol alone (Gallo *et al.,* 1989; Hernandez and Lehman, 1990; Gottlieb *et al.,* 1990; Tenney *et al.,* 1993), maximum activity under these conditions is presumably achieved when all of the available functional pol is complexed with UL42.

We prepared substantially purified proteins (>85%) by passage of bacterial extracts containing the GST or GST fusion proteins over glutathione agarose, followed by elution with 5 mM glutathione, an example of which is shown in Fig. 4A. The activity of pol, prepared by *in vitro* transcription/translation, was determined by the incorporation of [^{3}H]TMP into trichloroacetic-acid-insoluble radioactivity in the absence and in the presence of increasing amounts of affinity purified GST or GST–UL42 fusion proteins. Results for all of the mutants are summarized in Fig. 1, but those of several with activity are shown in Fig. 4B. The results demonstrate that GST alone has little or no effect on the activity of pol. Affinity purified GST fusion proteins which failed to stimulate the basal pol activity at least 2-fold at even the highest amount tested (600 nmol) were scored as negative for pol stimulation activity (summarized in Fig. 1). Thus, no pol accessory activity was observed for the large Region I deletion mutants (d129–163 and d137–142) or for most of the Region II deletion mutants (d202–337, d256–282, d274–288, and d282–283 (Fig. 1), in agreement with previous results from several laboratories (Monahan *et al.,* 1993; Digard *et al.,* 1993). However, the GST–UL42 (WT) protein stimulated the basal pol activity with approximately a linear dose-response when 200 nmol or less of fusion protein was added. Failure to further increase pol activity with additional fusion protein most likely indicates that all of the functional pol was bound when 200 nmol of GST–UL42 was added. A similar dose-response was observed with the Region II mutant, GST–d241–261, which, like the GST–UL42 wild-type protein, stimulated pol activity 8- to 10-fold at saturation (Fig. 4B). This level of stimulation is similar to that reported with authentic UL42 prepared by *in vitro* translation (Gallo *et al.,* 1989; Monahan *et al.,* 1993) or purified from insect cells infected with UL42-expressing baculovirus recombinants (results not shown). Similar dose–response curves were obtained for the GST–N339, GST–R134D, and GST–d270 proteins (results not shown), and they were, therefore, scored as ++ for their ability to stimulate pol activity (Fig. 1). The remainder of the GST–UL42 mutants tested (d140, i140, T142A, and i206) stimulated the basal pol activity at least 2-fold at the highest amount tested, but below 50% of the level observed for GST–UL42 (WT), and were scored as +, to indicate a significant, but reduced, ability to stimulate pol activity (Fig. 1).

The dose–response curves for two of the mutants with this intermediate ability to provide pol accessory function are shown in Fig. 4B. The T142A fusion protein stimulated the pol, but only fourfold at the highest amount tested. The dose–response curve in Fig. 4B indicates that stimulation of pol activity by the T142A protein reached maximum levels when 400 nmol of protein was added. Although we cannot rule out the possibility that

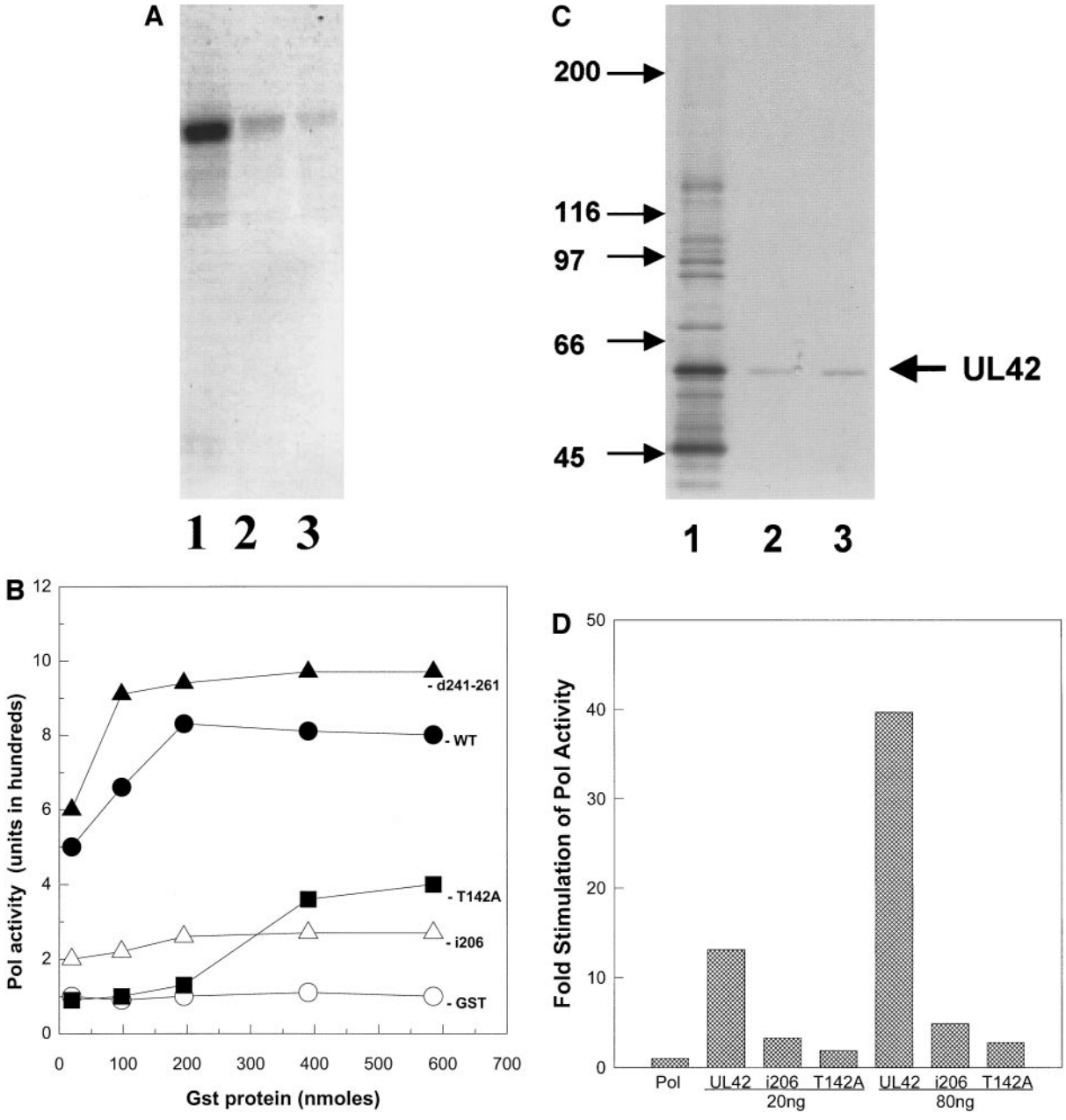

FIG. 4. Stimulation of pol activity by wild-type and mutant UL42 proteins. A. GST–UL42 (wild-type) protein was prepared from bacterial extracts by passage over glutathione agarose eluted with sequential one-column volumes of 5 mM glutathione. Eluates from the second to fourth fractions (lanes 1–3), which contain the bulk of the GST fusion protein, were subjected to SDS–PAGE using a 10–20% gradient polyacrylamide gel and the gel was stained with Coomassie blue. B. GST and GST fusion proteins, prepared as described in A, were dialyzed against B-2 buffer containing 50 mM NaCl and added to reaction mixtures 5 min prior to initiation of reactions with pol (100 units). Pol was prepared by coupled *in vitro* transcription/translation and its activity measured by its ability to incorporate [^{3}H]dTMP into trichloroacetic-acid-insoluble material in the absence and in the presence of the indicated amounts of GST or fusion protein, using activated calf-thymus DNA as a template as described under Materials and Methods. A unit of activity was defined as the amount of enzyme required for the incorporation of 1 fmol of dTMP into DNA per hour at 37°C. C. Pol and full-length wild-type UL42, i206, and T142A proteins were expressed by recombinant baculoviruses and purified to homogeneity by FPLC as described under Materials and Methods. The figure shows a silver-stained denaturing 10–20% polyacrylamide gel through which proteins from a wild-type UL42 preparation were separated by electrophoresis. Lane 1 contains 10 μg input nuclear extract and lanes 2 and 3 contain 0.6 μg of the two fractions containing the peak of pol stimulating activity from the Q–Sepharose column. D. Pol activity (5 mM) in the absence of UL42 or in the presence of 20 (3.9 nM) or 80 ng (15.7 nM) of wild-type or mutant protein was measured as described for B except that poly dA/oligo dT template was used. Pol stimulation activity is recorded as the ratio of pol activity found in the presence of wild-type or mutant UL42 protein to that found in the absence of UL42.

the larger amount of T142A protein required to maximally stimulate pol activity may indicate an altered stoichiometry with pol compared to wild-type UL42, we think it more likely that these results reflect a lower affinity of GST–T142A, compared to GST–UL42 (WT), for pol. This result would also be consistent with the somewhat re-

duced level of pol binding to affinity matrices charged with GST–T142A protein compared to that charged with the wild-type fusion protein (Table 1). Nevertheless, we cannot rule out the possibility that the reduced level of stimulation of pol activity achieved by the GST–T142A protein at apparently saturating amounts could have been due to mechanistic differences in binding to pol or DNA.

The GST–i206 protein was consistently capable of stimulating pol activity above basal levels, although at little more than twofold for all protein amounts tested. By contrast to GST–T142A, the poor ability of GST–i206 to stimulate pol activity is most likely not completely attributable to the altered ability of the protein to bind to pol for two reasons. First, GST–i206 binds pol at close to the levels observed with wild-type GST–UL42 (Table 1), and second, we observed little or no dose-response over the amounts of protein tested (Fig. 4B). However, it is possible that the manner in which the pol binds to the GST–i206 is distinct from that of the wild-type GST–UL42.

As indicated above, the GST fusion proteins all lacked UL42 AA 1–19 and 457–488. To eliminate the possibility that the reduced ability of the GST–T142A or GST–i206 proteins to stimulate pol activity reflected a conformational alteration as a result of the fusion with GST and/or the use of slightly truncated versions of the UL42 proteins, we expressed wild-type and mutant full-length, nonfusion UL42 proteins (i.e., containing AA1–19 and 457–488) using recombinant baculoviruses. These UL42 proteins, as well as pol, were purified from nuclear extracts of Sf-9 cells infected with the respective recombinant baculoviruses by conventional column chromatography as described under Materials and Methods. Protein preparations were considered >99% pure as judged by the absence of other polypeptide species following silver staining of gels of preparations separated by SDS–PAGE, an example of which is shown in Fig. 4C. To confirm their activities, the baculovirus-expressed UL42 proteins were tested in two different amounts for their abilities to stimulate the activity of pol in high-salt buffer as described above, except that an oligo dT/poly dA template was used. Using this template, wild-type UL42 stimulates pol activity 40- to 50-fold (Tenney *et al.,* 1993; Fig. 4D), thus allowing a determination of relative enhancement of activity over a larger range, compared with the use of activated calf-thymus DNA as template. In our hands, a substoichiometric amount of purified wild-type UL42, corresponding to a final concentration of 3.9 nM, stimulated pol activity (5 nM) 13-fold over the basal level, while an excess of wild-type UL42 (15.7 nM) increased the pol activity to nearly 40 times the basal level, using the homopolymeric template (Fig. 4D). By contrast, T142A and i206 stimulated pol activity in high salt, but only 2.8- and 4.9-fold over the basal level, respectively, even at the highest amount tested. We confirmed that this low level of stimulation reflected pol accessory function by the ability of these mutant proteins to increase the length of product, compared to that produced by pol alone, in the presence of cold DNA competitor (Chaudhuri and Parris, unpublished results). Although we observed similar lengths of product made by complexes of pol with T142A or i206, their average length was substantially shorter than that synthesized by complexes of pol with wild-type UL42. Taken together, these results confirm that the mutations *per se* in T142A and i206 are responsible for the reduced ability of the mutant proteins to provide pol accessory function.

Binding of WT and mutant UL42 proteins to DNA

It was possible that the poor ability of the T142A and i206 proteins to stimulate pol activity might reflect a reduced ability to bind to DNA. To quantify DNA binding ability, we selected as our target DNA molecule, a synthetic defined primer/template (P/T), shown to be suitable for kinetic analysis of pol activity in single-turnover experiments (Chaudhuri and Parris, unpublished results). The P/T consisted of a biotinylated 45-mer primer strand annealed to a 67-mer template strand, which produced a double-stranded portion 45 base pairs in length with a 22-nucleotide 5′ single-stranded overhang. The lengths of the double-stranded and single-stranded portions of the P/T were selected to correspond to the size of the respective regions footprinted by a single pol/UL42 heterodimer (Gottlieb and Challberg, 1994). The assay we employed measured binding of each purified UL42 protein to the P/T by detection of changes in surface plasmon resonance using the Biacore Biosensor 2000. This technology (Karlsson *et al.,* 1991; Haruki *et al.,* 1997) permits assessment of both the binding and the dissociation phases, as well a quantitative means for measuring binding affinities of the purified wild-type UL42, T142A, or i206 proteins for the immobilized P/T.

The P/T (120 response units; RU) was stably attached to the surface of a streptavidin gold chip via the biotin tag attached to the 5′ end of the primer strand. To ensure that the chip containing the immobilized P/T did not bind protein nonspecifically, various concentrations of a control, non-DNA-binding protein (bovine serum albumin; BSA) were passed over the chip surface. Even when 600 nM BSA was applied to the DNA-containing chip in low-salt buffer for 8 min, we noted very little binding of BSA (188 RU) above that bound to a control surface without DNA (Chaudhuri and Parris, unpublished results). By contrast, chips containing 120 RU of immobilized P/T bound more than 1500 RU of UL42 (Fig. 5A) or 1400 RU of pol (not shown) when 600 nM of the respective proteins was passed over the chip surface, thus validating the approach for detecting interactions of DNA-binding proteins with this P/T.

To measure association kinetics, different concentrations of UL42 protein were passed over DNA-bound

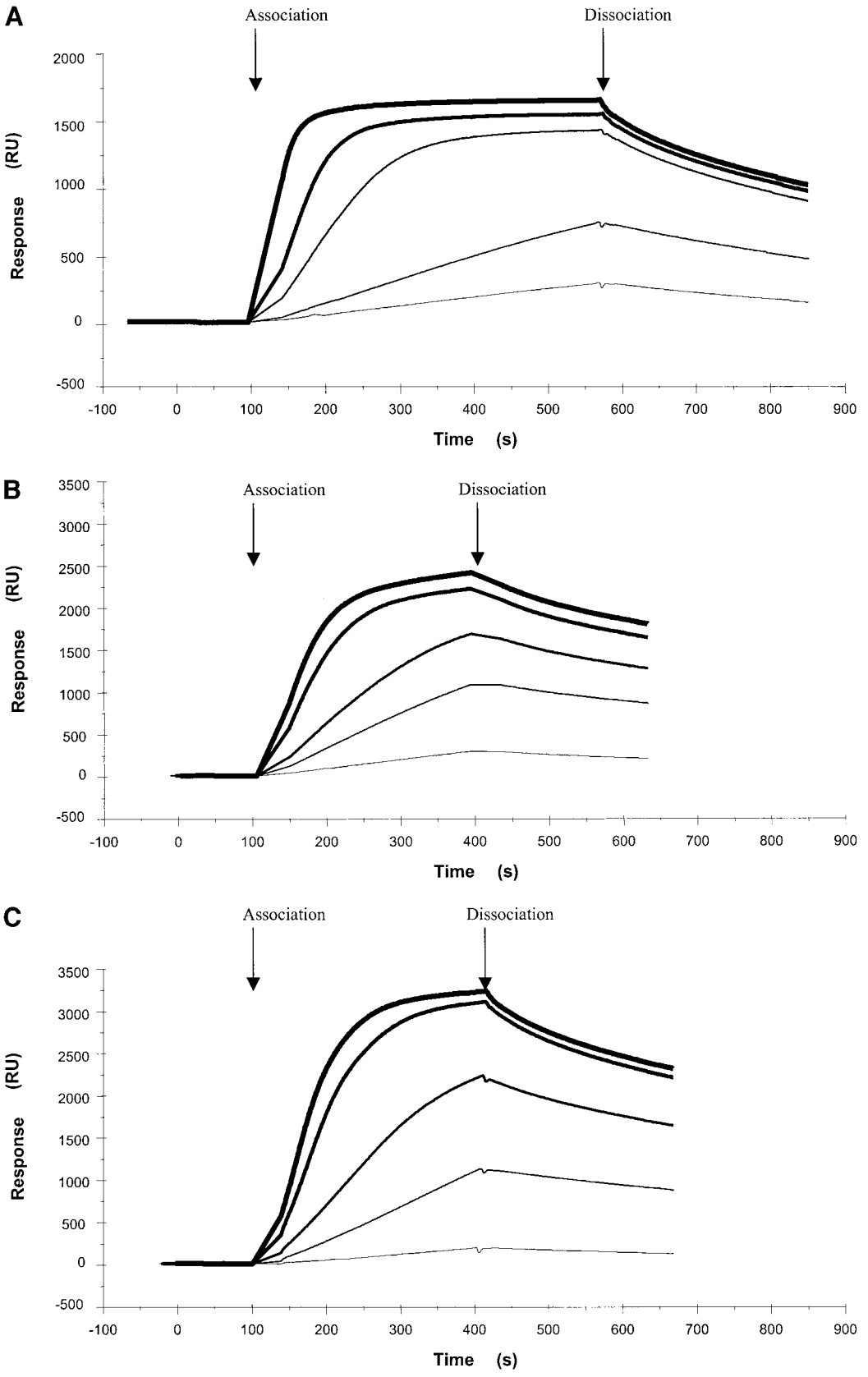

FIG. 5. Sensorgrams of purified UL42 proteins binding to synthetic P/T. Binding of proteins was measured with the BiaCore 2000. A surface of the streptavidin gold chip was left unbound and an additional surface was bound with 120 RU of biotinylated P/T as described under Materials and Methods. Increasing concentrations of the indicated purified proteins were passed over both surfaces and binding measured by changes in surface plasmon resonance recorded in arbitrary response units (RU). The sensorgrams shown represent binding to the DNA-bound surface, less any nonspecific binding to the control surface. The arrows indicate the beginning of the association and dissociation phases. A. Binding of wild-type UL42. Lines of increasing width indicate binding of 25, 50, 150, 300, and 600 nM concentrations of protein. B and C. Binding of T142A and i206, respectively. Lines of increasing width indicate binding of 50, 150, 300, 600, and 800 nM concentrations of mutant protein.

TABLE 2

Apparent Kinetic Constants for Binding of UL42 Proteins to Synthetic Primer/Template[a]

		Experiment 1			Experiment 2			Average		
Protein	Conc. (nM)	k_{on} (M^{-1} s^{-1})	k_{off}^{b} (s^{-1})	K_d^c (nM)	k_{on} (M^{-1} s^{-1})	k_{off}^{b} (s^{-1})	K_d^c (nM)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (nM) [Relative affinity]
WT UL42	600	8.54×10^4	2.12×10^{-3}		9.5×10^4	2.18×10^{-3}				
	300	8.13×10^4	2.12×10^{-3}		8.26×10^4	2.18×10^{-3}				
	150	9.20×10^4	2.12×10^{-3}		7.17×10^4	2.18×10^{-3}				
	Average	8.62×10^4	2.12×10^{-3}	24.6	8.31×10^4	2.18×10^{-3}	26.2	8.47×10^4	2.15×10^{-3}	25.4 [1.00]
i206	800	2.22×10^4	1.97×10^{-3}		3.08×10^4	2.49×10^{-3}				
	600	1.94×10^4	1.97×10^{-1}		3.11×10^4	2.49×10^{-3}				
	300	1.92×10^4	1.97×10^{-3}		2.39×10^4	2.49×10^{-3}				
	Average	2.02×10^4	1.97×10^{-3}	97.5	2.86×10^4	2.49×10^{-3}	87.1	2.44×10^4	2.23×10^{-3}	91.4 [0.28]
T142A	800	2.52×10^4	1.74×10^{-3}		3.57×10^4	1.84×10^{-3}				
	600	2.45×10^4	1.74×10^{-3}		3.11×10^4	1.84×10^{-3}				
	300	1.20×10^4	1.74×10^{-3}		2.85×10^4	1.84×10^{-3}				
	Average	2.06×10^4	1.74×10^{-3}	84.5	3.18×10^4	1.84×10^{-3}	57.9	2.62×10^4	1.84×10^{-3}	70.2 [0.36]

[a] Values were determined using the simple binding model A + B = AB and calculated using the BiaEvaluation 2.1 software.

[b] The apparent rate constant for dissociation was calculated from the first 20 s of the dissociation phase to minimize rebinding. The value used for each experiment represents that calculated following binding of the highest concentration of protein tested and was used to calculate the association rate constant for each concentration of protein tested using the nonlinear method.

[c] Calculated as k_{off}/k_{on}.

surfaces and control surfaces without DNA, and binding was recorded in response units. Dissociation kinetics were determined following the association phase by passage of the same buffer, without the UL42 protein, over the surfaces. The sensorgrams displayed in Figs. 5A–5C were produced by subtracting the response obtained by binding of the wild-type UL42, T142A, and i206 proteins, respectively, to a control surface without DNA, from the response obtained by binding to the DNA-containing surface. In initial experiments, we observed a reduced ability of the mutant UL42 proteins to bind to the P/T and, therefore, tested an even higher concentration (800 nM) of each mutant protein than the maximum concentration used for wild-type UL42 protein (600 nM). The apparent kinetic constants for each protein were calculated using BiaEvaluation 2.1 software as detailed under Materials and Methods and are shown in Table 2 for two independent experiments. The results demonstrate that the i206 and T142A mutant proteins were both capable of binding to the synthetic P/T, although with approximately a threefold reduced affinity compared to the wild-type UL42 protein. Dissection of the association and dissociation phases reveals that most of the reduced affinity of the mutant proteins for DNA is attributable to a reduced ability to associate with P/T, since there was little difference in the stability of protein:DNA complexes once formed. These results suggest that reduced inherent affinity for DNA cannot fully account for the reduced ability of these mutant proteins to stimulate pol activity.

Ability of mutant UL42 proteins to support ori-dependent DNA replication

All of the assays described above were used to define the function of UL42 *in vitro* in the absence of other viral proteins. However, UL42 is one of seven HSV-1 proteins required for ori-dependent DNA replication (ODR; Wu *et al.,* 1988; Stow, 1992), and it is likely that most or all of these proteins function in viral DNA replication as a complex. Thus, it is possible that deficiencies in DNA or pol binding observed *in vitro* might be compensated by interactions of UL42 and/or pol with other DNA replication proteins. Alternatively, mutations which alter these *in vitro* functions might produce an even more profound effect in complex activities such as ODR. We determined the abilities of UL42 mutant proteins to amplify ori-containing plasmids when the other DNA replication proteins were supplied by the UL42 null mutant, CgalΔ42. Plasmids containing the wild-type or indicated mutant UL42 genes downstream of the endogenous UL42 promoter were transfected into BHK cells together with plasmid T085, which contains a functional HSV-1 ori_s sequence, and cells were subsequently infected with CgalΔ42 (5 PFU/cell). Plasmids were scored for their abilities to support ODR based upon the presence of *Dpn*I-resistant vector sequences in cells harvested at 18 h postinfection (p.i.). An example of the results from one such assay is shown in Fig. 6. No *Dpn*I-resistant ori-plasmid DNA (large arrow) was detected in cells tranfected with empty vector followed by infection with CgalΔ42. However, amplification was observed in

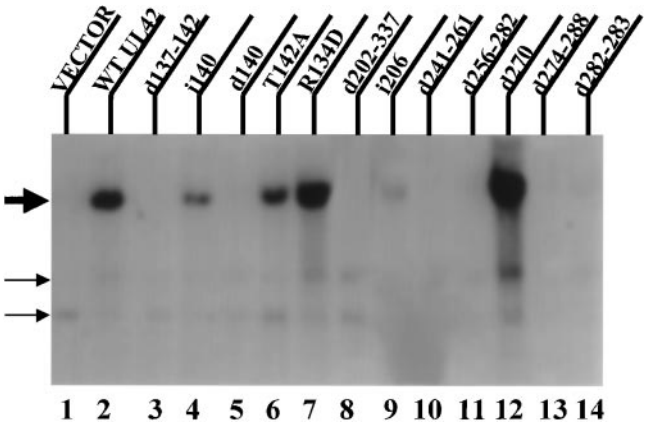

FIG. 6. Ori-dependent DNA replication supported by various UL42 mutant proteins. BHK cells, nonpermissive for the replication of the UL42 null virus, CgalΔ42, were transfected with the ori-containing plasmid, pT085 (0.4 μg), together with a plasmid (2 μg) encoding the indicated UL42 protein (lanes 2–14) or empty vector alone (lane 1). Cells were infected 29 h later with CgalΔ42 (m.o.i. of 5 PFU/cell) and harvested at 18 h p.i. Total DNA was prepared, subjected to digestion with *Eco*RI and *Dpn*I, and 5 μg of each sample was subjected to electrophoresis through a 1% agarose gel. The gel was blotted onto Nytran and probed with a [^{32}P]-labeled DNA fragment from pT085. The large arrow indicates the position of the *Dpn*I-resistant band indicative of plasmid amplification, while the smaller arrows indicate the positions of *Dpn*I-sensitive plasmid bands to control for plasmid input and DNA loading.

CgalΔ42-infected cells which were transfected with the wild-type UL42 plasmid, pLBN19A. Substantial amplification of plasmid sequences in this experiment was observed for i140, T142A, R134D, and d270.

Because of some variation in the efficiencies of ODR from experiment to experiment, we averaged the amount of amplification obtained with the mutant UL42 gene compared to that obtained with the wild-type gene in three independent experiments (Table 3). Moreover, to account for possible variations in transfection efficiencies among samples in a given experiment, results were considered valid only if blots revealed low-molecular-weight *Dpn*I-sensitive bands (Fig. 6, small arrows), corresponding to input T085 DNA, for test samples in an amount which was at least 50% of that present in cells transfected with the wild-type UL42 plasmid. The results demonstrate that i140, R134D, T142A, d270, and d282–283 support ODR to a level at least 50% of that supported by the wild-type UL42 gene. However, substantially reduced levels of ODR were consistently observed for the d140, i206, and d241–261 plasmids. No ODR was detected for plasmids encoding d137–142, d202–337, d256–282, or d274–288 (Table 3, Figs. 1 and 6).

Complementation of CgalΔ42 viral replication

Although the ability of mutant proteins to support ODR is one measure of the ability of these proteins to function in the context of the other essential viral DNA replication proteins, the true measure of their function in infected cells is best determined by their abilities to support productive viral replication. In the absence of HSV mutants with these lesions, we tested the plasmids containing the mutated UL42 genes for their respective abilities to complement the replication of the UL42 null mutant, CgalΔ42, in nonpermissive BHK cells. Test plasmids were introduced into cells by liposomal-mediated transfection and the cells were subsequently infected with CgalΔ42 (2.5 PFU/cell). The yield of progeny virus present in the combined intracellular and extracellular

TABLE 3

Ability of UL42 Mutant Proteins to Support ori-Dependent DNA Replication in Transient Assays

Mutant[a]	Amplification of ori plasmid (% of WT)[b] Expt. 1	Expt. 2	Expt. 3	Mean amplification ± SD[c]	Ability to support ODR[d]
Vector	0.0	0.0	0.0	0.0	−−
WT UL42	100	100	100	100	++
d129–163	0.0	0.0	0.0	0.0 ± 0.0	−−
d137–142	0.0	0.0	0.0	0.0 ± 0.0	−−
d140	9.9	1.6	12.0	7.8 ± 5.5	+
i140	100	i	100*	100	++
R134D	100	82	30	71 ± 36	++
T142A	67	64	i	66	++
d202–337	0.0	0.0	2.0	0.7 ± 1.2	−−
i206	49	44	23	39 ± 14	+
d241–261	17	22	5.0	15 ± 8.7	+
d256–282	0.0	0.0	0.0	0.0 ± 0.0	−−
d270	88	76	74	79 ± 7.6	++
d274–288	0.0	0.0	i	0.0	−−
d282–283	47	99	100*	82 ± 30	++

[a] The UL42 WT or mutant gene together with endogenous promoter was cloned into the vector pTZ19U.

[b] The ori-containing plasmid, pT085 (0.4 μg), was transfected with the indicated test plasmid (1 μg) into BHK cells. The cells were subsequently infected with CgalΔ42 and total cellular DNA isolated as described under Materials and Methods. Southern blots of DNA, digested with *Dpn*I and *Eco*RI and separated by electrophoresis, were probed with ori sequences and the bands quantified by phosphorimage analysis. Amplification was measured by the ratio of radioactivity in the band corresponding to the full-length, *Dpn*I-resistant T085 plasmid to the total radioactivity in that lane. Ratios were normalized to that obtained with the WT UL42 plasmid (scored as 100%). Values obtained which were greater than that obtained with the WT plasmids are denoted with an asterisk (*). Experiments were scored as indeterminate (i) if no amplification was observed, but for which the level of *Dpn*I-sensitive DNA in the sample was less than half of that in the wild-type lane.

[c] Means were calculated using all valid data and are presented with standard deviation (SD). Values for which no standard deviation is indicated represent the average of the remaining two experiments.

[d] Plasmids were scored as ++ for their ability to support origin-dependent DNA replication (ODR) if amplification was greater than 50% of the level obtained with the wild-type UL42 plasmid. A score of + was assigned if significant amplification was observed, but at a level below 50% of that obtained with the wild-type plasmid, and a score of − was assigned for plasmids yielding no significant amplification of the ori-containing plasmid.

TABLE 4

Complementation of CgalΔ42 Replication in BHK Cells

Plasmid[a]	Complementation index[b] ± SD[c] (*n*)[d]	Localization[e]
pLBN19A (WT)	100 ± 31.2 (14)	Nuclear
d129–163	0.2 ± 0.2 (6)	Nuclear
d137–142	0.4 ± 0.1 (6)	Nuclear
d140	145 ± 25.9 (6)	Nuclear
i140	11.2 ± 7.7 (12)	Nuclear
R134D	66.0 ± 12.3 (4)	Nuclear
T142A	86.0 ± 27.6 (4)	Nuclear
d202–337	0.1 ± 0.1 (12)	Nuclear
i206	13.0 ± 3.9 (11)	Nuclear
d241–261	<0.001 (4)	Nuclear
d256–282	<0.001 (4)	Nuclear
d270	102 ± 12.0 (2)	Nuclear
d274–288	0.2 ± 0.1 (6)	Nuclear
d282–283	23.0 ± 5.0 (5)	Nuclear
pTZ19U	0.1 ± 0.1 (14)	—
None	<0.001 (6)	—

[a] BHK cells were transfected with 2 μg of the indicated plasmid and infected with CgalΔ42 (2.5 PFU/cell) 29 h later. Cells were harvested 18 h p.i. Combined results from seven independent experiments, employing two to four replicate cultures for each test plasmid, are shown.

[b] Virus yields were determined by plaque assay in permissive V9 cells. Virus yields for transfections with the wild-type UL42 plasmid, pLBN19A ranged from 8.5×10^4 to 2.1×10^6 PFU/ml, while the yields with the vector alone ranged from 1.1×10^0 to 2.0×10^3 PFU/ml. The complementation index (CI) was calculated as

$$\mathrm{CI} = \frac{\left(\dfrac{\sum \text{yield with test plasmid}_{\text{expt. 1}}}{\text{ave. yield with WT}_{\text{expt. 1}}} + \dfrac{\sum \text{yield with test plasmid}_{\text{expt. 2}} \cdots}{\text{ave. yield with WT}_{\text{expt. 2}}}\right)}{n_{\text{expt. 1}} + n_{\text{expt. 2}} + \cdots} \times 100.$$

[c] Standard deviation.

[d] Combined number of replicate cultures independently transfected and assayed.

[e] Determined by indirect immunofluorescence with UL42 specific antibody 834.

fractions following a single cycle of replication (18 h) was determined by plaque assay in V9 cells which provide UL42 function *in trans* (Johnson *et al.,* 1991). Plasmids encoding the Region I point mutations R134D and T142A supported viral replication at 66 and 86%, respectively, when compared to that supported by the wild-type UL42-containing plasmid, pLBN19A (Table 4), although we do not consider these levels to be significantly different from those displayed by the wild-type plasmids. We also observed wild-type levels of complementation by the d270 plasmid. These experiments also confirm our previous report of significantly low, but detectable, levels of complementation by the i140 and i206 plasmids (Table 4; Reddig *et al.,* 1994) and indicate yet another UL42 mutation (d282–283) which produces a significantly lower level of complementation (23%) than the wild-type gene.

The complete failure of d241–261 to complement replication of CgalΔ42 (Table 4) and its poor ability to support ODR (Fig. 6, Table 3) were unexpected in view of the ability of the GST–d241–261 to bind to pol (Table 1) and double-stranded DNA (results not shown) and to stimulate the activity of pol *in vitro* at levels similar to those observed for wild-type UL42 (Fig. 4B). To rule out the possibility that a second-site mutation in the UL42 gene was obtained during the cloning of the mutation into the constructs used for complementation analysis, we derived an independent mutant construct by transferring the region containing the deletion mutation from the GST–d241–261 into the UL42 open reading frame in the pLBN19A plasmid. This plamid also failed to complement the replication of CgalΔ42 (not shown).

It was possible that negative or low-level complementation of the replication of the UL42 null mutant by some of the plasmid constructs was due to a lack of or inefficient availability of the mutant protein in nuclei. Therefore, we determined the localization of mutant proteins by indirect immunofluorescence using the UL42 antipeptide antibody 834. As previously described (Goodrich *et al.,* 1989), the wild-type UL42 expressed by pLBN19A localized exclusively to nuclei of Vero cells transfected with the plasmid, even in the absence of viral infection (Table 4). In fact, we found that each of the plasmids expressed a UL42 protein which localized exclusively to nuclei, indicating the presence of a functional nuclear localization or retention signal. Thus, negative or low-level complementation was not attributable to poor availability in cellular nuclei.

DISCUSSION

Previous UL42 domain mapping studies have suggested that discrete, independent domains for DNA binding and for pol binding within the essential N-terminal 315 amino acids of the protein do not exist (Digard *et al.,* 1993; Monahan *et al.,* 1993; Chow and Coen, 1995; Tenney *et al.,* 1993). We sought to identify mutations which could differentially affect one or more known properties of the protein and to determine the best *in vitro* assay to predict the ability of the various mutant proteins to function *in vivo* by further dissecting regions of the UL42 known to be important for its ability to function as a pol accessory protein. The studies reported herein are the first to examine the effects of single amino acid substitutions of Region I, defined by the deletion of AA 129–163, as well as the effects of smaller deletions within Region II, previously defined by the deletion of AA 202–337. Both regions have been reported to be essential for pol and DNA binding and for the ability to function in virus replication (Monahan *et al.,* 1993; Reddig *et al.,* 1994; Digard *et al.,* 1993).

Domains required for *in vitro* activities of UL42

The use of GST pull-down experiments with affinity matrices charged to saturation or near saturation with fusion proteins proved highly reliable in assessing the ability of the various mutant UL42 proteins to bind to pol, and such experiments were a significant improvement over coimmunoprecipitation analysis. Nonspecific interactions of the large Region I deletion mutant, d129–163, with other proteins such as β-galactosidase prevented us from using coimmunoprecipitation analysis to assess the ability of proteins with smaller alterations in this region to bind to pol (Thornton and Parris, unpublished results). Although we also observed low-level nonspecific interactions of GST–d129–163 and GST–d137–142 fusion proteins with pol in pull-down experiments (Fig. 3 and Table 1), binding in excess of this background level was determined with a high level of confidence ($P < 0.001$) for the Region I point mutant proteins, R134D and T142A.

Wild-type levels of pol binding activity were observed for the Region II mutant protein, GST–d241–261 (Table 1). The d241–261 mutation is significant in that it contains the largest deletion within the large N-terminal domain reported to date to retain DNA binding (Monahan and Parris, unpublished results), pol binding, and pol stimulation activities (Fig. 1). Digard and co-workers (1993) described a protein with a smaller deletion in this region, Δ242–250, and which also retained all three activities.

Despite the reproducibility and increased sensitivity of GST pull-down experiments to determine the ability of pol to physically interact with UL42, compared to coimmunoprecipitation analysis, we observed no significant binding of pol to GST–d140 or GST–i140 compared to that observed with the Region I deletion mutant protein, GST–d137–142. Nevertheless, affinity purified d140 and i140 GST fusion proteins were able to stimulate pol activity in high salt, albeit at a level substantially below that of the GST–UL42 (WT) (Fig. 1). In previous experiments using authentic, nonfusion UL42 proteins expressed by *in vitro* translation, we reported that d140 stimulated pol activity at a reduced level compared to that achieved with the wild-type protein (Reddig *et al.,* 1994), in excellent agreement with the results we obtained with GST–d140. However, the *in vitro* translated i140 failed to stimulate pol activity (Reddig *et al.,* 1994). We think it likely that GST–d140 and GST–i140, and their nonfusion counterparts, can bind to pol, but with substantially reduced affinity compared to GST–UL42 (WT). This would account for our failure to observe pol stimulation activity with small amounts of i140 protein, such as are produced by *in vitro* translation, but our success in demonstrating significant, but reduced, pol stimulation using large amounts of affinity purified GST–i140 protein (Fig. 1).

It is also possible that d140 and/or i140 cannot bind to pol with high affinity in the absence of DNA and that the ability to stimulate pol activity reflects, at least in part, the ability of these mutant UL42 proteins to bind to DNA. We did not directly measure the ability of either mutant protein to bind DNA. However, Chow and Coen (1995) have suggested that the inherent ability to bind to DNA may be a necessary attribute of a UL42 protein to provide pol accessory function, based on the impaired pol accessory function displayed by two mutant UL42 proteins which failed to bind DNA but retained pol binding activity. Although retention of DNA binding capability may be necessary in order for HSV-1 UL42 protein to stimulate pol activity, quantitative DNA binding results reported herein for purified i206 and T142A mutant proteins demonstrated a poor correlation between the effect of the mutations on the affinity of the encoded proteins for DNA and their effects on relative abilities of the proteins to stimulate pol activity under high-salt conditions compared to the wild-type protein (Table 2 and Fig. 4). Indeed, despite the fact that GST–i206 bound pol as well as did the wild-type GST–UL42 (Table 1) and displayed only a 4-fold reduced affinity for model P/T (Table 2), we observed approximately a 10-fold reduction, compared to that produced by the wild-type UL42 protein, in the level of pol stimulation by even excess UL42:pol ratios (Fig. 4D). That the ability of the pol accessory protein to bind to DNA is not sufficient for its ability to stimulate pol elongation activity is supported by a structure–function analysis of the Epstein–Barr virus (EBV) pol accessory protein, BMRF1 (Kiehl and Dorsky, 1995). These authors found that the EBV accessory protein deleted of amino acids 194–217 or 206–236 retained DNA binding activity but lost the ability to stimulate pol activity.

Regardless of whether binding to DNA facilitates the ability of UL42 to interact with pol, our results indicate that the pol stimulation assay with partially purified fusion proteins may be more sensitive for determining the ability of UL42 mutant proteins to provide pol accessory activity *in vitro* than stimulation assays using *in vitro* translation products or pol binding assays with GST fusion proteins linked to affinity matrices. The fact that partially purified GST–R134D and GST–N339 stimulated pol activity at a level at or above 50% of that achieved by the wild-type UL42 protein (Fig. 4B), while displaying significantly reduced pol binding activity in pull-down experiments (Table 1), provides additional support for the greater sensitivity of the pol stimulation assay. In only one case (GST–i206) did a mutant fusion UL42 protein score significantly better in the pol binding compared to the pol stimulation assay (Fig. 1, Table 1). Although we observed wild-type levels of pol stimulation activity for *in vitro* translation products of i206 in a previous study (Reddig *et al.,* 1994), the latter results did not take into account quantitative differences in the level of expression of proteins by *in vitro* translation. It is possible that significantly more translated i206, compared to the wild-type protein, was present in the previously published pol

stimulation assays. By contrast, the pol stimulation assays reported herein controlled for protein amount, utilized a range of protein concentrations, and used protein which was substantially purified. In independent assays with highly purified, nonfusion i206 protein, we also observed significantly reduced ability, compared to purified wild-type UL42, to stimulate pol activity using a more sensitive homopolymeric DNA substrate (Fig. 4D). Indeed, the reduced ability of i206 to stimulate pol activity *in vitro* compared to the wild-type UL42 protein, found in the studies within this report, is consistent with its compromised ability to function in two *in vivo* assays described in this report (see below) and in one of the *in vivo* assays described in the previous report (Reddig *et al.,* 1994).

Domains required for *in vivo* activities of UL42 mutant proteins

We measured activity of UL42 proteins *in vivo* by determining their abilities to support ODR (Fig. 6 and Table 3) or production of infectious progeny virus (Table 4) in complementations assays with the UL42 null mutant virus, CgalΔ42. We found that all mutant UL42 proteins which were unable to complement ODR by CgalΔ42 also failed to complement its productive replication (Fig. 1). Likewise, all those capable of complementing productive replication also complemented ODR. Even mutant UL42 genes which complemented the production of infectious progeny virus by the null mutant virus at a very low level (i140 and i206; Reddig *et al.,* 1994, and Table 4, this report) supported ODR at a reduced level compared to the wild-type gene (Fig. 1 and Table 3). Lack of correlation of these two *in vivo* complementation activities was found for only one mutant gene, d241–261. This mutant failed to increase the production of infectious progeny virus more than the vector or plasmid-less controls (Table 4 and results not shown), but complemented ODR, although at a low level (Table 3). Thus, we think it likely that these results represent quantitative rather than qualitative differences for assays with different levels of sensitivity. Although we think it less likely, the failure of d241–261 to complement the productive replication of CgalΔ42 may be indicative of one or more essential functions of UL42 in addition to those required for ODR.

Correlation of *in vitro* and *in vivo* activities of UL42 protein

We observed a nearly perfect correlation between the ability of a mutant UL42 protein to stimulate pol activity and its ability to function in ODR and virus replication. All but one of the mutant UL42 proteins which lacked *in vitro* activity also lacked the ability to provide UL42 function in infected cells. The Region II deletion mutant, d282–283, failed to significantly bind pol or stimulate pol activity, but could complement ODR and the production of virus progeny by the UL42 null mutant, CgalΔ42. Nevertheless, complemention of virus production by d282–283 was significantly less than for the wild-type gene (Fig. 1). Within the context of the infected cell, pol can bind to UL8, a member of the helicase/primase complex (Marsden *et al.,* 1997), as well as to the major single-stranded DNA-binding protein (Chiou *et al.,* 1985; Ruyechan and Weir, 1984; Vaughan *et al.,* 1984), and UL42 can bind to the ori-binding protein, UL9 (Monahan *et al.,* 1998). Thus it is possible that one or more of these protein contacts may stabilize interactions between d282–283 and pol and/or between the mutant protein and DNA. That high-level expression of a mutant UL42 gene, in the context of virus replication, can overcome the effect of low-affinity interactions *in vitro* is further suggested by the fact that we have been able to isolate i140 mutant virus which can productively replicate in Vero cells (Grinstead and Parris, manuscript in preparation), despite the mutant protein's poor ability to stimulate pol activity *in vitro* and its poor ability to complement CgalΔ42.

Discrepancies between the nearly wild-type *in vitro* activities of d241–261 and its negligible *in vivo* activities present an apparent paradox with the above interpretation. Control experiments demonstrated that these discrepancies are unlikely to be due to second-site mutations or improper cellular localization of the protein (Table 4). Thus, it is possible that the *in vivo* defect of d241–261 relates to another function of the UL42 or to its ability to perform this function in the context of the other proteins required for viral DNA replication. It is interesting to note that controlled proteolysis of UL42 protein within and close to the region encompassing 241–261 yielded two stable peptides in the presence, but not in the absence, of DNA (Hamatake *et al.,* 1993). The proteolytic fragments remained associated following chromatography and both were required for pol stimulation. Thus the region encompassing 241–261 may serve as a hinge between the N-terminal and C-terminal halves of the protein, whose interdigitation is required for conformational and functional integrity of UL42. Reduction in the length of the hinge region might lock the UL42 into a conformation inconsistent with its ability to interact with other proteins and/or to efficiently synthesize full-length viral DNA in the infected cell.

The importance of two noncontiguous regions for UL42 function was suggested by previous studies (Monahan *et al.,* 1993; Digard *et al.,* 1993). These regions were further confirmed and delineated by the results presented in this report. Within the N-terminal half, residues from 137–142 (Region I) were essential for all measured activities. Even minor perturbations within this region, such as the deletion of a single residue or substitution of a single amino acid, resulted in the alteration of one or more of the activities we measured. Within the C-terminal region we previously designated Region II, we found that the deletion of AA 256–282 or 274–288

destroyed all *in vitro* and *in vivo* activities, while deletion of residues 282 and 283 destroyed *in vitro,* but not *in vivo,* function. Thus, residues 274–282 further define a C-terminal domain which is essential for both *in vitro* and *in vivo* function.

Structure–function analysis of the EBV pol accessory protein also revealed two noncontiguous essential domains for pol stimulation activity (Kiehl and Dorsky, 1995). Similar to HSV-1 UL42, the two domains within the EBV pol accessory protein are separated by a short stretch of amino acids (215–233), the deletion of which results in a protein which retains at least some ability to stimulate its cognate pol's activity. This region of the EBV pol accessory protein may be analogous to 241–261 of HSV-1 UL42, although extensive homology is lacking.

Despite the exceptions noted above, we believe that the pol stimulation assay we have employed is sensitive and efficient enough to be used to predict the ability of a mutant UL42 protein to support viral DNA replication. Likewise, it should be possible to adapt the assay in order to screen for possible antiviral compounds capable of disrupting the ability of wild-type GST–UL42 to function in pol stimulation. The ability to demonstrate pol stimulation activity of different mutant UL42 proteins, qualitatively and quantitatively, using GST fusion proteins expressed in bacteria and affinity purified would make it possible to economically screen large numbers of compounds for their abilities to disrupt pol accessory function.

MATERIALS AND METHODS

Cells and viruses

Baby hamster kidney (BHK) cells and African green monkey kidney (Vero) cells were cultivated as previously described (Gallo *et al.,* 1988). V9 cells are stable transfectants of Vero cells capable of complementing mutants defective in UL42 function and were grown as indicated previously (Johnson *et al.,* 1991). Sf-9 insect cells obtained from Fred Hink (Ohio State University) were used to propagate recombinant baculovirus (*Autographica californica* nuclear polyhedrosis virus, AcNPV), which expresses the HSV-1 pol gene (gift of Robert Lehman, Stanford University). The Sf-9 cells and recombinant AcNPV were cultivated as previously described (Monahan *et al.,* 1993). The UL42 genes used in this study were derived from the KOS wild-type parental strain of HSV-1. The parental wild-type strain for the UL42 deletion mutant, CgalΔ42, was Cgal$^+$, a modified version of HSV-1 strain 17 syn$^+$ which expresses β-galactosidase (Johnson *et al.,* 1991). All stocks of HSV-1 were prepared by low-multiplicity passage (Parris *et al.,* 1978).

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 columns (Qiagen, Studio City, CA) according to the instructions of the manufacturer. All restriction enzymes which were used were purchased from Bethesda Research Laboratories (Gaithersburg, MD) except for *Bse*RI, which was purchased from New England Biolabs (Beverly, MA).

Plasmid pT085 (gift of Nigel Stow, Glasgow, Scotland) contained a functional HSV-1 origin (ori) of replication and was used in ori-dependent DNA replication assays. The HSV-1 pol (pT7-7.1; Dorsky and Crumpacker, 1988) and UL42 (pLBN19A; Gallo *et al.,* 1989) genes were cloned in phagemid vectors downstream of the T7 RNA polymerase promoter. The pol gene in pT7-7.1 lacks sequences encoding the first 67 amino acids to facilitate expression by *in vitro* translation, but the protein otherwise has properties that are indistinguishable from those of the full-length gene product (Dorsky and Crumpacker, 1988; Gallo *et al.,* 1989; Monahan *et al.,* 1993). The GST and GST fusion proteins were expressed from the pGEX-2T vector (Pharmacia Biotech, Piscataway, NJ) without or with the UL42 gene insert, respectively.

Construction of UL42 gene mutations

Figure 1 summarizes the UL42 gene mutations analyzed in the present study. The mutations d140, i140, i206, d129–163, d202–337, and N339 (Monahan *et al.,* 1993) and d137–142 and d274–288 (Reddig *et al.,* 1994) have been described elsewhere. Other deletion mutations within Region II were created by digestion of a plasmid containing the 398-bp UL42 *Sst*I fragment with *Pst*I (d282–283), *Sal*I (d270, d256–282), or *Not*I (d241–261), followed by digestion with exonuclease III for 5 or 10 min. The 5′ single-stranded overhangs were removed by digestion with mung bean nuclease and the plasmid was religated. The resulting *Sst*I fragment, deleted of residues encoding the indicated amino acids, was cloned into a pLBN19A derivative lacking the *Sst*I fragment. The point mutations in Region I, T142A and R134D, were constructed using an overlap extension PCR method (Ho *et al.,* 1989). Briefly, two pairs of primers were used to direct the synthesis of two DNA fragments with complementary ends possessing the desired mutation, followed by annealing of the two fragments and further PCR amplification with the outside primers prior to cloning (Table 5). The resulting mutations were confirmed by dideoxy sequencing.

The parental plasmid (pGST-42), from which all GST–UL42 constructs were derived, contained UL42 amino acid residues 20–456. The plasmid was created by cloning the 1308-bp *Mlu*I fragment, located within the UL42 open reading frame, into the *Bam*H1 site of pGEX-2T via a 10-mer *Bam*H1 linker (Table 5) to produce an in-frame fusion with the GST-encoding sequences. UL42 mutations (Fig. 1) were transferred by swapping a restriction fragment from the plasmid containing the mutation for

TABLE 5

Oligonucleotides used for Mutagenesis and DNA Binding Studies

Mutation/use	Oligonucleotide	Sequence[a]	Description
R134D	1	ccagcccgcgttgggatgac	Upper outside primer
	2	ccccgtttGAcacgctggtt	Upper mutagenic primer
	3	ctgaaccagcgtgTCaaacg	Lower mutagenic primer
	4	ccgggatggtgcgagagttg	Lower outside primer
T142A	1	ccagcccgcgttgggatgac	Upper outside primer
	5	ggacgtcgtcgCccatatgc	Upper mutagenic primer
	6	cgcatatggGcgacgacgtc	Lower mutagenic primer
	4	ccgggatggtgcgagagttg	Lower outside primer
GST fusion with UL42	7	cgggatcccg gccctagggc	Double-stranded *Bam*HI linker
DNA primer/template	8	gccactacgacaccttgatcgcctcgcagccgtccaaccaactca	Primer strand
	9	atttgctgacctttgttctggatgagttggttggacggctgcgaggcgatcaaggtgtcgtagtggc	Template strand

[a] Sequences are shown in the 5′ and 3′ direction for single-stranded sequences. For the double-stranded sequence, the top strand is in the 5′ and 3′ orientation. Uppercase letters indicate nucleotides which differ from the wild-type UL42 sequence.

the analogous fragment from the pGST-42 plasmid using standard cloning techniques. GST and GST fusion proteins were expressed and quantified as previously described (Monahan *et al.,* 1998).

Pol binding assay

Affinity matrices containing various amounts of GST, GST–wild-type UL42 [GST–UL42 (WT)], or GST–mutant UL42 proteins were obtained by charging glutathione agarose beads (Sigma, St. Louis, MO) with clarified bacterial extracts containing various concentrations or GST proteins ranging from 0.98 to 9.8 μM as described previously (Monahan *et al.,* 1998). The HSV-1 pol was expressed by recombinant AcNPV in Sf-9 cells labeled metabolically with [^{35}S]L-methionine as previously described (Monahan *et al.,* 1993), or by coupled *in vitro* transcription/translation in rabbit reticulocyte lysates in the presences of [^{35}S]L-methionine, as indicated in the figure legends. For the former, high-salt nuclear extracts were prepared as previously described (Monahan *et al.,* 1993) and applied to charged columns. For the latter, pT7-7.1 DNA was transcribed and translated using a kit according the manufacturer's specifications (Promega Biotech, Madison, WI). Translation products from approximately 100 ng of input DNA were diluted and applied to each column containing 125 μl of charged affinity matrix. Bound proteins were eluted with sequential one-column volumes of 5 mM glutathione. Eluates were analyzed by SDS–PAGE and the radioactivity in the pol bands was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and analyzed using ImageQuant software as described previously (Monahan *et al.,* 1998).

Purification of proteins

Partially purified GST proteins were obtained by affinity chromatography on glutathione agarose as described above. Following elution with 5 mM glutathione, the proteins were dialyzed against B-2 buffer (Gallo *et al.,* 1988) and stored in aliquots at −80°C. GST and GST fusion proteins were >85% pure as determined by Coomassie blue staining of SDS–polyacrylamide gels. Purified full-length UL42 proteins were obtained by conventional chromatography of nuclear extracts of Sf-9 cells infected with UL42 recombinant baculoviruses over DEAE–Sephaphose, Blue–Sepharose, and Q–Sepharose, essentially as described previously (Gallo *et al.,* 1988; Monahan *et al.,* 1993). Purity was assessed by SDS–PAGE, followed by silver staining.

Biosensor assays

The abilities of purified wild-type and select mutant UL42 proteins to bind to a model DNA primer/template were determined by changes in surface plasmon resonance as measured with the Biacore 2000 (Phamacia Biosensor AB). Specific details of the binding of pol, UL42, and pol/UL42 complexes to this P/T are discussed elsewhere (Chaudhuri and Parris, manuscript in preparation). The sequences of the primer (45-mer) and template (67-mer) strands are shown in Table 5 and were gel-purified by the manufacturer (Integrated DNA Technologies, Inc.). The primer strand, labeled at the 5′ end with biotin by the manufacturer, was mixed with an equimolar amount of the template strand, heated to 50°C, cooled to room temperature to anneal, and stored at −20°C.

Biosensor streptavidin gold chips were used to bind 120 response units of annealed P/T. Increasing concentrations of UL42 proteins were injected over DNA-bound and control (no DNA) surfaces of the chip at a flow rate of 5 μl/min for 5–8 min as indicated in the figures. Proteins were in buffer containing 50 mM Tris–Cl, pH 7.5, 50 mM KCl, 2% glycerol, and 5 mM 2-mercaptoethanol.

Binding of protein to the chip surface results in a change in surface plasmon resonance, recorded as an increase in RU. Following the association phase, dissociation of the protein, shown as a decrease in RU, was determined by passage of the same buffer, without protein, over the surfaces of the chip. Any residual bound protein was removed by passage of the same buffer adjusted to a KCl concentration of 1 M. This regeneration procedure had no effect on the amount of P/T bound to the chip surface. Apparent kinetic constants were calculated using the BiaEvaluation 2.1 software (Pharmacia Biosensor AB) and used the simple binding model A + B = AB. A single apparent rate constant for dissociation (k_{off}) was used for each protein to calculate the apparent rate constants for association (k_{on}) at each individual concentration tested. The k_{off} used in the calculations was the rate obtained during the first 20 s of dissociation for the highest concentration tested in order to minimize any effects of rebinding which can occur when few P/T sites are occupied.

DNA polymerase assays

The activity of HSV-1 pol, prepared by coupled *in vitro* transcription/translation of 100 ng of pT7-7.1, was determined in high-salt buffer (100 μl final volume) containing 100 mM Tris–HCl (pH 8.0), 125 mM KCl, 4 mM $MgCl_2$, 5 mM dithiothreitol, 5 μg bovine serum albumin, 0.2 mM each of dATP, dCTP, and dGTP, 50 μCi [^{3}H]TTP (43–82 Ci/mmol, ICN), and either 5 μg maximally activated calf-thymus DNA or 1 μg poly dA/oligo dT_{18} (1:1) as template, essentially as described previously (Monahan *et al.,* 1993). A unit of activity was defined as the amount of enzyme required for the incorporation of 1 fmol of dTMP into DNA per hour at 37°C. Activity in the presence of various amounts of GST fusion or full-length UL42 proteins was determined by mixing the pol with the UL42 proteins for 5 min at 37°C prior to initiation of reactions with the pol/UL42 mixture. The ability of UL42 protein to stimulate pol activity was determined by comparison of pol activity in the presence and absence of UL42 and was considered significant if the pol activity in the presence of UL42 was at least twice that of pol in the absence of UL42.

Ori-dependent DNA replication assays

Subconfluent monolayers of BHK cells in 35-mm culture dishes were transfected with 0.4 μg of ori-containing plasmid pT085 (Stow and McMonagle, 1983) together with 1 μg of empty vector or test plasmid containing a wild-type or mutated UL42 gene using 5 μg lipofectase (GIBCO-BRL, Gaithersburg, MD). After 4 h at 37°C, the medium was changed and the cells were infected 12 h later with CgalΔ42 (5 PFU/cell). Cells were harvested at 24 h p.i. and total cellular DNA was extracted using the Easy-DNA kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions.

One-fourth of the DNA was cleaved with *Eco*RI (to linearize the plasmids) and *Dpn*I (to degrade input methylated plasmid DNA) and the products were separated by electrophoresis through 1% agarose gels. The DNA was denatured and transferred to Nytran (Schleicher and Schuell, Keene, NH) membranes and probed with the 516-bp *Eco*RI–*Hin*dIII fragment from pT085. The DNA probe was labeled to a specific activity of approximately 1.2×10^8 cpm/μg using an oligolabeling kit (Pharmacia Biotech). Blots were hybridized as described previously (Sambrook *et al.,* 1989) overnight at 65°C in hybridization buffer containing 6× SSC (SSC is 150 mM NaCl, 15 mM Na citrate, pH 7.0). Blots were then washed several times in 1× SSC for 15 min at room temperature and at a final stringency of 0.5× SSC containing 0.1% SDS at 65°C for 15 min. Autoradiographs were prepared by exposure of the blots to Kodak X-OMAT film. The amount of plasmid amplification was quantified with the aid of a Molecular Dynamics PhosphorImager (Sunnyvale, CA) and ImageQuant software.

Complementation analysis

The ability of mutated UL42 genes to complement the replication of the UL42 null mutant, CgalΔ42, was determined as described previously (Reddig *et al.,* 1994). Replicate cultures of BHK cells were transfected with 2 μg of test plasmid followed by infection with CgalΔ42 (2.5 PFU/cell). After a 1-h period, unadsorbed virus was removed with a 2-min acid–glycine wash (Reddig *et al.,* 1994). At 18 h p.i., combined intracellular and extracellular virus was prepared by sonic disruption of cells. The yield of progeny in each sample was determined by plaque assay in V9 cells, permissive for CgalΔ42 replication, and in Vero cells, which are nonpermissive for replication of the virus. For these experiments, the complementation index (CI) for a particular plasmid refers to the percentage of the yield of progeny virus produced by the plasmid containing wild-type UL42 and was calculated as described in Table 4. Two to seven independent experiments were performed for each test plasmid with the exception of d270. Complementation was scored as ++ if the CI was >25, + if the CI was between 1 and 25, and − if it was <1. The percentage of recombinants in the samples was determined by the ratio of PFU scored on Vero cells compared to that scored on V9 cells. Because the relative production of recombinants never exceeded 0.24% of the progeny in any experiment, no correction for recombination was needed.

Indirect immunofluorescence

The localization of the mutant UL42 proteins was determined 24 h following transfection of Vero cells with 2

μg of the appropriate plasmid by the $CaPO_4$ technique essentially as described previously (Goodrich *et al.,* 1989). Briefly, UL42 was detected in acetone-permeabilized cells using the UL42-specific anti-peptide antibody 834 (Monahan *et al.,* 1993) as the primary antibody and fluorescein-isothiocyanate-conjugated goat anti-rabbit IgG as the secondary antibody. Immunofluorescence was viewed using the Zeiss Photomicroscope III equipped with a 25X Neofluar objective.

ACKNOWLEDGMENTS

We thank Jeff Henderson for constructing and sequencing the UL42 mutations, d270 and d256-282, Bob Lehman for the gift of pol-expressing recombinant baculovirus, Nigel Stow for the T085 plasmid, and members of the Parris laboratory for many helpful discussion. We are grateful for the expert statistical analysis supplied by Donn Young within the Biostatistics Core of the Comprehensive Cancer Center. This work was supported in part by Grant GM34930 from the National Institutes of Health. During part of this work, SJM was supported by Postdoctoral Fellowship F32 AI08566 and KET by Predoctoral Fellowship F31 GM15019 from the National Institutes of Health. Support services were provided by the Ohio State University Department of Molecular Virology, Immunology, and Medical Genetics and by the Comprehensive Cancer Center Core Grant from the National Cancer Institute (P30 CA16058).

REFERENCES

Challberg, M. D. (1991). Herpes simplex virus DNA replication. *Semin. Virol.* **2,** 247–256.

Chiou, H. C., Weller, S. K., and Coen, D. M. (1985). Mutations in the herpes simplex virus major DNA-binding protein gene leading to altered sensitivity to DNA polymerase inhibitors. *Virology* **145,** 213–226.

Chow, C. S., and Coen, D. M. (1995). Mutations that specifically impair the DNA binding activity of the herpes simplex virus protein UL42. *J. Virol.* **69,** 6965–6971.

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

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

Dorsky, D. I., and Crumpacker, C. S. (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.

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

Gallo, M. L., Jackwood, D. H., Murphy, M., Marsden, H. S., and Parris, D. S. (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.

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

Goodrich, L. D., Rixon, F. J., and Parris, D. S. (1989). Kinetics of expression of the gene encoding the 65-kilodalton DNA-binding protein of herpes simplex virus type 1. *J. Virol.* **63,** 137–147.

Gottlieb, J., and Challberg, M. D. (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., Marcy, A. I., Coen, D. M., and Challberg, M. D. (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.

Haffey, M. L., Stevens, J. T., Terry, B. J., Dorsky, D. I., Crumpacker, C. S., Wietstock, S. M., Ruyechan, W. T., and Field, A. K. (1988). Expression of herpes simplex virus type 1 DNA polymerase in *Saccharomyces cerevisiae* and detection of virus-specific enzyme activity in cell-free lysates. *J. Virol.* **62,** 4493–4498.

Hamatake, R. K., Bifano, M., Tenney, D. J., Hurlburt, W. W., and Cordingley, M. G. (1993). The herpes simplex virus type 1 DNA polymerase accessory protein, UL42, contains a functional protease-resistant domain. *J. Gen. Virol.* **74,** 2181–2189.

Haruki, M., Noguchi, E., Kanaya, S., and Crouch, J. (1997). Kinetic and stoichiometric analysis for the binding of *Escherichia coli* ribonuclease H1 to RNA–DNA hybrids using surface plasmon resonance. *J. Biol. Chem.* **272,** 22015–22022.

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

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77,** 51–59.

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

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

Johnson, P. A., Best, M. G., Friedman, T., and Parris, D. S. (1991). 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.

Karlsson, R., Michaelsson, A., and Mattson, L. (1991). Kinetic analysis of monoclonal antibody–antigen interactions with a new biosensor based analytical system. *J. Immunol. Methods* **145,** 229–240.

Kiehl, A., and Dorsky, D. I. (1995). Bipartite DNA-binding region of the Epstein–Barr virus BMRF1 product essential for DNA polymerase accessory function. *J. Virol.* **69,** 1669–1677.

Marchetti, M. E., Smith, C. A., and Schaffer, P. A. (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.

Marcy, A. I., Olivo, P. D., Challberg, M. D., and Coen, D. M. (1990). Enzymatic activities of overexpressed herpes simplex virus DNA polymerase purified from recombinant baculovirus-infected cells. *Nucleic Acids Res.* **18,** 1207–1215.

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

Marsden, H. S., McLean, G. W., Barnard, E. C., Francis, G. J., MacEachran, K., Murphy, M., McVey, G., Cross, A., Abbotts, A. P., and Stow, N. D. (1997). The catalytic subunit of the DNA polymerase of herpes simplex virus type 1 interacts specifically with the C terminus of the UL8 component of the viral helicase-primase complex. *J. Virol.* **71,** 6390–6397.

Monahan, S. J., Barlam, T. F., Crumpacker, C. S., and Parris, D. S. (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.* **67,** 5922–5931.

Monahan, S. J., Grinstead, L. A., Olivieri, W., and Parris, D. S. (1998). Interaction between the herpes simplex virus type 1 origin-binding protein and DNA polymerase accessory proteins. *Virology* **241,** 122–130.

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

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

Reddig, P. J., Grinstead, L. A., Monahan, S. J., Johnson, P. A., and Parris, D. S. (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 Weir, A. C. (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.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning. A Laboratory Manual," Second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

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., and McMonagle, E. C. (1983). Characterization of the TRS/IRS origin of DNA replication of herpes simplex virus type 1. *Virology* **130,** 427–438.

Tabor, S., Huber, H., and Richardson, C. C. (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., Hurlburt, W. W., Micheletti, P. A., Bifano, M., Hamatake, R. K., and Cordingley, M. G. (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.

Tsurumi, T., Daikoku, T., Kurachi, R., and Nishiyama, Y. (1993). Functional interaction between Epstein–Barr virus DNA polymerase catalytic subunit and its accessory subunit in vitro. *J. Virol.* **67,** 7648–7653.

Vaughan, P. J., Banks, L. M., Purifoy, D. J. M., and Powell, K. L. (1984). Interactions between herpes simplex virus DNA-binding proteins. *J. Gen. Virol.* **65,** 2033–2041.

Weller, S. K. (1991). Genetic analysis of HSV genes required for genome replication . *In* "Herpesvirus Transcription and Its Regulation" (E. K. Wagner, Ed.), pp. 105–135. CRC Press, Boca Raton, FL.

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