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Effects of Substitutions of Arginine Residues on the Basic Surface of Herpes Simplex Virus UL42 Support a Role for DNA Binding in Processive DNA Synthesis

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The way that UL42, the processivity subunit of the herpes simplex virus DNA polymerase, interacts with DNA and promotes processivity remains unclear. A positively charged face of UL42 has been proposed to participate in electrostatic interactions with DNA that would tether the polymerase to a template without preventing its translocation via DNA sliding. An alternative model proposes that DNA binding by UL42 is not important for processivity. To investigate these issues, we substituted alanine for each of four conserved arginine residues on the positively charged surface. Each single substitution decreased the DNA binding affinity of UL42, with 14- to 30-fold increases in apparent dissociation constants. The mutant proteins exhibited no meaningful change in affinity for binding to the C terminus of the catalytic subunit of the polymerase, indicating that the substitutions exert a specific effect on DNA binding. The substitutions decreased UL42-mediated long-chain DNA synthesis by the polymerase in the same rank order in which they affected DNA binding, consistent with a role for DNA binding in polymerase processivity. Combining these substitutions decreased DNA binding further and impaired the complementation of a *UL42* null virus in transfected cells. Additionally, using a revised mathematical model to analyze rates of dissociation of UL42 from DNAs of various lengths, we found that dissociation from internal sites, which would be the most important for tethering the polymerase, was relatively slow, even at ionic strengths that permit processive DNA synthesis by the holoenzyme. These data provide evidence that the basic surface of UL42 interacts with DNA and support a model in which DNA binding by UL42 is important for processive DNA synthesis.

Replicative DNA polymerases are highly processive enzymes that synthesize long stretches of DNA without dissociating from the template. Many replicative polymerases depend on accessory proteins called processivity factors, which confer processivity on their cognate polymerases by slowing their dissociation from DNA. Among these are the "sliding clamp" processivity factors (18), exemplified by proliferating cell nuclear antigen (PCNA), the processivity factor for eukaryotic replicative DNA polymerases. All sliding clamps adopt a common structure, in which a dimer or trimer of the sliding clamp protein forms a ring that encircles DNA (20, 21, 23, 28). The association of the sliding clamps with DNA requires the activity of accessory proteins known as clamp loaders (19), which open the clamp ring and reclose it on DNA in an ATP-dependent reaction. The sliding clamp then can tether the catalytic polymerase core to DNA, thus ensuring processivity without impeding the movement of the polymerase.

The DNA polymerase that replicates herpes simplex virus (HSV) is a heterodimer of a 137-kDa catalytic subunit (UL30, also called Pol) and a 60-kDa processivity subunit, UL42 (15,

24, 25). The extreme C terminus of Pol is necessary for interaction with UL42, long-chain DNA synthesis, and viral replication (9, 22, 29, 30); in turn, peptides corresponding to this portion of Pol are sufficient for specific interaction with UL42 (2, 36). In contrast to the sliding clamps, UL42 binds DNA as a monomer with high affinity and requires neither ATP nor accessory proteins for its productive association with DNA (12, 14, 15, 26, 33). UL42 increases the affinity of the polymerase for primer/template DNA 10- to 20-fold, primarily due to a decrease in the dissociation rate (14, 33). A four-amino-acid insertion at residue 203 or 206 of UL42 abrogates DNA binding without affecting binding to Pol (7). Each of these insertions prevents the stimulation of long-chain DNA synthesis by UL42, suggesting that the processivity of the HSV DNA polymerase depends on the affinity of UL42 for DNA (7).

These observations have been used to support a hypothesis (15) that UL42 tethers Pol to DNA via its direct DNA binding activity. This raised a paradox in that the binding of UL42 to DNA would be expected to impede polymerase translocation. However, despite the increased affinity of holoenzyme for DNA relative to that of Pol, the two forms of the polymerase translocate on DNA at similar rates, demonstrating that direct DNA binding by UL42 does not slow the translocation rate of Pol (33). A resolution of the paradox was offered by the observation that UL42 can diffuse (slide) on DNA (27). A possible explanation for the way UL42 could slide along DNA while maintaining a high-affinity interaction comes from a crys-

tallographic analysis of UL42 (36). This study revealed a large basic face on UL42 on the side of the molecule opposite from the Pol binding site, which was proposed to provide an electrostatic "tractor field" that could slide along the phosphate backbone of DNA (36). Several positively charged residues on this face are conserved among alphaherpesvirus homologs of UL42, and a docking model placed this basic region directly across the region of double-stranded DNA (dsDNA) located immediately upstream from Pol (36). While the four-amino-acid insertions described above lie outside of this basic region, these insertions may distort the structure of the basic face of UL42, without affecting Pol binding. Regardless, if the basic surface of UL42 is important for DNA binding, one would predict that the substitution of uncharged residues for basic residues would decrease the UL42-DNA interaction.

An alternative to the hypothesis that UL42 tethers Pol to DNA has also been advanced based on measurements of binding affinities to and rates of dissociation from short primer-templates at various ionic strengths. The values obtained, especially at higher ionic strengths, implied rather weak binding that appeared to be inconsistent with tethering, leading to the proposal that an interaction between Pol and UL42 results in a conformational change that allows UL42 and Pol to wrap around DNA together (4).

To address the hypothesis that the basic surface of UL42 is important for DNA binding, we have constructed mutants in which each of four conserved arginines that contribute to the overall positive charge of this surface was substituted with alanine. We tested the effects of these substitutions on the ability of UL42 to bind the C terminus of Pol and to bind DNA. Because we found that the substitutions exerted various effects on DNA binding without affecting binding to Pol, we were also able to correlate the effects of the mutations on DNA binding and long-chain DNA synthesis, a measure of processivity. We also used a revised mathematical model to assess the rate of dissociation of UL42 from an internal site on DNA at ionic strengths that promote processive DNA synthesis by the holoenzyme. The results of these studies allowed us to address the hypothesis that the binding of UL42 to DNA is important for its function in processivity.

MATERIALS AND METHODS

Construction of mutants. The R113A, R182A, R279A, R280A, and R279A/R280A mutants were constructed by a two-step PCR method (8). To create each mutation, each of two complementary internal primers containing the altered codon(s) was paired with an outside primer to amplify a segment of the UL42 open reading frame within pMBP-PP-UL42ΔC340 (a gift of Hong-ming Chen and James Hogle). pMBP-PP-UL42ΔC340 encodes a fusion of maltose-binding protein (MBP) and a truncated form of UL42 that retains biochemical and biological activity (2, 10), with an intervening cleavage site for PreScission protease. All oligonucleotides were obtained from Integrated DNA Technologies; the sequences of these primers are given in supplementary Table 1, available online at http://coen.med.harvard.edu. Reactions used Vent DNA polymerase in ThermoPol buffer (both from New England Biolabs), 200 μM deoxynucleoside triphosphates, 4 mM MgSO₄ (including 2 mM MgSO₄ supplied in ThermoPol buffer), 5% dimethyl sulfoxide, 200 pmol of each primer, and 20 fmol template DNA. PCR products were separated by electrophoresis on a 4% 3:1 NuSieve gel and gel purified using a QiaQuick gel extraction kit (QIAGEN). The two products of the first round of PCR were combined in one tube and used as a template for a second round of PCR using the outside primers. The products of these reactions were then cloned into the following gel-purified fragments of pMBP-PP-UL42ΔC340: BamHI/NheI (R113A), NheI/SphI (R182A), or SphI/HindIII

(R279A, R280A, R279A/R280A). All constructs were sequenced to confirm the presence of the mutation(s) and the absence of undesired mutations.

The R113A/R182A double mutant was constructed by excising fragments containing the R113A and R182A mutations from sequence-confirmed mutant plasmids using the BamHI and NheI or NheI and SphI restriction enzymes, respectively. These two fragments were then ligated into a BamHI-SphI vector fragment and transformed as before. Similarly, the R113A/R182A/R279A/R280A quadruple mutant was constructed by cloning the R113A and R182A fragments into a BamHI-SphI vector fragment derived from the R279A/R280A plasmid.

For transient complementation assays, a 5.1-kbp BamHI-EcoRI fragment including *UL42* was isolated from viral DNA and cloned into pGEM7Zf(+) to generate pBE5.1. From this plasmid, a 1.1-kbp SacII fragment, containing portions of *UL43* and *UL44*, was deleted, resulting in pHC700, which served as the wild type (wt). The quadruple mutant form of this plasmid was derived by deleting a 737-bp PstI fragment and replacing it with the corresponding fragments from the quadruple mutant plasmid described above. The mutant derivative was sequenced to confirm the presence of the desired mutations and no others.

Purification of proteins. Baculovirus-expressed HSV Pol, purified as previously described (34), was generously provided by K. Kumura-Ishii.

Wild-type and mutant UL42Δ340 proteins (which from here on will be referred to as UL42) were expressed in and purified from *Escherichia coli* BL21(DE3)pLysS (Novagen) as described previously (2), except that a 1-ml HiTrap heparin column (Amersham) was used in place of a DNA column for increased reproducibility and convenience. In addition, the protein was eluted from the heparin column using a 10- to 500-mM NaCl gradient in elution buffer rather than being batch eluted with 500 mM NaCl.

The mutant proteins containing two or four arginine-to-alanine substitutions did not bind to the heparin column (data not shown), necessitating an alternate method of purification (see Results). These mutant proteins were therefore purified as a complex with a peptide corresponding to the C-terminal 36 residues of Pol (peptide A) using a variation of the protocol described in reference 36. Briefly, *E. coli* BL21(DE3)pLysS cells expressing a given UL42 mutant and cells expressing a glutathione *S*-transferase (GST)-peptide A fusion were grown as described in reference 36. These cells were mixed and lysed by freezing/thawing and sonication, and the MBP-UL42:GST-peptide A complex was purified on a glutathione-Sepharose column (Amersham) as described in reference 36. The MBP and GST fusion partners were removed by PreScission protease (36). Because these mutants did not bind to either heparin or DNA columns, the DNA column used in a second purification step by Zuccola et al. (36) was omitted. The final Q Sepharose column used in this protocol was found to be sufficient to purify the UL42:peptide A complex free of both fusion partners, the PreScission protease, and uncleaved MBP-UL42:GST-peptide A complex.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays (EMSAs) were performed as described previously (27), but using the temperatures, salt concentrations, and amounts of protein and DNA indicated herein. The 30-bp DNA was formed by annealing oligonucleotide 5′-AGCTAGCTAC AGTCAGCTGACTACAGCTAG (Integrated DNA Technologies) and its complement and was radiolabeled and gel purified as previously described (27). The sequences and preparation of the longer DNAs have been described previously (27). For the determination of apparent K_d values, the amounts of bound and free DNA probe were quantified by phosphorimaging, and these values were fit with ProFit software (QuantumSoft) to the equation $[PD] = [PD]_{max}[D]/(K_d + [D])$, where $[PD]$ is the concentration of bound DNA in nM and $[D]$ is the concentration of free DNA in nM. The rates of dissociation of UL42 from different lengths of DNA were determined as described previously (27), except that binding was at 37°C and the binding buffer contained either 50 mM ammonium sulfate or 75 mM NaCl. The half-life of UL42 on each length of DNA in each condition was measured two to six times, and the mean values obtained were used to determine $k_{off(ends)}$ and $k_{off(internal)}$ (k_{off} is the rate of dissociation.) and sliding rates.

Isothermal titration calorimetry. Isothermal titration calorimetry (ITC) experiments were performed as described in reference 2 by using UL42 concentrations of 5.6 to 7.9 μM and peptide concentrations of 130 to 380 μM.

Assays of UL42-mediated long chain DNA synthesis. Polymerase assays were performed as described previously (2, 7, 16) using 10 μg/ml poly(dA)/oligo(dT) template and 50 μM [α-³²P]dTTP (5 Ci/mmol). Reactions used 200 fmol Pol and 800 or 1,200 fmol UL42. Reaction products were analyzed on 4% alkaline agarose gels, and the incorporation of radiolabeled dTTP into products longer than 18 b was quantified using a phosphorimager.

Complementation assay. *UL42* plasmids were tested for their ability to complement a *UL42* null mutant virus (CgalΔ42, a generous gift of P. Johnson and

TABLE 1. Summary of UL42 mutants

Mutation	K_d for peptide A (μM)[a]	Apparent K_d for DNA (nM)[b]	Stimulation of long-chain DNA synthesis ($\pm$ SD)[c]
None (wt)	1.5 $\pm$ 1.6	3.6	1
R113A	1.7 $\pm$ 0.03	80	0.19 $\pm$ 0.05
R182A	2.2 $\pm$ 0.38	51	0.38 $\pm$ 0.05
R279A	1.5 $\pm$ 0.09	86	0.22 $\pm$ 0.03
R280A	1.4 $\pm$ 0.69	110	0.074 $\pm$ 0.02

[a] From ITC (Fig. 3).
[b] From EMSAs (Fig. 2).
[c] Data from three sets of measurements, including those shown in Fig. 4, were quantified and averaged and are presented relative to the value obtained for wt UL42, which is set as 1.

D. Parris) in a transient transfection assay as described previously (7). Briefly, each plasmid (pHC700 and the quadruple form of this plasmid) was transfected into Vero cells, which were subsequently infected with the null mutant virus. The titers of progeny virus were determined on the permissive cell line, V9 (kindly provided by P. Johnson and D. Parris), or on V42.3 cells, which contain the pBE5.1 plasmid (C. Jiang and C. B. C. Hwang, unpublished results), and also on Vero cells to check for recombinants. The percent complementation of the mutant plasmid was calculated as [(titer on V9 or V42.3 − titer on Vero)$_{\text{mutant}}$/titer on V9 or V42.3 − titer on Vero)$_{\text{wt}}$] × 100%.

Calculation of $k_{\text{off(ends)}}$ and $k_{\text{off(internal)}}$ and the sliding rate of UL42. The rate at which UL42 dissociates from DNA [$k_{\text{off(observed)}}$] is the sum of two rates (27): the rate at which UL42 diffuses (slides) to a DNA terminus and falls off [$k_{\text{off(ends)}}$], and the rate at which it dissociates from internal sites [$k_{\text{off(internal)}}$]. The sliding of UL42 to a DNA terminus is analogous to the case of a particle traveling on a finite, linear path. The average time required for such a particle to reach one end of the path via a random walk is $b^2/12D$, where b is the length of the path (in unit steps of the random walk) and D is the diffusion coefficient (1). Dividing D by the square of the step length yields the number of base pairs covered per unit of time, i.e., the sliding rate (35). Thus, assuming that the step length of UL42 is 1 base pair, D is equivalent to the sliding rate of UL42. For UL42, then, $k_{\text{off(ends)}}$ is simply the inverse of the average time of this random walk, so $k_{\text{off(ends)}} = 12D/b^2$. Adding $k_{\text{off(internal)}}$ and $k_{\text{off(ends)}}$ and applying the relationship $t_{1/2} = \ln2/k_{\text{off}}$ yields the result that, for any linear DNA of length b, $t_{1/2} = \ln2/(12D/b^2 + k_{\text{off}})$. For each DNA, $t_{1/2}$s were calculated as previously described (27), the data were fit to the equation, and the values for D and $k_{\text{off(internal)}}$ were obtained using MacCurveFit software (Kevin Raner Software).

RESULTS

Construction and purification of UL42 mutants. If UL42 binds to DNA via its positively charged face, then reducing the amount of positive charge should reduce DNA binding. Zuccola et al. (36) identified four arginines (residues 113, 182, 279, and 280) that are located on this surface of UL42 and conserved among alphaherpesviral homologs of UL42. These residues are highlighted in a sequence alignment of UL42 and its homologs from certain equine, bovine, porcine, and human alphaherpesviruses (Fig. 1A). While the basic charge at positions 279 and 280 of HSV UL42 is not strictly conserved, two of the three amino acids at positions homologous to UL42 residues 279, 280, and 282 carry a positive charge in other alphaherpesviruses, with the third residue being a glutamine in all cases. The positions of these conserved residues in the model of Zuccola et al. (36) are shown in Fig. 1B.

We substituted each of the codons for these arginines (Arg113, 182, 279, and 280) with an alanine codon and introduced each of these mutations singly into an expression vector for the MBP-UL42ΔC340 fusion protein. (This fusion protein will be referred to as UL42 below. UL42ΔC340 is a truncated form of UL42 that retains biological and biochemical activity [2, 10]. A truncation that removes even more residues does not

affect viral replication in cell culture [13].) A substantial portion of the UL42 produced in bacteria is inactive, and the inactive fraction must be removed prior to biochemical analysis (reference 36; K.G. Bridges, J. C. W. Randell, and D. M. Coen, unpublished data). For experiments reported here examining the effects of single substitutions, mutant UL42 proteins were purified using successive amylose and heparin columns. Importantly, inactive UL42 fails to bind to heparin (which mimics DNA via its negative charge) and thus is not retained on the affinity column.

Effects of single UL42 substitutions on binding to DNA. We first measured the relative affinities of wild-type and singly substituted UL42 proteins for a 30-bp dsDNA by measuring the amount of DNA bound by UL42 over a range of DNA concentrations in mobility shift assays. The amounts of free and bound DNA at each DNA concentration were quantified and plotted (Fig. 2). The plots were fit to the equation [PD] = [PD]$_{\text{max}}$[D]/(K_d + [D]), where [PD] is the concentration of bound DNA in nM and [D] is the concentration of free DNA in nM, to determine an apparent K_d for each wild-type and mutant protein. The apparent K_d of the wild-type protein for binding to DNA was 3.6 nM, similar to that determined previously for an ~30-bp DNA (33). The mutant proteins yielded apparent K_d values that were 14- to 30-fold higher than that of wt UL42 (Table 1). The R182A mutant had the smallest defect in DNA binding, while the R280A mutant had the greatest defect, with faint but quantifiable bound DNA detectable when relatively high concentrations of UL42 were present (Fig. 2). The R113A and R279A mutants each showed an intermediate defect in DNA binding. Thus, each substitution of alanine for a conserved arginine residue on the basic face of UL42 affected DNA binding.

The single UL42 mutations do not affect binding to the Pol C terminus. To establish that the effects of the mutations on DNA binding were not due to global changes in protein structure, we used ITC to measure the affinity of wt and the UL42 mutants for peptide A, which corresponds to the C-terminal 36 residues of Pol (Fig. 3 and Table 1). For wt protein, the K_d, of 1.5 $\pm$ 1.6 μM (Table 1) was in good agreement with the value obtained previously by Bridges et al. (1.4 $\pm$ 0.8 μM) (2). All of the UL42 mutants tested possessed K_ds for peptide A very similar to that of wt protein (Fig. 3 and Table 1), indicating that the mutations do not quantitatively affect the binding of UL42 to the C terminus of Pol and therefore do not globally affect the structure of UL42.

Effect of UL42 mutations on long-chain DNA synthesis by Pol. We next asked whether the magnitude of the defects in DNA binding by the UL42 mutant proteins correlated with defects in processive DNA synthesis by the polymerase holoenzyme. If DNA binding contributes to processivity, then the degree of each mutation's effect on DNA binding should be reflected in the degree to which the processivity of the Pol/UL42 holoenzyme is reduced. We tested the ability of the wt and the single-mutant proteins to stimulate long-chain DNA synthesis by Pol, a measure of holoenzyme processivity, by measuring the incorporation of radionucleotides on an oligo(dT)-primed poly(dA) template after incubation with the various Pol:UL42 complexes. As previously observed (2, 7, 16), only short products were detectable after the incubation of this template with 200 fmol Pol in the absence of UL42 (Fig. 4, lane

A

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PRV  MSLFDDGLEDLDRHPTHAHHPAQVIHDGPFVLEDGEPLQRTGMLVLSDEHLEHARAAIAPLAAHLAHAFLVFSEAGLLVHASVRGEQVYVTLAPDQFSTFVWS----GPQAVFLGNVDGS  116
BHV  -------MLQPPSHLRADEGEARLTAG----LSDGGNGNPIASASLIGRQLADVSALLSPFGASLRNAFLVFSREGMLVHSGLYDEQVYVAVPAEKFTSFQWAPARDDARAVFLANVDSR  109
EHV  --------MALPRAMRPGGSHPNNFLFNTLPVIDNPVERQRAMAVFERESLRDAFEMLTPIAPSLKNAFLIFNEDGLLIHTSVGGEQVYIPIQTNNMESYSWK---KAPPAVFLANVDGR  109
HSV  -------MTDSPGGVAPASPVEDASDASLGQPEEGAP----CQVVLQGAELNGILQAFAPLRTSLLDSLLVMGDRGILIHNTIFGEQVFLPLEHSQFSRYRWR----GPTAAFLSLVDQK  105

PRV  GGVLDALKVDRRRTVFNVTFEVYGAFPARLLTRRAYFA--------DAGVLATGPGSPSVACVYKHEFNDYCIMLPSRAPDVSLTLSRPQVAKLA-AVAKGAAAGTTFALARGLDFSVSS  227
BHV  RGLLDAFRADKTRTVQNVAFLITGEPPASVLTQVTYFR--------EADALLQAS-------LVKHELGEYSIMLPTRGADLAVSLSRPQLSKLQ-AVAKDPNEAVVFAYRRARCLSAES  213
EHV  RGLLDAFKAKTQTNVSKVVFEIENYSPSRILTQTVFSARDQTEEDTEMGSDAEGATQTVSSRLVKHEFNNYALMLPTRQPDVSMSLSKAQLNKIL-GVCKQAGDPITFQCLFDDTLQVRS  228
HSV  RSLLSVFRANQYPDLRRVELAITGQAPFRTLVQRIWTTTS------DGEAVELAS-----ETLMKRELTSFVVLVPQGTPDVQLRLTRPQLTKVLNATGADSATPTTFELGVNGKFSVFT  214

PRV  SAGVVTFPARDRDG------------TAVLERASQRRQG--------VDAVGATEPFAMTLETAHGLLTLLQRLRAGNAELTFNFFTTPRQAPLFSVTTCGPVRATTFFFCAP------  320
BHV  ASGRASFAARHEGGESGAELQLALAPSAGSERIFARAQGGGRRAALLEVRCSGGAPDFRLELESPSGFRRLLQKVRQVGGDALVRFYLAPASAAMMSVSTAAPEGLTVFFFCRP------  327
EHV  GDRQVVFSVDYQHADNCG----VESSSSLLEKMPMKTKKSAPEP----IRGISGRRLFTLVLDEDTNFKQLIQKLKLKNAGAVLNFFLDPDSIPMIGLSTKQPFSVMMFFMCSY------  334
HSV  TSTCVTFAAREEGVSSST-------STQVQILSNALTKAGQAAANAKTVYGENTHRTFSVVVDDCS-MRAVLRRLQVGGG---TLKFFLTTP-VPSLCVTATGPNAVSAVFLLKPQKICLD  323

PRV  --------ADPATVPAAPEGAAATVA-------AACGAGA--------------SAAPAAGDKRPAAPRMYTP--IAKRPRAASGEGGHAYGDLF-------------------------  384
BHV  --------ATATASADDADDCAEPAPRLPAKRPAASGQPR--------RGAGAGIQTDGAHPVAVSGGRARKRPRADAGAPGPSSGPDRGGEGGETP----------------  408
EHV  --------PTQPCQVGFSPAAFSSTP-------MGAGVKR-------------RASEEEESDQPPKKLFPDGKLFKSNFVLLMDKTGAKIPCPEQPMHF------------------  405
HSV  WLGHSQGSPSAGSSASRASGSEPTDSQDSASDDAVSHGDPEDLDGAARAGEAGALHACPMPSSTTRVTPTTKRGRSGGEDARADTALKKPKTGSPTAPPPADPVPLDTEDDSDAADGTAAR  443

PRV  -----------------------------------------
BHV  -----------------------------------------
EHV  -----------------------------------------
HSV  PAAPDARSGSRYACYFRDLPTGEASPGAFSAFRGGPQTPYGFGFP  488
```
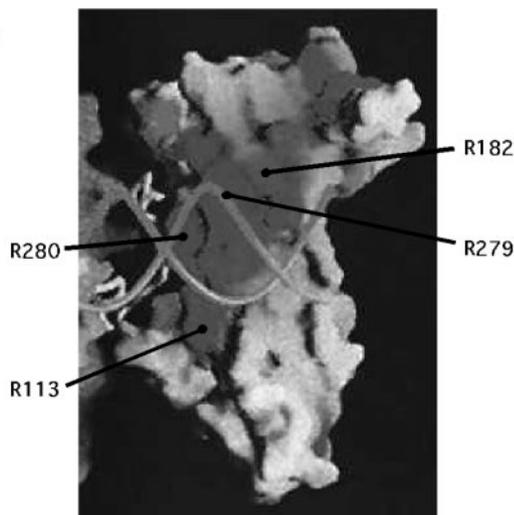
B

FIG. 1. Location of conserved basic residues of UL42. (A) Alignment of alphaherpesvirus UL42 homologs showing conserved basic residues predicted to mediate DNA binding. Amino acid sequences of HSV UL42 homologs from pseudorabiesvirus (PRV), bovine herpesvirus type 1 (BHV), equine herpesvirus (EHV), and HSV were aligned using CLUSTAL. Shaded boxes indicate positions predicted by a previous study (36) to be involved in DNA binding. (B) Space-filling diagram of back face of UL42 with hypothetical relationship to DNA backbone (modified from reference 36). Locations of conserved basic residues are labeled.

1). No extension of the oligo(dT) primer was seen when only UL42 was included in the reaction (lane 2). Long-chain DNA synthesis was seen only when both Pol and wt UL42 were included in the reaction (lanes 3 and 4). Furthermore, this long-chain synthesis by Pol/UL42 was reduced by all of the single-amino-acid changes studied (Fig. 4, lanes 5 to 12; Table 1). Of the four mutations, the R280A mutation had the greatest effect on long-chain DNA synthesis, while the R182A mutation had the least effect. The order of the mutants' effects on long-chain DNA synthesis (R280A > R279A ≅ R113A > R182A) was identical to the order of their effects on DNA binding, suggesting that the affinity of UL42 for DNA governs the stimulation of long-chain DNA synthesis by the polymerase holoenzyme.

Cumulative effects of UL42 mutations. Each single arginine-to-alanine substitution on the basic face of UL42 had a detrimental effect on DNA binding, yet even the most detrimental mutation increased the apparent K_d by a factor of only 30, so that the apparent K_d is still submicromolar. That each substitution had a measurable but limited effect on DNA binding is consistent with DNA binding being mediated by a diffuse region of positive charge rather than by one or two residues. This model predicts that a further reduction in the overall charge of this surface would further reduce the affinity of UL42 for DNA. We thus purified mutant proteins containing both of the R113A and R182A substitutions, both of the R279A and R280A substitutions, or all four arginine-to-alanine substitutions.

We initially attempted to purify these mutant proteins by the protocol used for the single mutants. However, the multiply substituted proteins did not bind to the heparin column under the conditions used for these purifications. This provided the first indication that these mutants were more defective for DNA binding than those bearing only one arginine-to-alanine substitution were. We then turned to an alternate affinity purification method to purify these proteins. Wild-type and mutant UL42 proteins were purified using a column consisting of a GST-peptide A fusion bound to glutathione-Sepharose by using a variation of a protocol previously used to purify the UL42-peptide A complex prior to crystallization (36). UL42 proteins are eluted from this column as a complex with peptide A, and this complex is further purified by ion-exchange chromatography. Similar to what is seen in purification by DNA or heparin affinity chromatography, the inactive portion of the UL42 preparation (typically ~80% [data not shown]) does not bind to the immobilized peptide A and is therefore removed. The ability of the UL42 mutants to bind immobilized peptide A during purification provided a qualitative assay for Pol binding by the UL42 mutants bearing more than one substitution. All of the mutant proteins (and wt) could be purified by this protocol (Fig. 5A), suggesting that the mutations do not grossly affect the overall conformation of the protein.
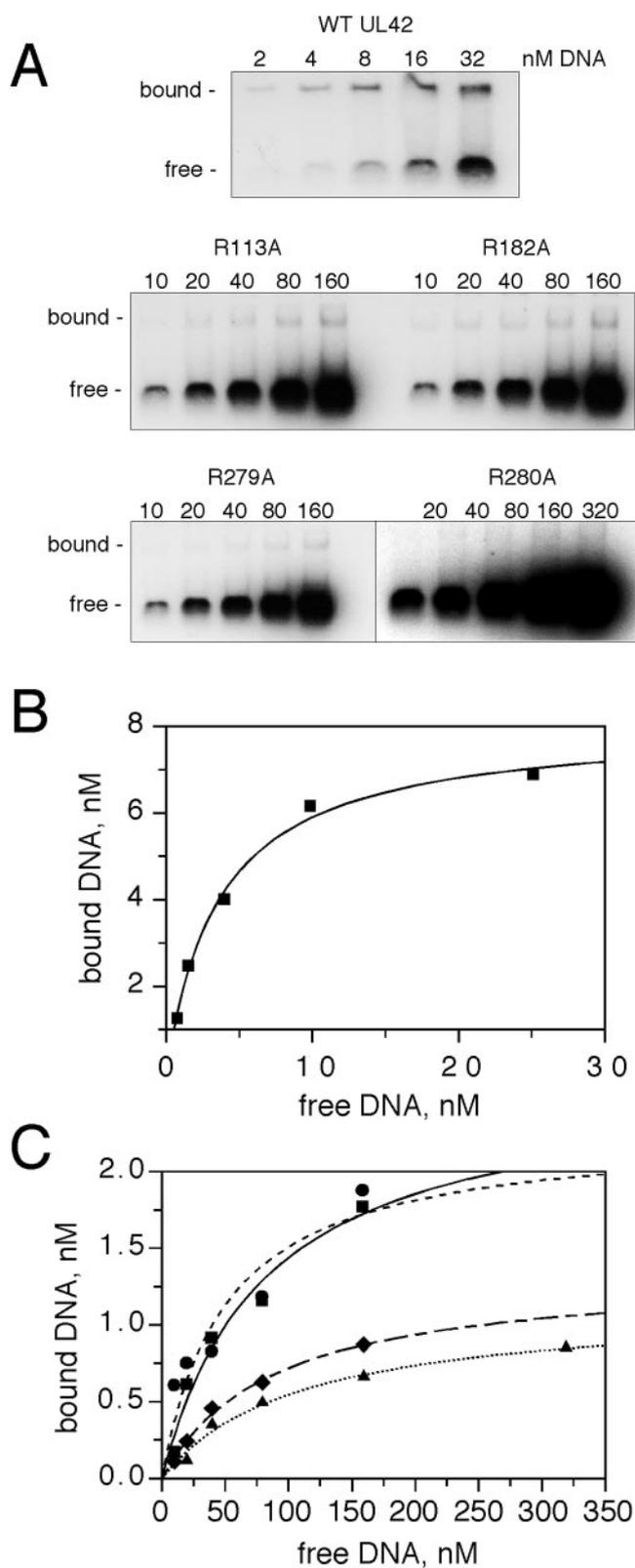
We then investigated whether the mutants bearing multiple

arginine-to-alanine changes were more defective for DNA binding than the single mutants. We tested the purified UL42-peptide A complexes for DNA binding using a mobility shift assay. Under the conditions used, which differ from those used for Fig. 2, wt protein bound to the probe such that two shifted complexes formed: C1, corresponding to one UL42 monomer bound per DNA, and C2, corresponding to two UL42 monomers bound (26). Consistent with the results shown in Fig. 2, each of the single-point mutations affected DNA binding (Fig. 5B). Again, of these mutations, the R113A and R182A mutations had the least effect on DNA binding (Fig. 5B, lanes 3 and 4), while the R279A and R280A mutations had the greatest effect (Fig. 5B, lanes 5 and 6). Furthermore, the R280A mutant was the most deficient for DNA binding, as no complex with DNA could be detected at the concentrations of protein and DNA used, even upon extreme overexposure of the gel (data not shown). The R113A/R182A double mutant was more defective for DNA binding than either the R113A or the R182A single mutant, as no binding of the R113A/R182A mutant could be detected in this assay (Fig. 5B, lane 7). This result indicates that the effects of these mutations are indeed cumulative. Similarly, no DNA binding could be detected by this assay with the other two mutants bearing multiple arginine to alanine mutations (lanes 8 and 9).

While wt and these UL42 mutants purified as a complex with peptide A can be used in DNA binding assays, the affinities of these UL42 preparations for peptide A cannot be determined without first removing the copurifying peptide. Attempts to remove the peptide by dialysis were unsuccessful (data not shown). As a result, we were not able to determine the affinity of the multiply substituted mutant proteins for the peptide or to test them for stimulation of long-chain DNA synthesis by Pol. We did, however, assess the effect of the quadruple substitution on complementation of a *UL42* null mutant virus in transiently transfected cells. The mutant was substantially impaired for complementation, permitting only 17% ± 8% of the yield of virus relative to that obtained when a wt *UL42* gene was transfected.

Dissociation rates of UL42 at ionic strengths that permit processive DNA synthesis. The strength of the interaction of UL42 with DNA and the way that UL42 dissociates from DNA are important factors for both the tethering and the alternative models for the way that UL42 promotes processivity. As pointed out by Chaudhuri and Parris (4), certain previous measurements (14, 33) of the affinity of UL42 for DNA were performed at low ionic strength (e.g., 10 to 50 mM NaCl) at room temperature. These conditions do not permit processive DNA synthesis by Pol/UL42 holoenzyme. Similarly, we previ-

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of DNA binding by these mutants). Bound and free probes were separated on a 4% native gel and exposed to a phosphorimaging screen for quantitation. (B) Determination of the apparent K_d of wt UL42 for DNA. The amounts of bound DNA as quantified in panel A were plotted against the amounts of free DNA, and these data were used to obtain a best fit for the equation $[PD] = [PD]_{max}[D]/(K_d + [D])$, where [PD] and [D] are the concentrations of bound and free DNA, respectively. (C) Determination of apparent K_d values of mutant UL42 proteins for DNA, determined from the data in panel A as for the wild type in panel B. Squares, R113A; circles, R182A; diamonds, R279A; triangles, R280A.

FIG. 2. DNA binding affinity of wild-type and mutant UL42 proteins. (A) Mobility shift analysis of DNA binding by UL42 mutants. Binding reactions used the indicated concentrations of a radiolabeled 30-bp DNA probe and 80 nM (wt), 400 nM (R113A and R279A), 200 nM (R182A), or 500 nM (R280A) UL42 (higher concentrations of DNA and protein were used for the mutants to permit quantification
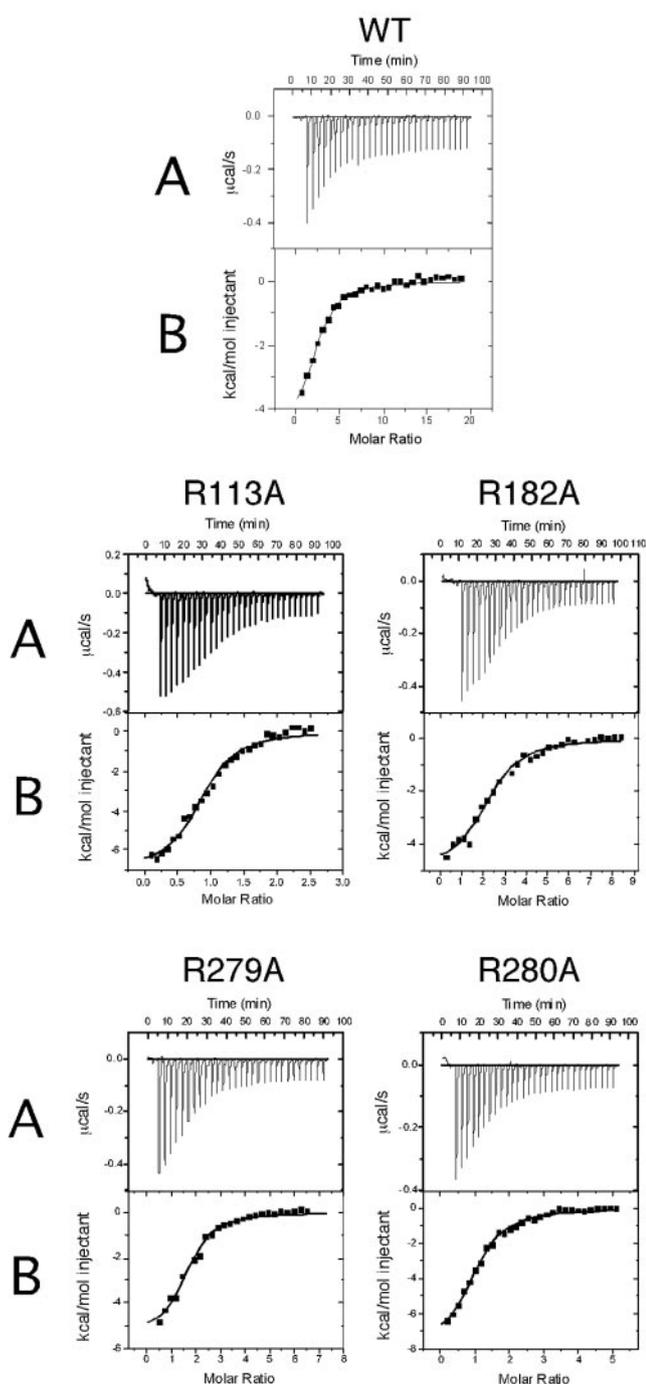
FIG. 3. ITC analysis of peptide A binding by UL42 mutants. Panels A illustrate the power applied to a sample cell containing either wt UL42 or a given mutant UL42 (named above each panel) during repeated injection of peptide A. Each peak was integrated to obtain a measure of the total amount of heat released per injection in kcal/mol of injectant. These data were plotted (panels B) against the molar ratio of peptide A to UL42 that had accumulated in the cell at the time of each injection. The heats of dilution for both peptide A and UL42 were subtracted from all data prior to analysis. K_d values for each protein are listed in Table 1.
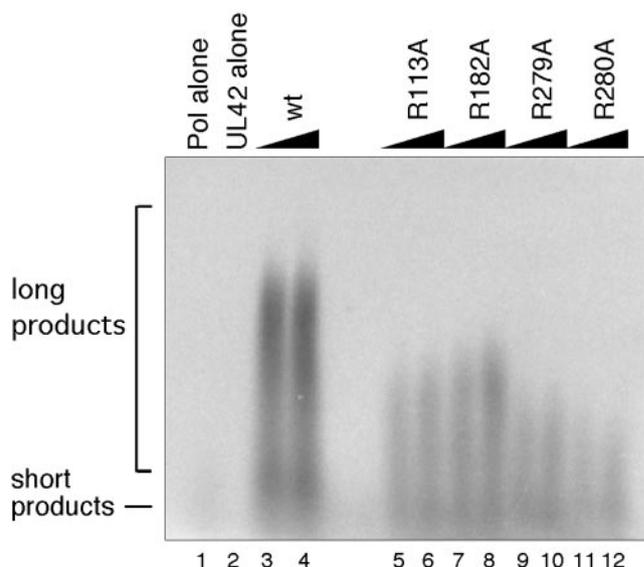

FIG. 4. Effect of UL42 mutations on long-chain DNA synthesis by HSV DNA polymerase. Long-chain DNA synthesis was measured by the incorporation of radiolabeled dTTP on a poly(dA)/oligo(dT) template. Products were separated on a 4% alkaline agarose gel and the products visualized by autoradiography. Lane 1 contained products synthesized by 200 fmol Pol in the absence of UL42, while lane 2 contained the products of a reaction containing 1,200 fmol UL42 in the absence of Pol. Reaction mixtures analyzed in lanes 3 and 4 contained 200 fmol Pol and 800 or 1,200 fmol wild-type UL42. Reaction mixtures analyzed in lanes 5, 7, 9, and 11 contained 200 fmol Pol and 800 fmol of the indicated UL42 mutant, while reaction mixtures analyzed in lanes 6, 8, 10, and 12 contained 200 fmol Pol and 1,200 fmol UL42 of the indicated mutant.

ously measured the dissociation of UL42 from various DNA templates of various lengths at 10 mM NaCl and room temperature as part of our study showing that UL42 can diffuse linearly (slide) on DNA (27). We therefore repeated these measurements of dissociation at 50 mM ammonium sulfate and 75 mM NaCl, and at 37°C. These conditions permit processive DNA synthesis by Pol/UL42 (6, 15); indeed, the latter conditions were those used to demonstrate that UL42 is a processivity factor (15). In these experiments, UL42 was incubated with a radiolabeled DNA of defined length. At time point zero, a vast excess of unlabeled DNA was added to ensure that any UL42 that dissociated would not rebind to the labeled template. Then, at various times, the binding reaction mixture was loaded onto a running polyacrylamide gel. Figure 6 shows plots of the half-lives obtained versus the lengths of DNA under both conditions. As observed previously at lower ionic strength and temperature (27), the half-lives increased with increasing lengths of DNA and then plateaued, consistent with UL42 dissociating not only from internal sites on each DNA template but also from the ends of the DNA via sliding.

If UL42 tethers polymerase to DNA, then its rate of dissociation from internal sites on DNA is highly relevant, as the holoenzyme ordinarily does not encounter DNA ends while replicating long DNAs such as the genome of HSV. Our previous mathematical model to assess sliding (27) did not permit a determination of this rate of dissociation; rather, we estimated this value from the half-lives observed with long DNA
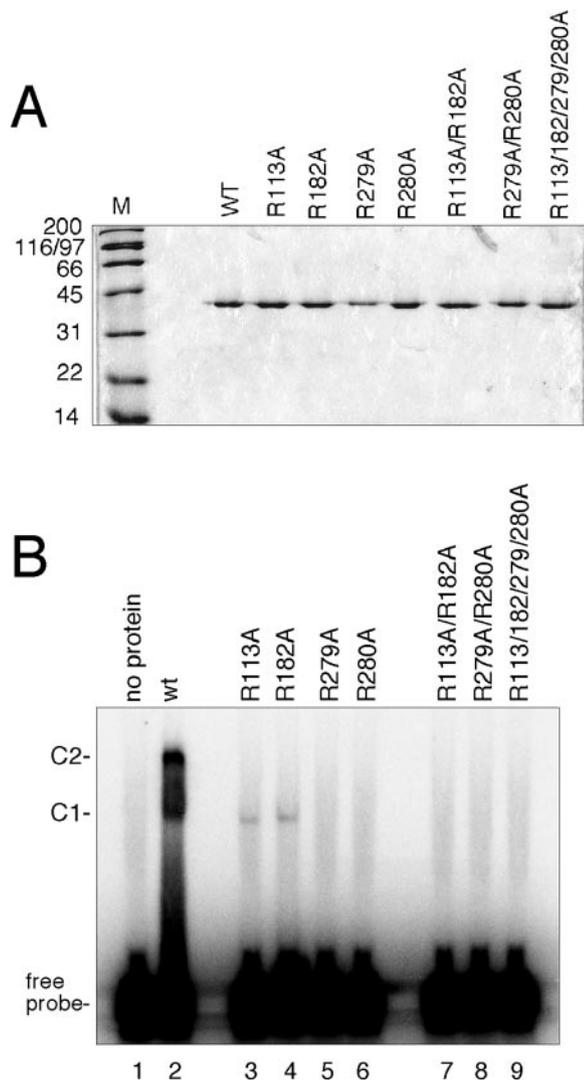
FIG. 5. Effects of multiple alanine substitutions on DNA binding by UL42. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of wild-type and mutant UL42 proteins purified by binding to GST-peptide A. M = molecular mass standards; molecular masses in kDa are indicated to the left of the gel. The molecular mass of UL42ΔC340 is 37 kDa. (B) DNA binding by purified UL42 proteins. EMSAs used 100 nM 30-bp DNA and 300 nM UL42:peptide A complex. Complexes labeled C1 and C2 are formed by the binding of either one or two UL42 monomers, respectively, to this 30-bp DNA. Lane 1 contained no UL42, while lane 2 contained wt UL42. Lanes 3 to 6 show DNA binding by the single mutants R113A, R182A, R279A, and R280A, while lanes 7 to 9 show DNA binding by the double mutants R113A/R182A and R279A/R280A and the quadruple mutant R113A/R182A/R279A/R280A, respectively.
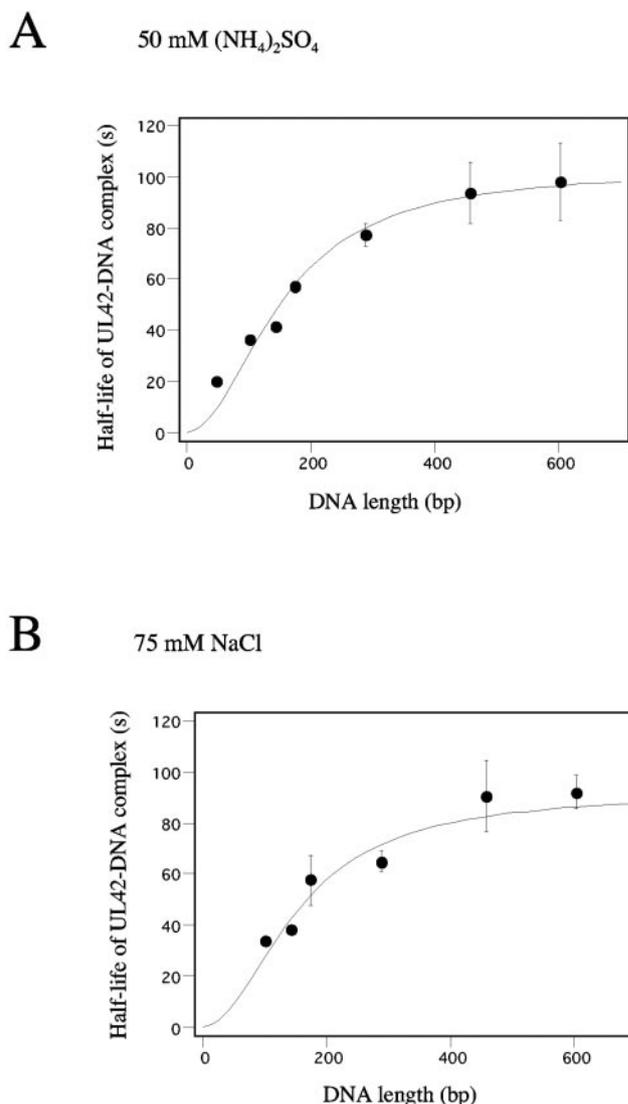

FIG. 6. DNA length dependence of half-life of the UL42-DNA complex at ionic strengths that permit processive DNA synthesis. The half-lives of UL42 binding to various lengths of DNA were determined as previously described (27) except at 37°C and 50 mM $(NH_4)_2SO_4$ (A) or 75 mM NaCl (B), and the values obtained are plotted against the lengths of DNA used. Each point (± standard error of the means) is from two to six independent measurements.

templates. Furthermore, as pointed out to us by E. P. Geiduschek (personal communication), our previous calculation of the sliding rate of UL42 failed to take into account the presence of two free DNA ends in our experiments and thus overestimated the rate of sliding. We therefore developed a revised model that incorporates both the rate of dissociation from internal sites and the rate of sliding. Dissociation from internal sites on the DNA occurs at a fixed rate independently of DNA length [$k_{\text{off(internal)}}$]. Dissociation from the ends of a linear

DNA occurs following sliding of the protein to a terminus and occurs at a rate that will vary depending on the length of the DNA [$k_{\text{off(ends)}}$]. Thus, the overall k_{off} is the sum of these two rates; that is, $k_{\text{off(observed)}} = k_{\text{off(internal)}} + k_{\text{off(ends)}}$.

$k_{\text{off(ends)}}$ is the rate at which UL42 encounters a DNA end and dissociates. An equation for an analogous situation has been derived previously by H. Berg (see Materials and Methods and reference 1). We used this equation to derive a formula for the relationship between the sliding and internal dissociation rates of UL42 and the half-life of the protein on any linear DNA of a given length (see Materials and Methods). We proceeded to analyze our data (Fig. 6) on the length-dependent dissociation rate of UL42 using this model. The

data fit the model very well (R^2 was more than 0.9 in both cases). We used these data and this model to calculate the rate of internal dissociation. (We also calculated the rate of sliding and consider these findings in the Discussion.) Under both conditions that permit processive DNA synthesis, we calculated a $k_{\text{off(internal)}}$ of $\sim 7 \times 10^{-3}$ s^{-1} [6.8 $\pm$ 0.4 $\times$ 10^{-3} s^{-1} for 50 mM (NH$_4$)$_2$SO$_4$ and 7.2 $\pm$ 0.5 $\times$ 10^{-3} s^{-1} for 75 mM NaCl]. Although this value is higher than that estimated previously under lower ionic strength and temperature (27), it is still relatively low. Below, we discuss the implications of this value for models regarding the way that UL42 promotes processivity.

DISCUSSION

We have used site-directed mutagenesis to test the hypothesis that DNA binding by the HSV processivity factor, UL42, is mediated by a strongly basic surface of the molecule, identified by crystallographic analysis. As the mutants studied here affected DNA binding to various degrees, we were also able to address the hypothesis that the DNA binding affinity of UL42 is critical for the processivity of the viral DNA polymerase. We discuss our results with these mutants and from measurements of dissociation of UL42 from DNA in terms of each of these hypotheses.

How does UL42 interact with DNA? Zuccola et al. (36) identified four conserved arginines on a basic surface of UL42 and postulated that DNA binding by UL42 is mediated by this surface. As the substitution of any of these arginines with alanine results in reduced DNA binding, we conclude that these four residues are indeed involved in DNA binding. These arginines could be positioned to interact with the negatively charged phosphates on DNA to facilitate both a high-affinity interaction and sliding on DNA, as we have suggested previously (27). Of the four arginines, Arg280 appears to play the largest role in DNA binding, as substituting alanine at this position reduces DNA binding the most. However, substitutions at any of the four positions led to a measurable reduction in DNA binding.

If, as proposed previously (36), UL42 interacts with DNA via an electrostatic tractor field provided by the basic surface, then not only would each arginine be expected to contribute to DNA binding, as observed, but the effects of the mutations would also be expected to be cumulative. Consistent with this expectation, the double- and quadruple-substitution mutants bound less avidly to the DNA surrogate, heparin, than did the single mutants. Similarly, the R113A/R182A mutant was more defective for DNA binding than either single mutant. We caution that this analysis was qualitative in terms of both the assays of DNA binding and the assays of binding to peptide A. Nevertheless, the data suggest that residues crucial for DNA binding are distributed across this basic surface.

The results presented here suggest, but do not demonstrate, that it is the positive charge of the Arg residues that is important for DNA binding. Preliminary results from experiments assessing the role of cations in the UL42-DNA interaction and studies of mutants in which Arg residues are replaced with Lys residues lend further support to this idea (G. Komazin and D. M. Coen, unpublished results).

There are several less-well-conserved arginines and lysines on this surface whose positive charge may also contribute to

DNA binding, specifically Arg51, Lys105, Arg106, Lys187, and Arg275. Full DNA binding by UL42 may depend on all of these residues, although not all of these residues may be in contact with DNA at any one time. For example, the movement of UL42 on DNA may require that negatively charged phosphates be transferred between positively charged residues on UL42. In addition, uncharged or negatively charged residues, such as the conserved residues Gln282 or Asp270, could modulate the strength of the UL42-DNA interaction. Too much net basic charge on the surface conceivably could slow sliding by UL42 and impair processive DNA synthesis. Alternatively, negative charge near the edges of the surface could "channel" DNA onto the region of positive charge. Testing these possibilities will require substantial further investigation.

To perform our studies of DNA binding, it was crucial to select for active protein. Interestingly, in three separate purifications, the fraction of the R182A mutant protein that bound to either a GST-peptide A column or a heparin column (data not shown) was much higher than that of the wild-type protein. Accordingly, the R182A mutant exhibited higher DNA binding activity in crude extracts or after partial purification on an amylose column than did wt protein (data not shown). Although we do not understand why such a large fraction of the R182A protein in crude extracts was active, these observations illustrate the necessity of first removing inactive protein prior to assaying the binding of UL42 proteins to DNA or to other proteins, including Pol. This issue may be relevant to a report of poor correlations between the effects of mutations on certain in vitro activities of mutant UL42 proteins and their effects in infected cells (31).

We previously suggested that the UL42-DNA interface could serve as a potential target of antiviral drugs, based on the observation that insertion mutations that abolish DNA binding also abolish viral replication (7). However, the fact that no single mutation completely abolishes binding and the wide distribution of important residues on this interface argue that a small molecule could not effectively disrupt the interaction.

Revised values for rates of sliding and polymerase translocation. In the course of analyzing the rates of dissociation of UL42 from DNA, we uncovered three sets of errors in previous studies from our lab (27, 33). The first, as noted by Chaudhuri and Parris (4), was that we had measured these rates (27) at low ionic strengths and temperatures that do not correspond to those at which UL42 promotes processivity or at which rates of polymerase translocation had been measured (33). Second, as noted above, we had overestimated the rate of sliding (27) due to a failure to take the presence of two free DNA ends into account in our calculations. Third, we improperly estimated rates of polymerase translocation by using a single time point rather than the slopes of the curves generated by plotting nucleotide incorporation versus time (33). This led to an overestimation of these rates. Additionally, in our previous publication (33), we stated only estimations of the 75th-percentile rates, rather than the median rates. As outlined below, these errors offset each other, so that the main conclusions and suggestions derived from these previous studies remain correct.

Figure 6 presents the half-lives of UL42 on various lengths of DNA under two conditions where UL42 promotes processivity (6, 15), one of which (50 mM ammonium sulfate, 37°C)

we have previously used to measure the rate of polymerase translocation (33). Using the data in Fig. 6 and our revised mathematical model that takes the presence of two free ends of DNA into account, we calculate that D, the rate of linear diffusion (sliding) under both conditions tested, was ~14 bp/second [13 ± 2.2 bp/s for 50 mM $(NH_4)_2SO_4$ and 15 ± 2.8 bp/s for 75 mM NaCl]. When median rates of polymerase translocation at 50 M ammonium sulfate are properly calculated from slopes of the curves (Fig. 6C in reference 33), the value for Pol alone is 11 nucleotides (nt)/second, and the value for holoenzyme is 19 nucleotides/second. (Thus, as previously concluded [33], UL42 does not slow the rate of translocation). Although the rate of sliding of UL42 calculated here (~14 bp/second) is lower than that previously calculated (27), it is similar to the rate of translocation of Pol measured under the same conditions (11 to 19 nucleotides/second). This then is consistent with the suggestion that UL42 can freely slide downstream with Pol during DNA replication (27). As emphasized previously (27), further studies are needed to test this suggestion more directly.

How does UL42 promote processivity? There are two hypotheses explaining how UL42 promotes processivity of HSV DNA polymerase. The tethering hypothesis (15) proposes that UL42 tethers Pol to DNA via its direct DNA binding activity. An alternative hypothesis (4) proposes that UL42 does not increase Pol processivity by virtue of its affinity for DNA but rather promotes a conformational change that allows UL42 and Pol to wrap around DNA together.

Biochemical evidence consistent with the tethering model includes higher-affinity binding and more-extensive nuclease protection of primer-template DNA by holoenzyme than by Pol alone (14, 27, 33). Genetic evidence was initially provided by two four-codon insertion mutations in UL42 that abolish both detectable DNA binding and long-chain DNA synthesis by Pol without qualitatively affecting the Pol-UL42 interaction (7). The results presented here substantially extend this correlation in that the rank order of effects of four different point mutations on the strength of DNA binding was the same as that for the effects on the ability of UL42 to support long-chain DNA synthesis. Interestingly, mutation of conserved basic residues in PCNA on the inner, DNA-proximal face of its ring reduces overall DNA synthesis by the associated polymerase without affecting the length of the products synthesized (11). Thus, these basic residues of PCNA appear to play a role in the loading of the sliding clamp but not in the processivity of the polymerase. In contrast, the UL42 mutations described here affect the lengths of the products of primer elongation. Thus, our results are wholly consistent with predictions from the tethering hypothesis.

The primary evidence in support of the alternative hypothesis comes from surface plasmon resonance (SPR) studies (4) of the binding of UL42 to DNA at differing salt concentrations. At higher salt concentrations, where stimulation of Pol by UL42 on activated calf thymus DNA templates is observed (4, 17), the K_d of UL42 for a 67-nt/45-nt primer/template DNA determined by SPR ranged from ca. 100 nM to ca. 100 μM. It was suggested that this binding is too weak to adequately tether Pol to DNA (4). Instead, it was proposed that UL42 induces conformational changes in Pol to promote high-affinity interactions with DNA.

A technical issue regarding the evidence in support of the alternative hypothesis concerns the primer/template DNA used, which contained only 45 base pairs of dsDNA (4). As shown here and previously (27), UL42 rapidly dissociates from such short primer/templates due to sliding to the end of the DNA molecule, such that the SPR studies would greatly overestimate the parameter most relevant for tethering—the $k_{off(internal)}$—and thus also overestimate the K_d. In an effort to address this issue, an additional SPR experiment, in which the free end (i.e., that not bound to the SPR chip surface) was blocked with a protein, was performed, and the same off-rate as in the absence of the block was observed (4). However, the protein block was separated from the double-stranded segment of DNA by 22 nt of single-stranded DNA (ssDNA). It seems plausible that UL42, which binds only weakly to ssDNA (33), would have dissociated from the ssDNA once it slid to it, irrespective of the protein block. Thus, it is possible that the SPR study might have measured only the rate at which UL42 diffuses to the ds-ssDNA junction rather than the true dissociation rate from an internal DNA site, which would be more representative of the state of UL42 in the replisome.

To examine the off-rate from these more relevant internal sites, we examined the rates of dissociation of UL42 from various lengths of DNA and analyzed the data using a mathematical model that permits the calculation of $k_{off(internal)}$. At a temperature (37°C) and two salt concentrations that permit processive DNA synthesis, the $k_{off(internal)}$ was ~7×10^{-3} s^{-1}, which corresponds to a $t_{1/2}$ of ~100 seconds. Although these values by themselves would limit processive DNA synthesis to only 1,000 to 2,000 bp at an average incorporation rate of 11 to 19 nucleotides/second, it is important to emphasize that processivity entails the likelihood that the polymerase holoenzyme will dissociate from DNA after each catalytic cycle. Thus, when UL42 dissociates from DNA in the context of the holoenzyme, it would still be bound to Pol, which itself binds tightly to primer-template (5, 14, 33) and therefore would not diffuse away. Immediate rebinding of UL42 to the template would ensure that Pol remained tethered on DNA. In effect, the two proteins can be thought of as processivity factors for each other. Only when both proteins simultaneously dissociate will DNA synthesis terminate. These concepts have been recently illustrated by results showing that a protein that binds DNA weakly (K_d in the micromolar range) without sequence specificity is able to substantially increase the processivity of DNA polymerases from two different families (32). These results, and perhaps the interactions of other replication proteins with DNA, likely explain the ability of the quadruple *UL42* mutant to support HSV replication, albeit weakly, in transient complementation assays.

The alternative model invokes conformational changes in Pol and/or UL42 to wrap the holoenzyme around DNA. There are attractive features of this model. Indeed, the segment of Pol that is known to interact with UL42 does change conformation upon binding to UL42. However, it becomes more ordered and less flexible (3, 36) and thus less likely to permit wrapping of Pol around DNA. Additionally, using limited proteolysis, we have looked for evidence of other conformational changes in Pol and UL42 upon binding to each other, in the presence or absence of DNA, and have found none (K. Weisshart, A. Kuo, and D. M. Coen, unpublished results). Although these were negative results, in the absence of compelling evi-

dence in favor of conformational changes that would wrap the holoenzyme around DNA, the simplest explanation for all the available data at present is that DNA binding by UL42 tethers Pol for processive DNA synthesis. Nevertheless, we certainly cannot rule out conformational changes such as those proposed in the alternative model. If they do occur, however, given the strong correlation between the effects of mutations on DNA binding and long chain DNA synthesis (this study and reference 7), it would be difficult not to also ascribe a role for DNA binding by UL42 in polymerase processivity.

ACKNOWLEDGMENTS

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