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Interaction of Herpes Simplex Virus Type 1 DNA Polymerase and the UL42 Accessory Protein with a Model Primer Template

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Genetic and biochemical studies have shown that the products of the herpes simplex virus type 1 (HSV-1) DNA polymerase (UL30) and UL42 genes are both required for viral DNA replication. A number of studies have previously suggested that these two proteins specifically interact, and more recent studies have confirmed that the viral DNA polymerase from HSV-1-infected cells consists of a heterodimer of the UL30 (Pol; the catalytic subunit) and UL42 polypeptides. A comparison of the catalytic properties of the Pol-UL42 complex with those of the isolated subunits of the enzyme purified from recombinant baculovirus-infected insect cells indicated that the Pol-UL42 complex is more highly processive than Pol alone on singly primed M13 single-stranded substrates. The results of these studies are consistent with the idea that the UL42 polypeptide is an accessory subunit of the HSV-1 DNA polymerase that acts to increase the processivity of polymerization. Preliminary experiments suggested that the increase in processivity was accompanied by an increase in the affinity of the polymerase for the ends of linear duplex DNA. We have further characterized the effect of the UL42 polypeptide on a defined hairpin primer template substrate. Gel shift and filter binding studies show that the affinity of the Pol catalytic subunit for the 3′ terminus of the primer template increases 10-fold in the presence of UL42. DNase I footprinting experiments indicate that the Pol catalytic subunit binds to the primer template at a position that protects 14 bp of the 3′ duplex region and an adjacent 18 bases of the single-stranded template. The presence of the UL42 polypeptide results in the additional protection of a contiguous 5 to 14 bp in the duplex region but does not affect the 5′ position of the Pol subunit. Free UL42 protects the entire duplex region of the substrate but does not bind to the single-stranded region. Taken together, these results suggest that the increase in processivity in the presence of UL42 is related to the double-stranded DNA-binding activity of free UL42 and that the role of UL42 in the DNA polymerase complex is to act as a clamp, decreasing the probability that the polymerase will dissociate from the template after each cycle of catalysis.

Herpes simplex virus (HSV) encodes a number of proteins that participate in the replication of its genome (5–8, 10, 11, 36, 49, 50, 60–63). Central among these proteins is the viral DNA polymerase. The HSV DNA polymerase has been studied extensively for a number of years, in part because it was one of the first virus-encoded enzymes to be detected in infected cells biochemically (47, 49) and also in part because it is the target for clinically important antiherpesvirus drugs (9, 17). The enzyme is now known to comprise two subunits: a 130-kDa polypeptide (Pol, the product of the UL30 gene) and a 60-kDa polypeptide (the product of the UL42 gene) (22, 25). Genetic studies using both conditionally lethal mutant viruses and transient replication assays have shown that both subunits are required for viral DNA replication (7, 8, 30, 36, 50, 62).

Sequence comparison studies have demonstrated that the *pol* gene polypeptide is a member of the α family of eukaryotic DNA polymerases (28, 37, 40). The *pol* gene polypeptide has been shown to contain a catalytically active DNA polymerase in the absence of other viral polypeptides by overexpression in yeast cells (23), in insect cells infected with a recombinant baculovirus (38), and by in vitro transcription-translation (16). The Pol polypeptide also contains an intrinsic 3′-5′ exonuclease proofreading activity (31, 38, 42) and a 5′-3′ exonuclease that can function as an RNase H activity (12, 38).

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The UL42 gene product was first identified as a second polypeptide of approximately 50 to 60 kDa associated with the DNA polymerase from HSV-infected cells (19, 45, 47, 59). Hydrodynamic analysis of purified polymerase has shown that the native form of the enzyme consists of a heterodimer of Pol and UL42 (22, 25). Mutational analysis of UL42 has demonstrated that the amino-terminal two-thirds of the polypeptide is sufficient for interaction with the polymerase as well as for overall function (14, 20, 24, 55). It has not been possible, however, to identify a single small peptide sequence or subdomain within the amino-terminal functional domain that is both necessary and sufficient for interaction with Pol (41). In contrast, mutational analysis of the Pol polypeptide has revealed a short peptide sequence located near the carboxy terminus of the protein that is sufficient for the interaction of Pol with UL42 (13, 56). Deletion of this sequence eliminated the ability of Pol to support DNA replication in vivo, even though the deletion was shown to have no effect on the activity of the enzyme in standard in vitro assays (13, 51, 56). It seems likely, therefore, that the interaction of Pol and UL42 is critical for DNA replication.

A comparison of the activities of the Pol polypeptide and the Pol-UL42 protein complex on singly primed single-stranded DNA substrates has shown that the presence of UL42 greatly increases the processivity of the DNA polymerase (22, 25). The mechanism by which UL42 effects this increase in processivity is not known. Mutational analysis of the two polypeptides has shown that there is a tight correlation between processivity and the ability of the two proteins to interact (13–15, 20, 24, 41, 55,

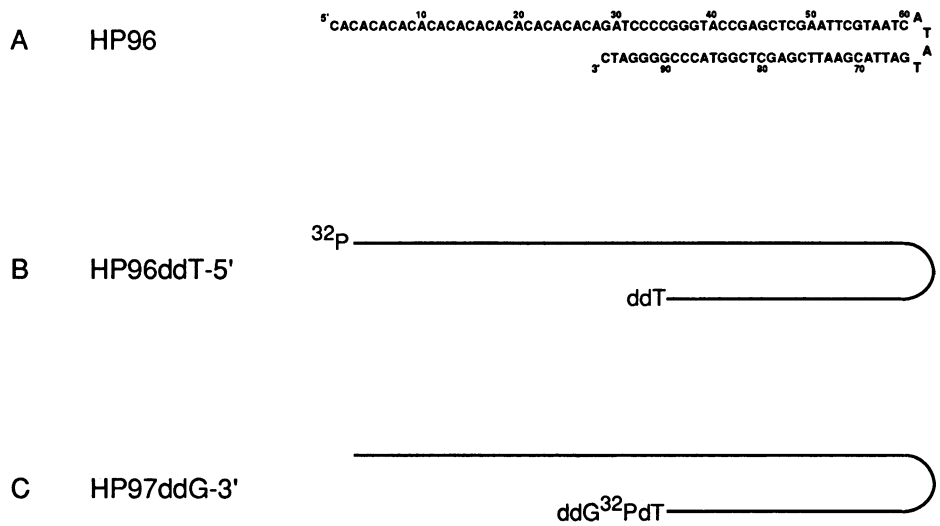

FIG. 1. Structures of the hairpin primer template DNAs. The preparation of the synthetic hairpin primer template (HP96) used to study protein-DNA interactions has been described in Materials and Methods and by Tsurimoto and Stillman (58). HP96 (A) consists of a 5′ single-stranded region 28 bases in length and a 31-bp double-stranded region flanked by a 6-base single-stranded loop. The hairpin DNA substrates (B and C) contain a dideoxyribonucleoside monophosphate (ddT for HP96 and ddG for HP97) at the 3′ terminus to prevent degradation of the substrate by the 3′-5′ exonuclease activity of the polymerase. HP96ddT-5′ (B) is the 96-base synthetic oligonucleotide illustrated in panel A and was labeled at the 5′ end with [γ-^{32}P]ATP and the Klenow fragment of *E. coli* Pol I. HP97ddG-3′ (C) was constructed by the addition of a single labeled nucleotide ([α-^{32}P]dTTP) to the 3′ end of HP96.

56). Biochemical analysis of the UL42 polypeptide revealed that UL42 has a high affinity for double-stranded DNA, to which it binds in an apparently noncooperative, sequence-independent manner (19, 59). On the basis of these observations, it has been proposed that UL42 increases the processivity of the DNA polymerase by acting as a clamp or tether (22). To gain further insight into the mechanism of action of UL42, we have used a model primer template previously described by Tsurimoto and Stillman (58) to investigate the interaction of the HSV DNA polymerase at a junction between an elongating DNA strand and the DNA template. Gel shift and filter binding studies showed that the affinity of the enzyme for the primer template is increased 10-fold by the presence of UL42. Footprinting experiments demonstrated that the Pol subunit binds to the primer template at a position which is approximately centered on the 3′ terminus of the priming strand in such a way as to make close contacts with both the single-stranded template ahead of the growing chain and the double-stranded product DNA. The presence of UL42 had no effect on the interaction with single-stranded template but increased the contacts of the enzyme with double-stranded DNA behind the 3′ end of the primer. These results provide additional support for the view that the increase in DNA polymerase processivity in the presence of UL42 is related to the double-stranded DNA-binding activity of free UL42 and that the role of UL42 in the DNA polymerase complex is to act as a sliding clamp, increasing the affinity of the HSV DNA polymerase for the 3′ end of the priming strand.

MATERIALS AND METHODS

Enzymes. The HSV DNA polymerase holoenzyme (Pol-UL42) and the Pol (UL30) and UL42 polypeptides were purified from *Spodoptera frugiperda* (SF9) cells infected with the appropriate recombinant baculoviruses as described previously (22). DNase I was obtained from United States Biochemical, and micrococcal nuclease was from Pharmacia.

Preparation of the hairpin primer templates. Synthetic hairpin DNA primer templates were constructed as described previously (58). A synthetic hairpin DNA (Fig. 1) of 96 nucleotides (nt) (HP96) was constructed by the ligation of two oligonucleotides, HP45 (5′-CACACACACACACACACACACACAGATCCCCGGGTACCGAG-3′) and HP51 (5′-CTCGAATTCGTAATCATATGATTACGAATTCGAGCTCGGTACCCGGGGATC-3′). Equimolar amounts (5 nmol) of HP45 and HP51 were heated to 90°C for 5 min and cooled slowly to room temperature. The annealed oligonucleotides were ligated with T4 DNA ligase (Life Technologies), and the 96-base DNA product was isolated from a 10% acrylamide gel containing 8 M urea. A longer hairpin DNA (HP105) was constructed in a similar manner except that the oligonucleotide HP9 (5′-TGTGTGTGT-3′) was added to HP45 and HP51 during the annealing and ligation reactions. The hairpin DNAs were labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase (United States Biochemical). HP96 was labeled at the 3′ end with [α-^{32}P]dTTP and the DNA polymerase I (Pol I) Klenow fragment (HP97). A single dideoxynucleoside triphosphate was added to the 3′ terminus of each hairpin construct to prevent degradation of the substrate by the 3′-5′ exonuclease activity of the HSV DNA polymerase (12). 5′-end-labeled HP96 was extended at the 3′ end with the addition of ddTTP (HP96ddT-5′), and 3′-end-labeled HP97 was extended at the 3′ end with the addition of ddGTP (HP97ddG-3′).

DNA synthesis reaction. Synthesis reaction mixtures contained 50 fmol of HP96 hairpin substrate, 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 50 mM NaCl, 50 mg of bovine serum albumin (BSA) per ml, and either 200 fmol of Pol-UL42 protein complex or 400 fmol of Pol polypeptide in a reaction volume of 25 μl. DNA synthesis was stopped with the addition of 25 μl of 1% sodium dodecyl sulfate–40 mM EDTA after incubation at 37°C for 30 min. The reaction products were precipitated with ethanol and fractionated on a 6% acrylamide–8 M urea sequencing gel. The gel was dried and exposed for autoradiography.

Footprinting. Footprinting reaction mixtures (10 μl) contained 50 fmol of 5′- or 3′-end-labeled primer template DNA, 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 50 mM NaCl, 50 mg of BSA per ml, and the indicated amount of polymerase and UL42 protein. Reaction mixtures were incubated at room temperature for 5 to 10 min, and the protein-DNA complexes were digested with either 40 mg of DNase I per ml, or 4×10^{-4} U of micrococcal nuclease per ml for 1 min at room temperature. The digestions were terminated with the addition of 12 μl of stop solution (100 mg of sonicated calf thymus DNA per ml, 20 mM EDTA, 4 mM EGTA, 0.6 M sodium acetate), extracted once with phenol-chloroform (1:1), precipitated with ethanol, and redissolved in 5 μl of formamide-dye solution. The reaction products were fractionated on acrylamide sequencing gels containing 7 M urea, 40% formamide, and Tris-borate-EDTA buffer. The gels were fixed in a solution of 10% methanol–5% acetic acid, dried, and autoradiographed.

Gel shift assay. Gel shift binding reactions were identical to those described for footprinting. Reaction mixtures containing HP96ddT-5′ DNA and the indicated quantity of protein were incubated for 5 min at room temperature, and the products were fractionated on a 4% acrylamide gel containing 25 mM Tris-HCl and 0.19 M glycine (33) at pH 8.3. After gel electrophoresis, the gels were dried and autoradiographed. The amount of protein-bound DNA was determined by using a Betagen BetaScope 630 blot analyzer.

RESULTS

Construction and characterization of a model primer template. To characterize the interaction between Pol, UL42, and a substrate for DNA synthesis, we used a model primer template previously described by Tsurimoto and Stillman (58). The basic substrate (HP96), constructed from two synthetic oligonucleotides by using DNA ligase, is 96 bases in length. The 3′-terminal 34 nt are complementary to the 34 nt immediately upstream so that the oligonucleotide can self-anneal, or hairpin, to form the structure shown in Fig. 1A. The key features of this structure are a 28-base single-stranded region at the 5′ end, a 3′-terminal duplex region of 31 bp, and a 6-base loop. The virtues of this substrate for quantitative and qualitative analysis of binding are that it contains a short single-stranded region and a unique 3′ terminus. Thus, binding studies are not complicated by the presence of additional specific and/or nonspecific protein binding sites. To verify the structure of HP96 and to determine whether it can serve as a substrate for the HSV DNA polymerase, unlabeled HP96 was incubated with purified Pol or Pol-UL42 under standard DNA polymerase assay conditions in the presence of [α-^{32}P]dTTP. The reaction products were analyzed by denaturing polyacrylamide gel electrophoresis. As shown in Fig. 2, incubation of HP96 with the HSV DNA polymerase under polymerizing conditions resulted in a single major product 124 nt in length, or 28 nt longer than HP96. The size of the product is that expected if DNA polymerase elongated the 3′ end of HP96 to form a fully duplex structure. We conclude from this experiment that HP96 is a legitimate substrate for the HSV DNA polymerase. Two variations of HP96 were used to study the protein-DNA interaction of the HSV DNA polymerase and the primer template DNA (Fig. 1B and C). Both substrates contain a 3′ dideoxynucleotide to prevent degradation of the DNA by the 3′-5′ exonuclease activity of the polymerase (12). HP96 was labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase to produce HP96ddT-5′ (Fig. 1B) or at the 3′ end with [α-^{32}P]dTTP and the DNA Pol I Klenow fragment to form HP97ddG-3′ (Fig. 1C).

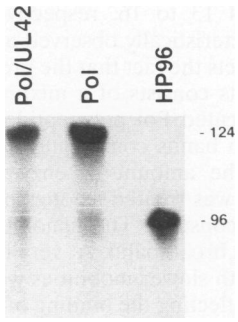

FIG. 2. HP96 is a substrate for Pol and Pol-UL42. Synthesis reaction mixtures (25 μl) contained 50 fmol of HP96 and 200 fmol of either Pol-UL42 or Pol under conditions described in Materials and Methods. The products of the reaction were fractionated on a 6% acrylamide–8 M urea gel, which was dried and autoradiographed. Sizes in bases are shown on the right. Fully duplex substrate is 124 bases in length.

UL42 increases the affinity of the HSV DNA polymerase for the primer template. A gel electrophoretic mobility shift assay was developed to determine the binding affinities of Pol, UL42, and the Pol-UL42 complex for primer template DNA. As shown in Fig. 3, incubation of 5′-^{32}P-labeled HP96 (HP96ddT-5′) with either Pol or UL42 resulted in a protein-DNA complex with reduced electrophoretic mobility relative to DNA incubated in the absence of protein. Protein-DNA complexes containing Pol or UL42 alone had similar electrophoretic mobilities but were qualitatively different (Fig. 3,

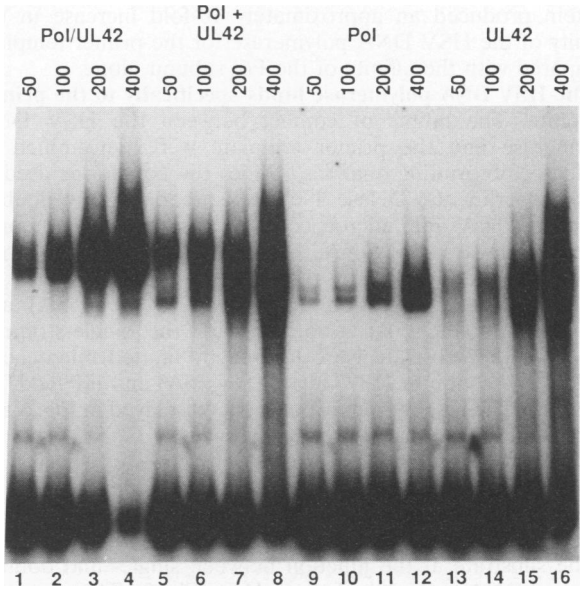

FIG. 3. Gel shift assay. The reaction conditions for the gel shift assay are as described in Materials and Methods. Binding reaction mixtures contained 50 fmol of HP96ddT-5′ and the indicated quantity of enzyme. Binding mixtures were incubated at room temperature for 5 min, and the products were separated on a nondenaturing 4% polyacrylamide gel. Lanes: 1 to 4, Pol-UL42 heterodimer; 5 to 8, 200 fmol of Pol and the indicated amount of UL42 protein; 9 to 12, Pol; 13 to 16, UL42 monomer.

lanes 9 to 12, and 13 to 16, respectively). The Pol-HP96 complex was characteristically observed as a doublet of bands that very likely reflects the fact that the preparation of Pol used in these experiments consists of a mixture of full-length and proteolytically truncated Pol polypeptides. Since the relative intensity of the two bands comprising the doublet remained unchanged when the amount of enzyme or substrate was varied, the doublet was treated as one species for determination of the affinity constant. The major UL42-HP96 complex migrated as a single broad band. At very high ratios of protein to DNA, species with slower mobilities were observed (Fig. 3, lane 16), possibly reflecting the binding of two or more protein monomers per substrate. Binding reactions that contained the Pol-UL42 heterodimer produced a complex that migrated more slowly than that observed with either subunit alone, strongly suggesting that the heterodimer does not dissociate upon binding DNA (Fig. 3, lanes 1 to 4). A band with an electrophoretic mobility identical to that seen with the Pol-UL42 heterodimer was observed in binding reactions in which HP96 was incubated with the individual subunits of Pol and UL42 (Fig. 3, lanes 5 to 8). In binding reactions that contained a molar excess of UL42 compared with Pol (Fig. 3, lanes 7 and 8), the pattern of bands appears qualitatively to be a simple addition of the patterns seen with Pol-UL42 and UL42 alone.

Binding saturation curves were obtained for Pol-UL42, Pol, and UL42, using gel shift assays similar to those shown in Fig. 3. In these assays, a fixed molar amount of protein was incubated with increasing concentrations of HP96ddT-5′ (not shown). The binding isotherms shown in Fig. 4A demonstrate that saturation of the Pol-UL42 complex occurred at a significantly lower substrate concentration than did saturation of free UL42 or the Pol subunit alone. Binding dissociation constants (K_D) of 7.1×10^{-9} M for Pol, 7.8×10^{-10} M for Pol-UL42, and 1.1×10^{-9} M for free UL42 were determined by Scatchard analysis of the gel shift and filter binding data (Fig. 4B). These results showed that the presence of UL42 protein produced an approximately 10-fold increase in the affinity of the HSV DNA polymerase for the primer template compared with the affinity of the Pol subunit alone.

The HSV DNA polymerase binds specifically to the primer template. The points of contact between the HSV DNA polymerase and the primer template were determined by nuclease footprinting analyses. Under the conditions used in these experiments, DNase I cleaves predominately double-stranded DNA and micrococcal nuclease cleaves predominately single-stranded DNA. Thus, protein contacts with the double-stranded region of the substrate were detected by limited DNase I digestion of HP96ddT-5′ (Fig. 5A) and HP97ddG-3′ (Fig. 5B). Contacts with the single-stranded region of the substrate were located by limited micrococcal nuclease digestion of HP97ddG-3′ (Fig. 6A) and HP96ddT-5′ (Fig. 6B). The results from experiments displayed in Fig. 5 and 6 are summarized diagrammatically in Fig. 7.

The polymerase subunit (Pol) alone protected specific sequences in both the double-stranded and single-stranded regions of the substrate. The protected sequence of double-stranded DNA was 14 bp in length, extending from the 3′ end of the substrate at the junction between single- and double-stranded DNA toward the hairpin loop (Fig. 5). The protected sequence of single-stranded DNA was the 18 nt extending from the primer template junction toward the 5′ end of the template strand (Fig. 6). When the double-stranded region of HP96 was extended at the 3′ end by 9 bases, the size of the protected regions did not change, but their location was displaced toward the 5′ end of the substrate (not shown). Thus, it seems likely that the polymerase interacts specifically with the substrate by

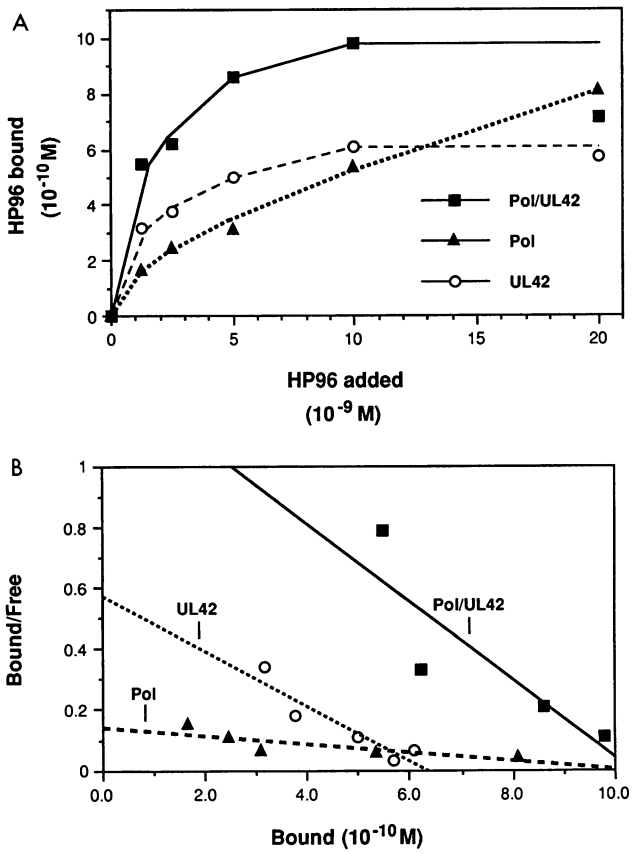

FIG. 4. Quantitation of binding to the hairpin primer template. Binding reaction mixtures (20 μl) contained increasing quantities of HP96ddT-5′ and a fixed amount of Pol/UL42 (100 fmol), Pol (200 fmol), or UL42 (400 fmol). (A) Binding isotherm. The amount of HP96 bound in a gel mobility assay was quantitated by using a Betagen BetaScope analyzer and was plotted against the amount of HP96 DNA added to each binding reaction. Saturation of binding occurred at a substantially lower substrate concentration with Pol-UL42 and UL42 than with the Pol polypeptide. (B) Scatchard analysis of binding. The ratio of the amount of HP96ddT-5′ bound by an enzyme relative to the amount of HP96ddT-5′ unbound (free) was plotted against the quantity of HP96ddT-5′ bound, where the slope = $-1/K_D$. The K_Ds for Pol-UL42, Pol, and UL42 were 7.8×10^{-10}, 7.1×10^{-9}, and 1.1×10^{-9} M, respectively.

virtue of recognition of a specific structure (the primer template junction) rather than by recognition of a specific sequence. Moreover, this specific interaction involves close contacts with both the single-stranded template DNA and with the double-stranded product of polymerization (Fig. 7).

UL42 alone did not protect any specific region of the substrate. At relatively high protein concentrations, however, UL42 did protect the entire double-stranded region of the template (Fig. 5). At these same concentrations, UL42 afforded little if any protection to the single-stranded region of the substrate (Fig. 6). These results are in accord with previous studies of UL42 in which it was shown that this protein has nonspecific double-stranded DNA-binding activity (19, 59).

In contrast to the results obtained with free UL42 in the absence of Pol, the presence of UL42 in binding reactions containing Pol resulted in protection of specific sequences of the substrate (Fig. 5). In particular, the region of duplex DNA protected by Pol was expanded by 5 nt on the top strand and 14

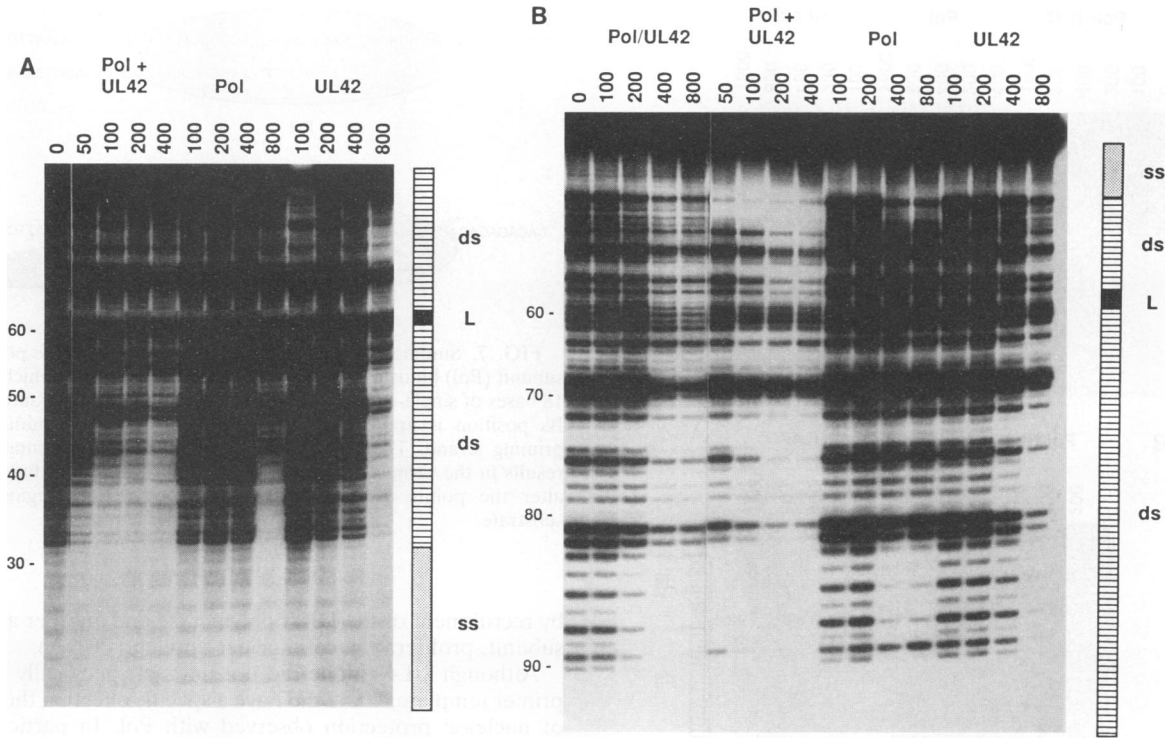

FIG. 5. DNase I footprints of HP96ddT-5′ and HP97ddG-3′. The binding reaction conditions were as described in Materials and Methods. Reaction mixtures contained 50 fmol of HP96ddT-5′ (A) or HP97ddG-3′ (B) and the indicated amount (in femtomoles) of Pol-UL42, Pol, or UL42 protein. The DNA-protein complexes were digested with 40 μg of DNase I per ml for 1 min at room temperature, and the products of the reactions were analyzed on an 8% (A, HP96ddT-5′) or 6% (B, HP97ddG-3′) polyacrylamide gel containing 7 M urea and 40% formamide. The numbers at the left correspond to the nucleotide numbers of the HP96 substrate illustrated in Fig. 1. Lanes labeled Pol + UL42 contained a fixed amount of Pol (800 fmol) and the indicated amount of UL42. ds, L, and ss, double-stranded, loop, and single-stranded regions of HP96, respectively.

nt on the bottom strand by the addition of UL42 at concentrations at which little or no nonspecific protection was observed in the absence of Pol (compare UL42 lanes alone with Pol/UL42 lanes in Fig. 5). The same pattern of protection was observed by the addition of isolated Pol and UL42 peptides and by the addition of the Pol-UL42 heterodimer (Fig. 5B). The footprint of Pol-UL42 on single-stranded DNA was essentially identical to that seen with Pol alone, and the addition of UL42 to reactions containing Pol did not alter the pattern of protection (Fig. 6). The simplest interpretation of these results is that the extension of the nuclease protection pattern seen by the addition of UL42 to Pol results from an interaction of UL42 with the substrate that is directed to a specific site by protein-protein interactions between Pol and UL42. Alternative explanations, which cannot be ruled out, are considered in Discussion.

DISCUSSION

In this study, we used both nuclease protection experiments and gel mobility shift assays to analyze the interaction of the HSV DNA polymerase with a model primer template. The combined application of these two techniques has provided the basis for developing a picture of how the polymerase interacts with its nucleic acid substrate as it carries out DNA synthesis, as well as insight into the function of the processivity subunit, UL42.

As one might reasonably expect from the enzymatic properties of the protein, our data suggest that the catalytic subunit of the polymerase (Pol) interacts with a primer template in a structural rather than sequence-specific fashion. Nuclease protection experiments indicate that the polymerase interacts with its nucleic acid substrate in such a way that it is approximately centered on the 3′ end of the primer, making close contact with about 18 bases of single-stranded template and about 14 bp of product duplex DNA. These results are generally in keeping with those obtained for other DNA polymerases. For example, the structure of a DNA polymerase-DNA complex has been determined for the Klenow DNA polymerase fragment of *Escherichia coli* Pol I by X-ray diffraction analysis (18). The most recent model for the interaction of this enzyme with DNA predicts a structure in which 11 to 12 bp of duplex DNA and 3 to 6 bases of single-stranded template are held within a deep cleft in the protein (2). In the case of human DNA polymerase δ, footprinting experiments, which, like those reported here, used the model primer template HP96, showed that this polymerase protected 24 to 26 bp of duplex DNA and 4 to 6 bases of the single-stranded template (58). One possible explanation for the greater extent of protection of single-stranded template DNA seen in the case of the HSV DNA polymerase may relate to the fact that this enzyme, unlike either the Klenow fragment or polymerase δ, has an intrinsic 5′-3′ exonuclease activity. Clearly, analysis of additional DNA polymerases both lacking and possessing a 5′-3′ exonuclease will be necessary to assess the merit of this speculation.

Gel mobility shift experiments indicated that like Pol, the accessory subunit UL42 alone binds tightly to the HP96 substrate. Nuclease protection experiments showed, however,

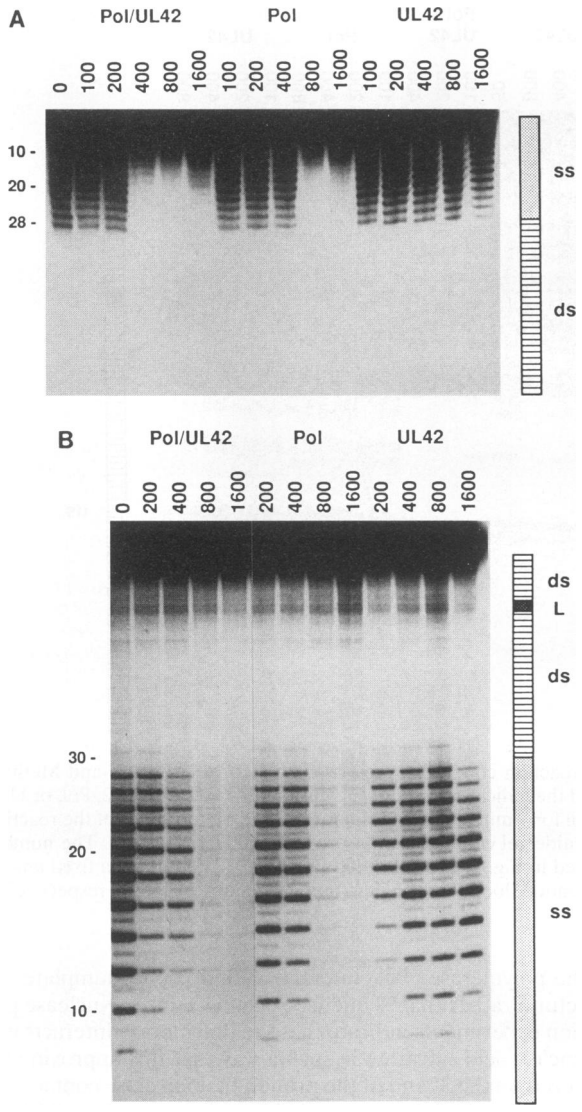

FIG. 6. Micrococcal nuclease footprints of HP97ddG-3′ and HP96ddT-5′. Binding reaction conditions were the same as those described in the legend to Fig. 5. Reaction mixtures contained HP97ddG-3′ (A) or HP96ddT-5′ (B) and the indicated quantity of enzyme. DNA-protein complexes were digested with 4×10^{-4} U of micrococcal nuclease per ml for 1 min at room temperature and analyzed on a 6% (HP97ddG-3′, A) or 12% (HP96ddT-5′, B) polyacrylamide sequencing gel containing 7 M urea and 40% formamide.

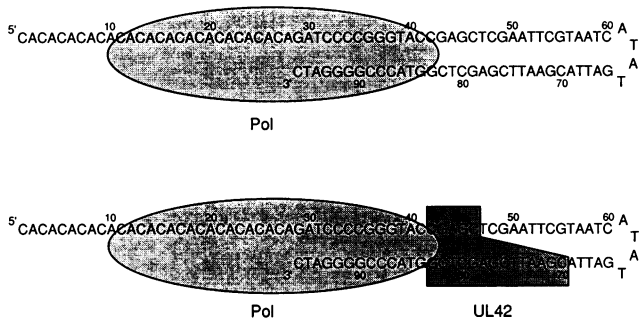

FIG. 7. Summary of nuclease protection of HP96. The polymerase subunit (Pol) binds to the primer template at a position which protects 18 bases of single-stranded DNA and 14 bp of double-stranded DNA. Its position is approximately centered on the 3′ terminus of the priming strand. The addition of UL42 to reactions containing Pol results in the protection of additional duplex sequences but does not alter the points of contact in the single-stranded region of the substrate.

that unlike Pol, UL42 does not interact specifically with a primer template junction. Instead, we found that UL42 interacted nonspecifically with the double-stranded portion of the HP96 substrate and did not interact to a detectable degree with the single-stranded region. These findings are consistent with previous studies on the DNA-binding properties of UL42, which showed that this protein is a double-stranded-specific DNA-binding protein (19, 59). We conclude from these findings that UL42 does not have a function analogous to that of the class of polymerase accessory proteins exemplified by the eukaryotic accessory protein replication factor C (RF-C) (4, 46, 57, 58). This protein interacts specifically with a primer template junction and appears to function in DNA replication by recruitment of the DNA polymerase and another accessory subunit, proliferating cell nuclear antigen (PCNA).

Although UL42 alone did not interact specifically with the primer template, UL42 did have a specific effect on the pattern of nuclease protection observed with Pol. In particular, we found that the presence of UL42 increased the extent of protection of double-stranded product DNA but had no effect on the protection of single-stranded template DNA. There are at least two explanations for this finding. First, it is possible that the binding of UL42 to Pol causes a conformational change in Pol that alters and extends its interaction with double-stranded DNA. Second, it is possible that the extension of the nuclease protection pattern seen in the presence of Pol and UL42 results from an interaction of UL42 with the substrate that is directed to a specific site by protein-protein interactions between Pol and UL42. Although there is no direct experimental evidence that bears on this subject, we favor the idea that the double-stranded DNA-binding activity of UL42 is an important aspect of its function. We therefore propose that the extension of nuclease protection observed following the addition of UL42 reflects a direct interaction between the double-stranded product DNA and UL42 as depicted schematically in Fig. 7.

Quantitative analysis of the interaction between the DNA polymerase subunits and HP96 by the gel mobility shift assay showed that in addition to altering the details of the nuclease protection pattern of the enzyme, UL42 also served to increase the affinity of Pol for the model primer template. The K_D of Pol for the substrate was 7.1×10^{-9} M, and the K_D for Pol/UL42 was 7.8×10^{-10} M, a difference of nearly 10-fold. Again, there are two ways in which this difference can be interpreted: UL42 may cause a structural change in the Pol polypeptide that alters its affinity for the primer template, or UL42 may increase the affinity of Pol by acting as a tether or clamp as depicted in Fig. 7. For the reasons mentioned above, we favor the latter view. According to this model, the increase in the affinity of Pol for substrate is due to the contribution of binding free energy derived both from UL42-DNA interaction and from UL42-Pol interaction. We have determined that the affinity of UL42 for HP96 is 1.1×10^{-9} M. The affinity of the Pol-UL42 interaction has been measured to be within a range of 1×10^{-8} M (24) and 5×10^{-9} M (21). According to the calculations of Ackers et al. (1), the free energy derived from these two interactions

should easily account for the increase in the affinity of the polymerase for the primer template in the presence of UL42.

Interpretation of both the nuclease protection experiments and the mobility shift assays reported in this paper are complicated by the fact that the preparation of Pol used in these experiments contains an approximately equimolar mixture of Pol and a partially proteolyzed derivative of Pol that is shorter by approximately 10 kDa (see Fig. 2 in reference 22). Two considerations suggest that the presence of this proteolytic derivative has no effect on our overall conclusions. First, the pattern of nuclease protection observed with Pol in the presence of added UL42 was identical to that seen with the purified Pol-UL42 heterodimer, which is not contaminated by proteolyzed Pol. Thus, the differences between the footprints of Pol and Pol-UL42 cannot be attributed to the presence of the contaminant. Second, the presence of two closely spaced bands in the gel mobility shift assays using Pol suggest that the proteolyzed derivative also binds to the HP96 substrate. An analysis of a number of substrate titration experiments showed that the affinities the two forms of Pol for HP96 were identical. It therefore seems unlikely that the missing fragment of Pol polypeptide has any effect on substrate binding by Pol itself. Close inspection of the data in Fig. 3 does suggest, however, that the shortened derivative of Pol may not interact as well as full-length Pol with UL42 (compare the intensities of the two Pol bands in the presence and absence of UL42). For this reason, data from binding experiments in which Pol and UL42 were mixed in vitro were not used in the analysis of the affinity of Pol-UL42 for HP96. In this context, it is of interest that mutational analyses of Pol have shown that a short region at the carboxy terminus of the polypeptide is both necessary and sufficient for interaction with UL42 (13, 15, 56), while deletion of this region had no effect on Pol catalysis. It seems likely, therefore, that the shortened derivative of Pol contaminating our purified preparations is due to proteolytic removal of a segment of the polypeptide from the carboxy terminus. It may be that the absence of UL42 bound to this region of Pol renders the carboxy-terminal segment more susceptible to proteolytic cleavage.

Taken together, our data are consistent with the idea that UL42 increases the processivity of the HSV DNA polymerase by acting as a sliding clamp, increasing the affinity of Pol for its nucleic acid substrate and decreasing the probability that Pol will dissociate from its substrate following a round of catalysis. In this regard, UL42 is like several other DNA polymerase accessory factors that have been studied (for a review, see reference 32), such as the β subunit of *E. coli* Pol III (35, 43, 52), the eukaryotic replication factor PCNA (3, 34, 44, 48, 54, 57), the gene 45 protein for T4 phage DNA polymerase (26, 29), and the thioredoxin subunit of the T7 DNA polymerase (27, 39, 53). Although the overall functions of these various proteins appear to be similar in that they increase the processivity of the cognate DNA polymerase, there are important differences in mechanism. For example, the β subunit of Pol III does not have a high intrinsic affinity for DNA. Rather, in the presence of γ complex and ATP, the β subunit is assembled onto DNA as a dimeric torus that is sterically retained on duplex DNA (35). PCNA is thought to function in an analogous fashion in which the ATP-dependent assembly of a trimeric PCNA torus is accomplished by the primer-binding factor RF-C (57). Similarly, the processive form of the T4 DNA polymerase requires the presence of the gene 45 protein. The assembly of the polymerase-gene 45 complex requires ATP and is mediated by T4 genes 44 and 62 (26, 29). The mechanism by which UL42 acts can be distinguished from that of β, PCNA, and gene 45 protein in a number of ways. First, UL42 has high intrinsic affinity for DNA. Second, it forms a tight heterodimeric complex with the DNA polymerase, and there is no evidence that UL42 is assembled into a multimeric torus around DNA. Finally, the function of UL42 does not require the participation of a primer recognition protein, nor does it require ATP. UL42 appears to be most similar to the thioredoxin subunit of the T7 DNA polymerase, which is thought to function by causing a decrease in the dissociation rate of the polymerase from the primer template (27, 39, 53). A key question that now remains to be answered is the mechanistic basis for sliding along DNA by proteins like UL42 that have a high intrinsic affinity for DNA. It seems likely that the answer to this question will require detailed structural information.

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