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The Herpes Simplex Virus Type 1 UL42 Gene Product: a Subunit of DNA Polymerase That Functions To Increase Processivity

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Genetic experiments 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, and a number of studies have suggested that these two proteins specifically interact. We have confirmed and extended these findings. The viral DNA polymerase from HSV-1-infected cells has been purified as a complex containing equimolar quantities of the UL30 (Pol, the catalytic subunit) and UL42 polypeptides. Sedimentation and gel filtration analyses of this complex are consistent with the idea that the complex consists of a heterodimer of Pol and UL42. A complex with identical physical and functional properties was also purified from insect cells coinfected with recombinant baculoviruses expressing the two polypeptides. Therefore, the formation of the Pol-UL42 complex does not require the participation of any other HSV-encoded protein. We have compared the catalytic properties of the Pol-UL42 complex with those of the isolated subunits of the enzyme purified from recombinant baculovirus-infected insect cells. The specific activity of the catalytic subunit alone was nearly identical to that of the complex when assayed on activated DNA. When assayed on a defined template such as singly primed M13 DNA, however, the combination of Pol and UL42 utilized fewer primers and formed larger products than Pol alone. Template challenge experiments demonstrated that the Pol-UL42 complex was more highly processive than Pol alone. Our data are consistent with the idea that the UL42 polypeptide is an accessory subunit of the DNA polymerase that acts to increase the processivity of polymerization.

The genome of herpes simplex virus type 1 (HSV-1) is a 153-kb linear double-stranded DNA molecule containing three *cis*-acting origins of replication (43–45, 49, 53) and at least 72 open reading frames (27). Genetic studies have shown that seven viral genes are essential for HSV origin-dependent DNA replication (4–9, 24, 39, 40, 51, 52, 54, 55), and biochemical functions have been ascribed to the products of several of these genes: *UL9* encodes an origin recognition protein (32); *UL5*, *UL8*, and *UL52* encode the three subunits of a helicase-primase complex (11); *UL29* encodes a single-stranded DNA-binding protein (ICP8) (9, 36, 52); *UL30* encodes a DNA polymerase (7, 8, 39); and *UL42* encodes a double-stranded DNA-binding protein that has been reported to stimulate the activity of the DNA polymerase (13, 14, 35). Although genetic studies strongly suggest that no other HSV gene products are essential for viral DNA replication, it is possible that one or more as-yet-unidentified host cell proteins might also play an essential role.

The HSV DNA polymerase was first detected as a novel enzyme activity present in extracts of HSV-infected cells. This activity was shown to be dependent on the product of the *pol* gene (UL30) by analysis of temperature-sensitive mutants and viral mutants with altered sensitivities to PP_i and nucleoside analog inhibitors (7, 8, 39). DNA sequence analyses of the *pol* gene predict a polypeptide chain of 137 kDa (15, 41), and purified preparations of the enzyme from HSV-infected cells have been shown to contain a polypeptide of approximately that size (31, 37). Sequence comparison studies have demonstrated that the *pol* gene polypeptide is a member of the eucaryotic DNA polymerase α family of DNA polymerases (29). The *pol* gene polypeptide has been shown to be catalytically active in the absence of other viral polypeptides by overexpression in yeast cells (16), by in vitro transcription-translation (12), and by overexpression in insect cells infected with a recombinant baculovirus (25). In addition to DNA polymerase activity, the Pol polypeptide also has an intrinsic 3′-5′ exonuclease proofreading activity (19, 25, 31) as well as a 5′-3′ exonuclease activity capable of functioning as a RNase H (10, 25).

It was reported some time ago that HSV DNA polymerase activity is associated with a second polypeptide of about 50 to 60 kDa (37, 48). This polypeptide has since been shown to be the product of the *UL42* gene (14, 35). The nature and stoichiometry of the association between Pol and UL42 is not known. There is evidence that the UL42 protein stimulates the DNA polymerase activity of the Pol polypeptide, although the mechanistic basis for this stimulation has not been explored (13). UL42 is more abundant than Pol in HSV-infected cells and can be purified of detectable DNA polymerase activity (13, 14, 48). This form of the UL42 protein binds double-stranded DNA in an apparently noncooperative, sequence-independent manner (14, 48). The relationship between the DNA-binding activity of UL42 and DNA replication is not known. UL42 is a phosphoprotein (26), but again, the functional significance of phosphorylation has not yet been studied.

In this report, we have examined both the physical and functional interactions between the Pol polypeptide and UL42 protein. We have shown that the HSV DNA polymerase can be purified from virus-infected HeLa cells as a heterodimer of the Pol and UL42 polypeptides. We have purified the individual components of this heterodimer from

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insect cells infected with recombinant baculoviruses, and have additionally shown that the Pol-UL42 heterodimer can be purified from insect cells coinfected with the two recombinant baculoviruses. Finally, by comparing the catalytic properties of these purified proteins on singly primed M13 DNA templates, we have shown that the UL42 protein acts to increase the processivity of the DNA polymerase.

MATERIALS AND METHODS

Cells and viruses. Vero and ICP8-transformed U35 Vero cells (34) were propagated in Eagle's medium containing 10% fetal bovine serum. HeLa suspension cells were maintained in minimal essential medium containing 5% horse serum. Virus stocks of the KOS strain of HSV-1 were grown and assayed as described previously (6). *Spodoptera frugiperda* (Sf9) cells were maintained in TMNFH medium (GIBCO) containing 10% fetal bovine serum, and wild-type *Autographica californica* nuclear polyhedrosis virus (AcNPV) and recombinant baculoviruses were propagated as previously described (46).

Construction of the AcNPV/UL42 baculovirus recombinant. The baculovirus recombinant AcNPVUL42 contains the HSV-1 UL42-coding region inserted downstream of the baculovirus polyhedrin promoter and expresses the 51-kDa UL42 gene product. The plasmid pNN4, which was the source of the UL42-coding sequences, contains the entire *UL42* gene and approximately 300 bp of 5′ and 3′ flanking sequences inserted into the *Sma*I site of pBluescribe (54). A unique *Bgl*II site was engineered 8 bp upstream of the ATG start site of the UL42 open reading frame in pNN4 by oligonucleotide-directed mutagenesis (20). The UL42-coding region was removed by digestion with *Bgl*II and *Eco*RI and ligated into the *Bam*HI and *Eco*RI sites of pVL1392 (22) to produce the plasmid pVLUL42. Sf9 cells were cotransfected with AcNPV DNA and pVLUL42 plasmid DNA, and several viral plaques with altered morphology were isolated as previously described (46). Lysates of infected cells were screened for *UL42* sequences by DNA hybridization and for the expression of UL42 protein by Western immunoblot analysis of sodium dodecyl sulfate (SDS) protein gels using rabbit antisera as described previously (33).

Buffers. Buffer A contained 20 mM Tris hydrochloride (pH 8.0), 0.5 mM dithiothreitol (DTT), 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Buffer B contained 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.6), 0.5 mM DTT, 0.5 mM $MgCl_2$, 10 mM $NaHSO_3$, 0.5 mM phenylmethylsulfonyl fluoride, and 2 μg (each) of leupeptin and pepstatin A per ml. Buffer C was 20 mM HEPES (pH 7.6), 0.5 mM DTT, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Buffer D was 20 mM Tris hydrochloride (pH 7.5), 3 mM $MgCl_2$, 4% glycerol, 0.5 mM DTT, 0.1 mM EDTA, 50 μg of bovine serum albumin (BSA) per ml, 150 mM $(NH_4)_2SO_4$. Buffer E contained 20 mM HEPES (pH 7.6), 1 mM $MgCl_2$, 4% glycerol, 0.1 mM EDTA, 1 mM DTT, and 75 mM NaCl.

Protein purification. (i) HSV DNA polymerase. Six liters of HeLa suspension cells was infected with HSV-1 at a multiplicity of infection of 20. At 16 h after infection, the cells were harvested by centrifugation, washed with 200 ml of cold phosphate-buffered saline, and resuspended in 40 ml of hypotonic buffer (buffer B). The cells were allowed to swell on ice for 15 min and were lysed with a Dounce homogenizer. Nuclei were pelleted by low-speed centrifugation (2,000 × *g*) and were resuspended in 20 ml of buffer B, lysed with the addition of an equal volume of buffer B containing 3.4 M NaCl, and incubated on ice for 1 h. The nuclear extract was clarified by centrifugation at 100,000 × *g* for 1 h, and the supernatant fraction was removed and dialyzed extensively against buffer C containing 50 mM NaCl. Insoluble protein was removed from the nuclear extract by centrifugation, and the soluble protein fraction was applied to a 25-ml phosphocellulose column (5.1 by 2.5 cm) previously equilibrated in the same buffer. Proteins were eluted from the column with a 250-ml linear gradient containing 0.05 to 0.8 M NaCl in buffer C. Fractions containing HSV DNA polymerase (assayed as described below) were pooled and dialyzed against buffer C containing 0.1 M NaCl and applied to a 10-ml single-stranded DNA agarose column (Bethesda Research Laboratories) equilibrated in the same buffer. Proteins were eluted with a 100-ml linear salt gradient containing 0.1 to 0.8 M NaCl in buffer C. Fractions containing DNA polymerase activity were pooled and dialyzed against buffer A. The partially purified polymerase was further fractionated on a Mono Q HR 5/5 anion exchange column (Pharmacia) at 0.5 ml/min with a 40-ml (80 min) linear salt gradient from 0 to 1 M NaCl in buffer A. Fractions containing DNA polymerase activity were concentrated to 0.6 ml in Centricon 30 concentrators (Amicon) and loaded onto two 12.4-ml 15 to 35% glycerol gradients in buffer C–0.1 M NaCl–0.01% Nonidet P-40. After centrifugation in an SW40 Ti rotor for 56 h at 40,000 rpm, 0.3-ml fractions were collected from the bottoms of the tubes, assayed for DNA polymerase activity, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

For the purification of the isolated catalytic subunit (Pol), 40 225-cm^2 flasks of Sf9 cells were infected with the baculovirus BP58 (25) at a multiplicity of 10 PFU per cell; for the purification of recombinant Pol-UL42 complex, 40 225-cm^2 flasks of Sf9 cells were coinfected with BP58 and AcNPV/UL42 at multiplicity of 10 PFU of each virus per cell. In both cases, the infected cells were shaken off the flasks at 60 h after infection, washed in serum-free Grace's medium, and resuspended in 40 ml of buffer B. The isolation of nuclei and the subsequent purification of these proteins were identical to the procedure described above for the Pol-UL42 complex from HSV-1-infected cells.

(ii) UL42 protein. Approximately 90% of the UL42 protein was physically separated from the Pol-UL42 complex during early stages of Pol-UL42 purification. Fractions containing UL42 protein were identified by Western blot analysis of SDS protein gels using rabbit antisera to the UL42 gene product (33). The majority of free UL42 eluted before the Pol-UL42 complex during fractionation on phosphocellulose (see above). Fractions containing UL42 protein were pooled and dialyzed against 0.05 M NaCl in buffer C and reapplied to a 5-ml phosphocellulose column. Bound proteins were eluted from the column with a 50-ml linear gradient, as described above. Fractions containing UL42 were pooled and dialyzed against 0.05 M NaCl in buffer C and applied to a 3-ml double-stranded DNA cellulose column (Sigma). Proteins were eluted from the column with a 30-ml linear gradient containing 0.05 to 0.70 M NaCl in buffer C. Fractions containing UL42 protein were pooled and dialyzed against buffer A and applied to a Mono Q HR 5/5 column (Pharmacia). The column was eluted as described above for the Pol-UL42 complex by using a linear salt gradient from 0 to 0.5 M NaCl in buffer A. To remove residual polymerase activity, UL42-containing fractions were pooled and concentrated to 0.6 ml with Centricon 30 concentrators (Amicon)

and sedimented through 12.4-ml glycerol gradients as described above for Pol-UL42.

UL42 protein from recombinant baculovirus-infected insect cells was purified from 40 225-cm^2 flasks of Sf9 cells infected with AcNPV/UL42 at a multiplicity of 10 PFU/cell. Nuclear extracts were prepared 60 h after infection as described above and routinely contained 15 mg of UL42 per 10^9 cells. The procedure for the purification of the recombinant UL42 protein was identical to that described for UL42 from HSV-infected cells except that the second phosphocellulose step was omitted.

(iii) ICP8. ICP8 was purified from U35 Vero cells (34) by a modification of the method of O'Donnell et al. (31). Nuclei were prepared from 40 roller bottles of U35 Vero cells 18 h after infection with HSV-1 at a multiplicity of infection of 5 and extracted in buffer B and high salt as described for the Pol-UL42 complex. The clarified nuclear extract was dialyzed against 0.1 M NaCl in buffer C and fractionated through a 15-ml single-stranded DNA agarose column. The column was eluted with a 150-ml linear salt gradient containing 0.1 to 1.5 M NaCl in buffer C. Column fractions were analyzed by immunoblotting of protein gels with rabbit antisera to ICP8 (33). Fractions containing ICP8 were dialyzed against buffer C and applied to a 4-ml heparin agarose column (Sigma) equilibrated in the same buffer. Proteins were eluted from the column with a 40-ml linear salt gradient containing 0 to 0.75 M NaCl in buffer C. Fractions containing ICP8 were pooled and concentrated to a volume of 0.3 ml and layered onto a 15 to 35% glycerol gradient, as described for the purification of the Pol-UL42 complex.

Determination of hydrodynamic properties. The sedimentation coefficient ($S_{20,w}$) of each purified protein was determined from the rate of sedimentation of the protein during the glycerol gradient sedimentation step of purification (see above). The proteins with known sedimentation coefficients that were employed as standards on parallel gradients were as follows: BSA, 4.4S; alcohol dehydrogenase, 7.6S; β-amylase, 8.9S. The Stokes radius of each purified protein was determined by gel filtration analysis on a Superose 12 10/30 column (Pharmacia) according to the method of Ackers (1). The standards of known Stokes radii used to calibrate the columns were as follows: BSA, 3.64 nm; alcohol dehydrogenase, 4.52 nm; β-amylase, 5.14 nm.

DNA templates. Singly primed single-stranded DNA substrates were produced by hybridizing 50 pmol of synthetic oligonucleotide primer to 5 pmol of the appropriate phage DNA (M13mp19 or φX174) in a reaction volume of 100 μl containing 50 mM Tris hydrochloride (pH 7.5), 5 mM $MgCl_2$, and 100 mM NaCl. The hybridization mixture was incubated at 90°C for 5 min and allowed to cool slowly to room temperature for 1 h. Excess primer was removed by gel filtration on a 10-ml Bio-Gel A15m column (26 by 0.7 cm; Bio-Rad) equilibrated in a solution containing 10 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, and 50 mM NaCl. The oligonucleotides used were 5′-GGCGCATAACGATACCA CTGACC, which is complementary to nucleotides 2067 to 2045 of φX174 DNA, and 5′-CAGTCACGACGTTGTAA AACGACGGCCAGT, which is complementary to nucleotides 6300 to 6271 of M13mp19 DNA.

DNA polymerase assays. DNA polymerase activity was assayed during purification in 50-μl reaction mixtures containing 5 μg of activated calf thymus DNA (Sigma); 0.5 mM ATP; 50 μM (each) of dGTP, dATP, and dTTP; and 5 μM [α-^{32}P]dCTP (5×10^3 to 15×10^3 cpm/pmol) in buffer D. DNA synthesis was initiated with the addition of 1 μl of the column fractions. The reactions were terminated with the addition of 2 ml of cold 10% trichloroacetic acid containing 400 μg of BSA after incubation at 37°C for 30 min, and the amount of incorporation of acid-insoluble radiolabeled nucleotide was measured in a liquid scintillation counter. One unit of polymerase activity was defined as the amount of enzyme required to incorporate 1 pmol of [α-^{32}P]dCTP into acid-insoluble material in 30 min at 37°C.

The specific details of DNA synthesis on singly primed M13 or φX single-stranded circular DNA are given in the figure legends. In general, standard reaction mixtures (50 μl) contained 20 to 50 fmol of substrate DNA (as circles); 0.5 mM ATP; 60 μM (each) of dGTP, dATP, and dTTP; 20 μM [α-^{32}P]dCTP (3,000 to 8,000 cpm/pmol); 50 μg of BSA per ml; and 10 to 2,000 fmol of DNA polymerase. The reactions were incubated at 37°C and terminated with the addition of an equal volume of 1% SDS–40 mM EDTA–60 μg of sonicated calf thymus DNA per ml. After precipitation in ethanol, the reaction products were analyzed by gel electrophoresis in 1% alkaline or neutral agarose gels or in 6% acrylamide-urea gels followed by autoradiography.

RESULTS

HSV DNA polymerase is a heterodimer of Pol and UL42. It has been reported that the HSV DNA polymerase is associated with UL42 polypeptide (14, 37, 48). To gain further insight into the nature of this association, we purified the virus-specific DNA polymerase from HSV-infected HeLa cells. During the course of purification, we assayed for DNA polymerase activity by using activated DNA as a primer-template and for Pol and UL42 antigen by immunoblot analysis with monospecific antisera (33) raised against either a bacterial fusion protein (Pol) or a peptide predicted from the deduced amino acid sequence (UL42) (Fig. 1; Table 1). At all steps in purification, DNA polymerase activity copurified with Pol antigen, as expected (data not shown). During the early stages of purification the majority of the UL42 protein was separated from DNA polymerase activity (Fig. 1A and B). A fraction of UL42, however, eluted with the polymerase and was observed as a small shoulder of the major UL42 peak during the second chromatographic purification step (heparin agarose chromatography; Fig. 1B). Glycerol gradient sedimentation of the peak polymerase fractions following Mono Q chromatography produced two distinct peaks of UL42 containing approximately equal amounts of antigen (Fig. 1C). The faster-sedimenting form of UL42 protein cosedimented with the DNA polymerase activity. These results suggest that a fraction of the UL42 protein forms a stable complex with the polymerase catalytic subunit. The sedimentation rate of the more slowly sedimenting form of UL42 corresponded to the molecular mass expected for monomeric UL42 protein (51 kDa [28]). We estimate that approximately 5 to 10% of the infected-cell UL42 protein copurifies with the DNA polymerase.

The polypeptide composition of the purified DNA polymerase was examined by SDS-PAGE followed by staining with Coomassie blue (Fig. 2A, lane 4) and by immunoblot analysis (Fig. 2B, lane 1). In the stained gel, three major polypeptides, as well as several minor species, are apparent. The larger major polypeptide, with an observed molecular weight (MW) of 140,000, corresponds to the Pol polypeptide; the smallest major polypeptide, with a MW of 60,000, corresponds to UL42; the third major polypeptide, with a MW of ~70,000, does not precisely cosediment with DNA polymerase activity (data not shown) and is present in other preparations of the polymerase in different amounts relative

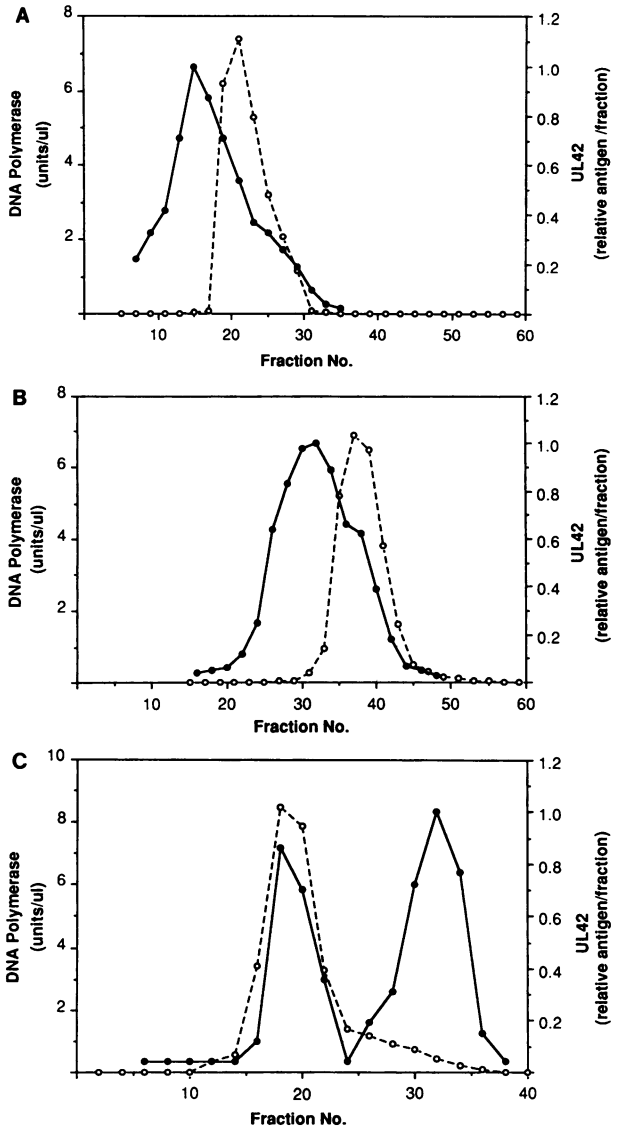

FIG. 1. Copurification of UL42 and DNA polymerase activity. DNA polymerase was purified from HSV-infected HeLa cells, as described in Materials and Methods. DNA polymerase activity (○) was assayed by using activated DNA as a template, and UL42 antigen (●) was quantitated by densitometric analysis of Western blots of each fraction; the values plotted refer to relative UL42 antigen per fraction, normalized to the peak fraction in each panel. (A) Phosphocellulose chromatography; (B) single-stranded DNA agarose chromatography; (C) glycerol gradient sedimentation.

to the other two polypeptides. We believe that this polypeptide is not a component of the DNA polymerase. The minor polypeptides in the range from 75 to 130 kDa were shown by immunoblot analysis to be related to the Pol polypeptide (Fig. 2B) and probably represent products of limited proteolysis. Densitometric scanning of the stained gel indicated that Pol and UL42 are present in an apparent molar ratio of 1:1.1. The MW of the purified enzyme (Table 2) was estimated from its sedimentation coefficient as determined by glycerol gradient sedimentation analysis and from its Stokes radius as determined by gel filtration analysis. The observed value of 181 kDa is in close agreement with the value predicted from the deduced amino acid sequences of Pol (137 kDa) and UL42 (51 kDa) assuming that the structure of the native enzyme is a heterodimer of Pol and UL42. We cannot at present rule out the possibility that one or more of the small, minor polypeptide species present in the most purified preparations is also a subunit of the enzyme. We think it unlikely, however, since a complex of Pol and UL42 with identical physical and enzymatic properties, but with different contaminating polypeptides, has been purified from insect cells infected with recombinant baculoviruses expressing Pol and UL42 (see below).

TABLE 1. DNA polymerase purification

Fraction	Total activity (units, 10^3)	% Recovery	Sp act (units/μl)	Relative purification
Pol-UL42				
Nuclear extract	660	100	2.3	
Phosphocellulose	458	69	10.6	4.6
Single-stranded DNA agarose	337	51	166	72
Mono Q	280	42	170	73
Glycerol gradient	192	29	571	248
Pol				
Nuclear extract	2,600[a]			
Phosphocellulose	341	100	12.7	
Single-stranded DNA agarose	186	54	75.5	5.9
Mono Q	164	48	214	16.8
Glycerol gradient	107	31	348	27
Pol-UL42(bv)[b]				
Nuclear extract	1,800[a]			
Phosphocellulose	810	100	26.1	
Single-stranded DNA agarose	378	46	87.5	3.3
Mono Q	134	16	37.2	1.4
Glycerol gradient	107	13	238	9.1

[a] Nuclear extract also contains baculovirus DNA polymerase activity (25); yield and relative purification are calculated relative to phosphocellulose.
[b] bv, Purified from recombinant baculovirus-infected Sf9 cells.

Construction of recombinant baculovirus expressing UL42. To obtain a source of UL42 protein free of other HSV-encoded proteins, we constructed a recombinant baculovirus in which the UL42-coding sequences were expressed at high levels under the control of the polyhedrin promoter. A restriction fragment from the plasmid pNN4 (54) was cloned into M13mp19, and oligonucleotide-directed mutagenesis was used to create a site for the restriction enzyme *Bgl*II 8 bp upstream from the amino terminus of the UL42 open reading frame. A *Bgl*II to *Eco*RI fragment containing the complete UL42 open reading frame was then inserted between the *Bgl*II and *Eco*RI sites of the plasmid vector pVL1392 (22). The resulting plasmid was cotransfected into Sf9 cells with infectious AcNPV DNA, and recombinant viruses were selected on the basis of altered plaque morphology and the presence of UL42 DNA sequences. One such recombinant, named AcNPV/UL42, was selected for further study. Figure 3 shows the results from SDS-PAGE analysis of extracts prepared from Sf9 cells infected with AcNPV/UL42. An abundant polypeptide with an electrophoretic mobility identical to that of UL42 purified from HSV-infected HeLa cells (apparent MW, 60,000) was observed in an extract of the nuclei of such cells (Fig. 3, lane 4). This polypeptide was not observed in a parallel control extract of Sf9 cells infected with the recombinant baculovirus BP58 (Fig. 3, lane 2) (25); moreover, it reacted strongly with monospecific anti-UL42

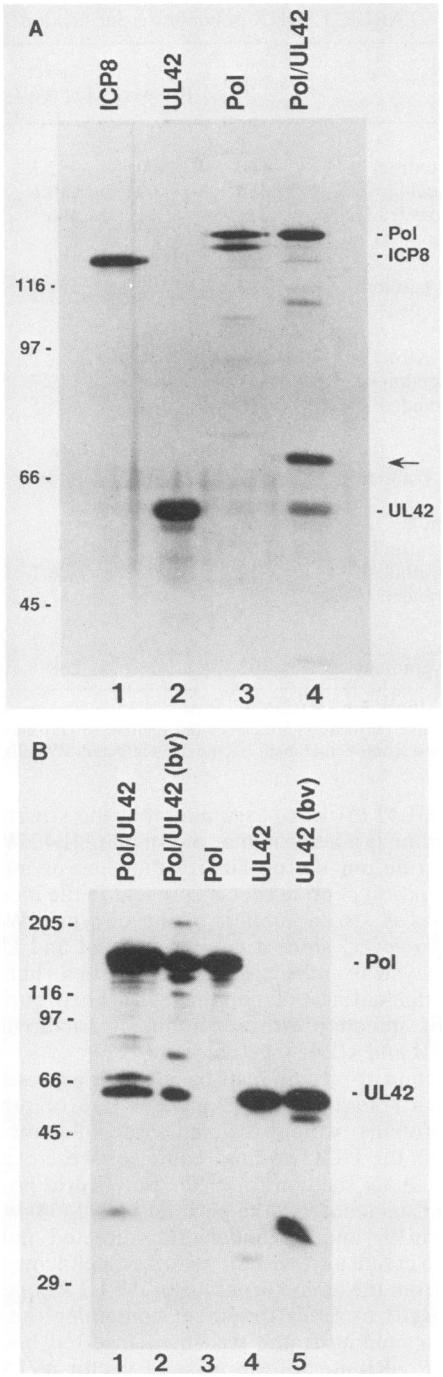

FIG. 2. SDS-PAGE analysis of purified proteins. Electrophoresis was performed as previously described (33) by using a resolving gel of 7.5% acrylamide. The numbers at the left of each panel indicate the electrophoretic mobility and size (in kilodaltons) of marker proteins run on the same gel: myosin, 205; β-galactosidase, 116; phosphorylase B, 97; BSA, 66; egg albumin, 45; carbonic anhydrase, 29. (A) Each lane contained 10 μg of the most highly purified fraction of the indicated protein. Following electrophoresis, the gel was stained with Coomassie brilliant blue. The arrow indicates a polypeptide of ~70 kDa which we consider a contaminant of the enzyme (see Results for details). (B) Each lane contained 1 μg of the most highly purified fraction of the indicated protein. Following electrophoresis, the resolved polypeptides were transferred to a polyvinylidinedifluoride membrane (Immobilon; Millipore) and probed with a mixture of antisera against Pol and UL42, as previously described (33).

TABLE 2. Hydrodynamic properties[a] of purified proteins

Protein	$S_{20,w}$ (10^{-13} s)	Stokes radius (nm)	MW	f/f_0[b]
Pol-UL42	8.0	5.16	181,000	1.18
Pol	6.8	4.49	133,000	1.14
UL42	3.0	4.25	56,000	1.44

[a] $S_{20,w}$ and Stokes radius were determined as described in Materials and Methods. Molecular weight was calculated from those values according to the equation: $MW = S_{20,w}6R\pi\eta r/k(1 - \nu\rho)$, where R is the ideal gas constant, η is the viscosity of water, r is the Stokes radius, k is the Boltzmann constant, ν is the partial specific volume of the protein, assumed to be 0.74 cm^3/g, and ρ is the density of water.

[b] The frictional coefficient f/f_0 equals $r/\{[3\nu(MW)/4\pi N]^{0.33}\}$, where $N = 6.02 \times 10^{23}$ mol^{-1}.

sera on immunoblot analysis (data not shown), confirming the identity of this polypeptide as UL42. We estimate that approximately 15 mg of UL42 polypeptide was produced in 10^9 AcNPV/UL42-infected cells, and at least 95% of this protein was recovered in soluble form in the high-salt nuclear extract. This represents approximately 10- to 15-fold overproduction relative to HSV-infected HeLa cells.

Formation of the Pol-UL42 heterodimer in Sf9 cells expressing Pol and UL42. The availability of recombinant baculoviruses expressing both Pol (25) and UL42 allowed us to ask

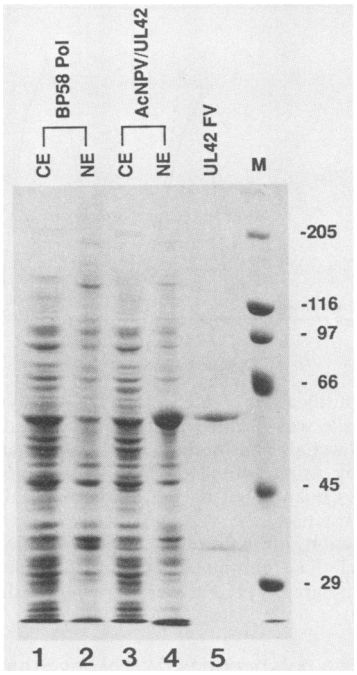

FIG. 3. SDS-PAGE analysis of Sf9 cells infected with AcNPV/UL42. Sf9 cells were infected with the recombinant baculovirus BP58 (expressing Pol) or AcNPV/UL42 at a multiplicity of infection of 10. At 60 h postinfection, cells were harvested and fractionated into cytoplasmic (CE) or high-salt nuclear (NE) extracts, as described in Materials and Methods. An aliquot of each extract corresponding to soluble protein from 5×10^5 cells was fractionated on an SDS-polyacrylamide gel containing 7.5% acrylamide. Following electrophoresis, the gel was stained with Coomassie brilliant blue. Six micrograms of glycerol gradient-purified UL42 (see Table 3) were loaded onto the last lane on the right. The numbers at the right indicate the electrophoretic mobilities and sizes (in kilodaltons) of marker proteins (M) run on the same gel.

TABLE 3. Purification of UL42

Fraction	UL42 antigen (mg)[a]	Total protein (mg)	Relative purification
Nuclear extract	3.2	411	
Phosphocellulose I	2.7	42.0	8.2
Phosphocellulose II	1.7	13.2	16.5
Double-stranded DNA cellulose	1.1	1.1	128
Glycerol gradient	0.9	0.9	128

[a] The values for UL42 antigen were determined by densitometric analysis of Western blots of glycerol gradient-purified protein, which is essentially homogeneous (Fig. 2), as a reference.

whether the formation of the complex between these polypeptides can occur in the absence of other HSV-encoded polypeptides. Sf9 cells were coinfected with BP58 and AcNPV/UL42, and HSV DNA polymerase was purified from a nuclear extract of these cells. During the course of purification, DNA polymerase activity was assayed by using activated DNA as primer-template; Pol and UL42 antigen were monitored by immunoblot analysis. As in the case of the DNA polymerase from HSV-infected HeLa cells, Pol polypeptide copurified with DNA polymerase activity and with a small fraction of the UL42 polypeptide, which was expressed at a much higher level than the Pol polypeptide in coinfected cells. SDS-PAGE and immunoblot analysis of the purified protein revealed that Pol and UL42 were present in approximately the same ratio as observed with the enzyme purified from HSV-infected cells (Fig. 2B). Moreover, the sedimentation coefficient and Stokes radius of the purified DNA polymerase (Table 2) and all catalytic properties tested (Table 1; see below) were also identical to that observed with the polymerase from HSV-infected cells. We conclude that no additional HSV-encoded proteins are required for the formation of functional Pol-UL42 heterodimer.

Purification of the isolated subunits of HSV DNA polymerase. To determine the contribution of the two subunits of the DNA polymerase to its overall function, we have purified the individual subunits. In the case of the large subunit, this was accomplished as previously described (25) by using the baculovirus expression system. The results of the purification are summarized in Table 1, and SDS-PAGE analysis of the purified protein is shown in Fig. 2. The MW of the purified Pol polypeptide was determined by sedimentation and gel filtration analyses (Table 2). The observed value of 133,000 is in excellent agreement with the size of the polypeptide predicted from the deduced amino acid sequence (137 kDa) and suggests that the isolated large subunit exists as a monomer in solution. The fact that the purified native Pol subunit has a smaller observed MW than the DNA polymerase purified from HSV-infected cells (or from insect cells also expressing UL42) lends further support to the conclusion that the HSV DNA polymerase contains more than one subunit.

The UL42 polypeptide was purified from two sources by using immunoblot analysis as an assay during purification. Since, as previously mentioned, UL42 is present in HSV-infected cells in large excess relative to the amount of Pol-UL42 complex, it was possible to purify UL42 free of detectable DNA polymerase from that source; the results of the purification are summarized in Table 3, and SDS-PAGE analysis of the purified protein is shown in Fig. 2. From a practical perspective, the major difficulty in purifying UL42 from HSV-infected cells was contamination by the HSV-encoded alkaline nuclease. The peak of nuclease activity substantially overlapped the peak of UL42 antigen in most of the purification procedures tested. The greatest separation of the two proteins was achieved by phosphocellulose chromatography; the peak of UL42 elution from the column occurred at 0.3 M NaCl, and the peak of nuclease activity occurred at 0.2 M NaCl. Two successive passes over phosphocellulose were required to obtain acceptable levels of separation. UL42 was also purified from Sf9 cells infected with the baculovirus AcNPV/UL42 by a nearly identical procedure; the major differences between the two procedures are a consequence of the facts that recombinant UL42 accounts for a higher percentage of the starting material and therefore required less purification and that nuclear extracts of baculovirus-infected Sf9 cells do not contain a nuclease with chromatographic properties similar to UL42. We did not observe any physical or biochemical differences between the two preparations of UL42. As shown in Table 2, the observed MW of purified UL42 was approximately 56,000, suggesting that isolated UL42 polypeptide is a monomeric protein like the Pol subunit.

Pol-UL42 utilizes a long single-stranded template more efficiently than Pol alone. As we have previously reported (25), the specific activity of the Pol-UL42 complex did not differ significantly from that of the purified catalytic subunit when assayed with activated DNA as primer-template (Table 1). On the basis of this result and by analogy with other DNA polymerase systems, it seemed possible that UL42 might act as an accessory subunit to increase the efficiency of polymerization on long single-stranded templates. This possibility was tested by comparing the activity of Pol-UL42 with that of Pol on singly primed M13 single-stranded DNA in the presence and absence of exogenous UL42 protein. Figure 4 shows an analysis by alkaline agarose gel electrophoresis of the products formed at various ratios of polymerase to primer-template in such an experiment. At all ratios of enzyme to template tested, the products synthesized by Pol-UL42 consisted almost exclusively of full-length M13 strands. Identical results were obtained by using Pol-UL42 purified from HSV-infected HeLa cells and Pol-UL42 purified from baculovirus-infected Sf9 cells. In either case, the size distribution of products was not altered by the addition of purified UL42 protein (data not shown). In contrast, at low ratios of the isolated Pol subunit to template, the predominant products were much smaller in size than full-length M13 strands, with the majority of product consisting of specific bands representing pause sites on the template. We presume that these pause sites represent sites of template secondary structure, but we have not characterized these sites in detail. Nearly 10-fold greater amounts of Pol than of Pol-UL42 were required to produce approximately equal amounts of unit-length product (compare Pol-UL42 at 25 and 50 fmol with Pol at 200 and 400 fmol). The addition of a constant amount (100 fmol) of purified UL42 to reactions containing Pol resulted in a marked change in the size distribution of the products synthesized. In those reactions containing less than 100 fmol of Pol in particular, the size distribution of products was comparable with that observed for an equal amount of Pol-UL42. Again, identical results were obtained with UL42 purified from HSV-infected HeLa cells and UL42 purified from recombinant baculovirus-infected Sf9 cells. Titration of the reaction with increasing amounts of UL42 protein (Fig. 5) revealed that the amount of UL42 required to achieve maximum stimulation of synthesis of full-length strands was dependent on and approximately equal (within a factor of two) to the amount of Pol in the reaction. UL42 protein purified from HSV-in-

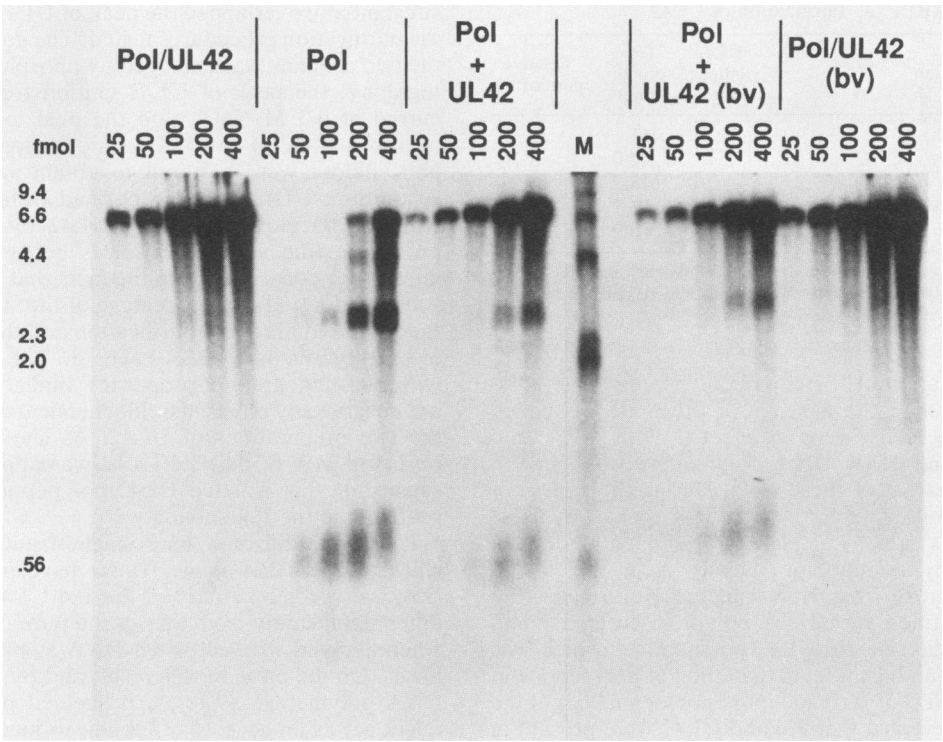

FIG. 4. DNA synthesis on singly primed M13 single-stranded circles. Reactions (50 μl) contained 25 fmol (as circles) of primed M13 single-stranded DNA; 0.5 mM ATP; 2.5 μg of BSA; 60 μM (each) of dATP, dGTP, and dTTP; 20 μM [α-^{32}P]dCTP (4,400 cpm/pmol); and 25 to 400 fmol of the indicated polymerase in buffer E. DNA synthesis was initiated by the addition of polymerase. In reactions that contain UL42 protein, 100 fmol of UL42 was added prior to the addition of DNA polymerase. Incubation was for 30 min at 37°C, and the reactions were terminated with the addition of an equal volume of 1% SDS–40 mM EDTA–60 μg of carrier DNA per ml. After precipitation with ethanol, the products were dissolved in 15 μl of 0.1 N NaOH–5% glycerol–1 mM EDTA–0.025% bromcresol green and fractionated on a 1% alkaline agarose gel. The radioactivity in the gel was visualized by autoradiography. Pol-UL42 and UL42, Proteins purified from HSV-infected HeLa cells; Pol, Pol-UL42 (bv), and UL42 (bv), proteins purified from recombinant baculovirus-infected Sf9 cells. Lane M, Phage lambda DNA digested with *Hin*dIII and end labeled with ^{32}P. The sizes of the *Hin*dIII restriction fragments (in kilobases) are indicated by the numbers at the left.

fected cells and UL42 purified from recombinant baculovirus-infected Sf9 cells were equally effective in stimulating the synthesis of full-length strands by Pol (data not shown). The effect of UL42 on the size of the product synthesized by Pol was not dependent on the presence of any combination of ribonucleoside triphosphates, nor was it inhibited by γ-thio-ATP (data not shown).

The effect of the HSV single-stranded DNA-binding protein (ICP8) on these reactions was examined (Fig. 6). Addition of ICP8 to levels sufficient to completely coat the template in the reaction had no significant effect on the activity of the Pol-UL42 complex on primed single-stranded M13 substrates; the addition of ICP8 to reactions containing Pol stimulated DNA synthesis approximately twofold, but did not affect the overall distribution of product length. In agreement with the published results of Ruyechan and Weir (42), we observed a twofold stimulation of the activity of either Pol or Pol-UL42 on the addition of ICP8 to assays utilizing activated DNA as template (data not shown). In contrast to the results of O'Donnell et al. (30), we conclude that ICP8-coated single-stranded DNA is an effective template for the HSV DNA polymerase.

UL42 increases the processivity of the HSV DNA polymerase. To gain some insight into the mechanistic basis for the increase in the size of the products synthesized by Pol in the presence of UL42, we examined the efficiency with which the enzyme utilizes primers. A molar excess of substrate

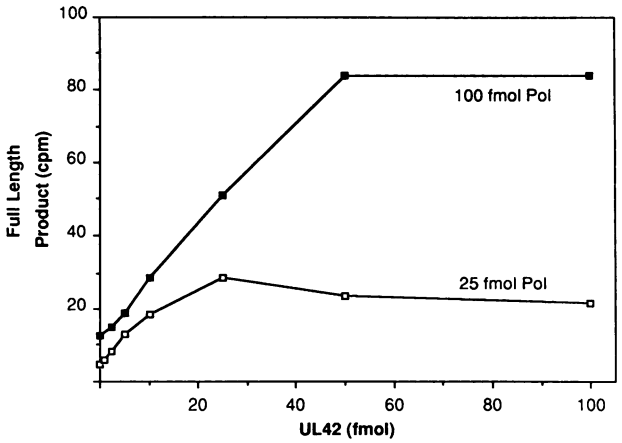

FIG. 5. Titration of Pol with UL42. Reactions contained 25 fmol of primed M13 DNA, 25 or 100 fmol of Pol, and the indicated amount of UL42 protein (purified from HSV-infected cells). Other reaction conditions were identical to those described in the legend to Fig. 4. Following incubation, the reactions products were fractionated on a 1% alkaline agarose gel, and the radioactivity in a band of product that migrated as unit-length M13 DNA strands was quantitated with a Betagen BetaScope 600.

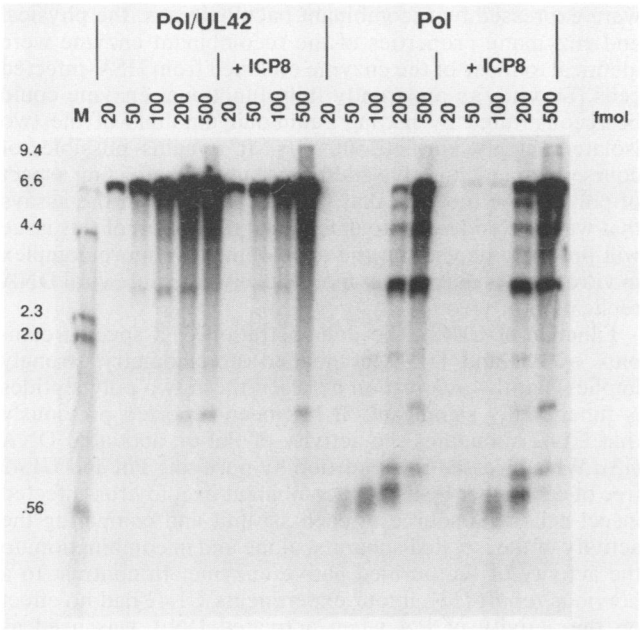

FIG. 6. ICP8-coated DNA is an effective template for HSV DNA polymerase. The reaction conditions and the treatment of the samples were as described in the legend to Fig. 4. Reactions contained 20 fmol of singly primed M13 single-stranded DNA, 20 to 500 fmol of Pol-UL42 or Pol, and 10 pmol of ICP8. Reactions were preincubated at 37°C for 3 min in the presence or absence of ICP8 prior to the addition of DNA polymerase.

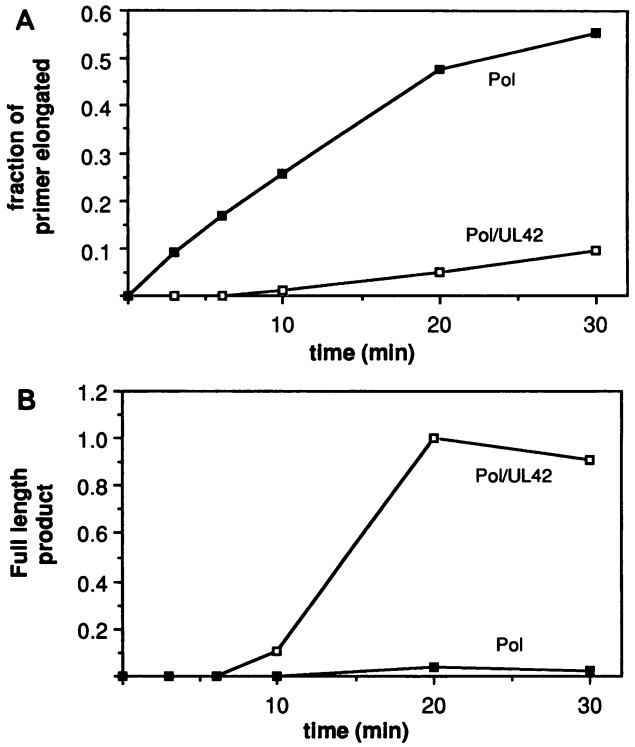

FIG. 7. Comparison of primer utilization by Pol and Pol-UL42. The replication reactions contained 300 fmol of single-stranded M13 circular DNA annealed to a single 5'-end-labeled primer (4,100 cpm/fmol), 0.5 mM ATP, 50 μg of BSA per ml, 60 μM each deoxyribonucleoside triphosphate, and 300 fmol of either Pol or Pol-UL42 in buffer E in a volume of 600 μl. Reactions were preincubated at 37°C for 5 min prior to the addition of DNA polymerase. Aliquots (100 μl) were removed at 0, 2, 5, 10, 20, and 30 min and added to an equal volume of 1% SDS–40 mM EDTA–60 μg of DNA carrier per ml to stop the reaction. Following precipitation with ethanol, the products of the reaction were fractionated by electrophoresis on a 6% acrylamide-urea sequencing gel to determine the fraction of primer that was elongated (A) and on a 1% alkaline agarose gel to determine the relative amount of primer that was elongated to produce unit-length M13 DNA strands (B). The quantitation of radioactivity on the gels was accomplished with a Betagen BetaScope 600.

consisting of 5'-^{32}P-labeled oligonucleotide primer annealed to M13 single-stranded circles was incubated with either Pol or with Pol-UL42, and the labeled products were analyzed (i) on an 8% acrylamide-urea gel to determine the fraction of primer that was elongated (Fig. 7A) and (ii) on an alkaline agarose gel to quantitate the synthesis of full-length M13 DNA strands (Fig. 7B). The results of this experiment show quite clearly that the Pol-UL42 complex utilizes significantly fewer primers than an equal amount of Pol alone but elongates them into full-length strands much more efficiently. These results suggest that UL42 acts to increase the processivity of the HSV DNA polymerase.

We directly compared the processivity of the Pol-UL42 complex with the isolated Pol subunit by means of a template challenge assay (Fig. 8). Singly primed M13 was preincubated with polymerase and three deoxyribonucleoside triphosphates to allow binding of the polymerase to the template-primer and limited (a single nucleotide) polymerization. An equivalent amount of a second substrate, primed ϕX174 DNA, was added to the reaction, and DNA synthesis was initiated by the addition of [α-^{32}P]dCTP. Aliquots were removed at various times, and the products of the reaction were fractionated on a neutral agarose gel. Under these conditions, a highly processive DNA polymerase would be expected to synthesize predominantly M13 DNA, while a completely nonprocessive polymerase would incorporate label equally into both substrates. The products of DNA synthesis in reactions containing Pol-UL42 consisted almost entirely of partially duplex M13 circles at early time points and fully replicated duplex M13 by 30 min (Fig. 8B). Very little, if any, fully replicated duplex ϕX174 DNA was observed after 30 min of incubation. In contrast, Pol alone synthesized approximately equimolar amounts of both M13 and ϕX174 and produced few fully duplex circles of either substrate.

DISCUSSION

Structure of HSV DNA polymerase. Two lines of evidence have previously suggested the existence of a specific association between Pol and UL42. First, purified preparations of the HSV-2-induced DNA polymerase were reported to contain two polypeptides (37, 48). The larger of these two polypeptides was shown to be the product of the Pol gene; the smaller polypeptide, with an estimated MW of ~55,000, was shown to be immunologically distinct from the Pol polypeptide and was identified as infected-cell-specific polypeptide (ICSP) 34/35. ICSP 34/35 was subsequently demonstrated by hybrid-selected translation to be equivalent to the product of the HSV-1 gene *UL42* (35). Second, immunoaffinity purification of UL42 with an anti-UL42 monoclonal antibody resulted in the specific enrichment of HSV DNA polymerase activity (14). The results reported in this paper

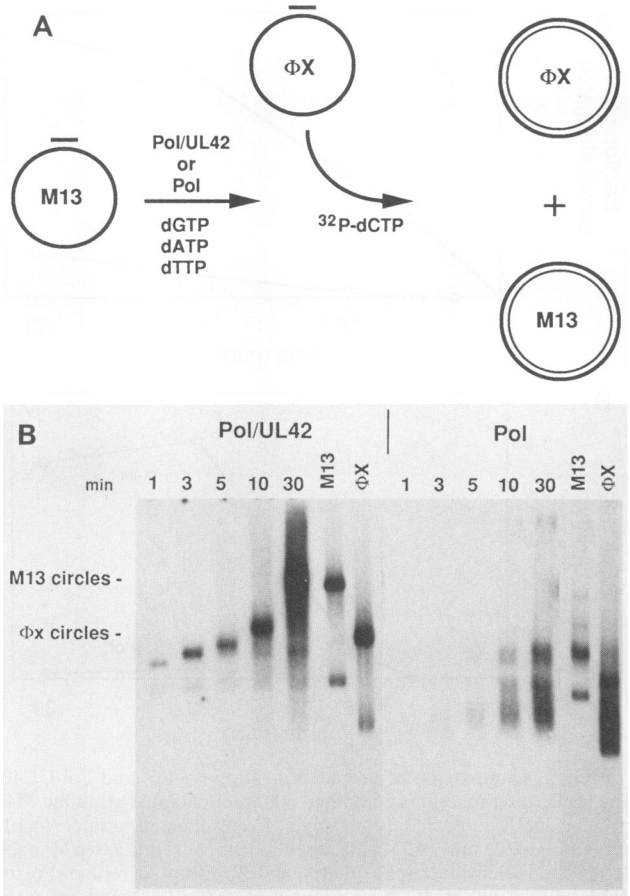

FIG. 8. UL42 increases the processivity of the HSV DNA polymerase. (A) Diagrammatic representation of the experimental strategy. See Results for details. (B) Reactions (500 μl) contained 400 fmol of singly primed M13 single-stranded DNA; 0.5 mM ATP; 50 μg of BSA per ml; 60 μM (each) dATP, dGTP, and dTTP; and either 80 fmol of Pol-UL42 or 200 fmol of Pol. The Pol-UL42 used in this experiment was purified from HSV-infected cells; identical results were obtained with Pol-UL42 purified from recombinant baculovirus-infected Sf9 cells (not shown). After preincubation at 37°C for 5 min, 400 fmol of singly primed ϕX single-stranded DNA was added and DNA synthesis was initiated by the addition of 50 μl of 0.2 mM [α-^{32}P]dCTP (7,100 cpm/pmol). Aliquots (100 μl) were removed at 1, 3, 5, 10, and 30 min, and following ethanol precipitation, the products of the reaction were fractionated on a 1% neutral agarose gel. The M13 and ϕX marker lanes contain the products of replication using 50 fmol of either Pol-UL42 or Pol and 100 fmol of the M13 or ϕX substrate alone.

both confirm and extend these previous reports. We have shown here that the HSV-1 DNA polymerase can be purified as a complex containing equimolar amounts of both Pol and UL42. Determination of the size of this complex on the basis of its hydrodynamic properties yielded a value (181,000) that is consistent with the idea that the structure of the native enzyme is a heterodimer of Pol and UL42.

Although the evidence that both Pol and UL42 are components of the native enzyme is unambiguous, our most highly purified preparations contain other polypeptides, and so we cannot rule out the possibility that one or more of these polypeptides might also be a specific component of the DNA polymerase. We view this possibility as unlikely for two reasons. First, we have also purified a complex between Pol and UL42 from insect cells in which the two subunits were expressed by recombinant baculoviruses; the physical and enzymatic properties of the recombinant enzyme were identical to those of the enzyme obtained from HSV-infected cells. Second, an apparently fully functional enzyme could be reconstituted by mixing equimolar amounts of the two isolated, highly purified subunits. It remains possible, of course, that any putative additional subunits affect an aspect of polymerase function that was not required in the assays that we have employed to date. Final resolution of this issue will probably depend on the development of more complex in vitro assay systems that more closely resemble viral DNA replication in vivo.

Function of UL42. The demonstration of a specific complex of Pol and UL42 of defined stoichiometry strongly implies that the association between these two polypeptides is functionally significant. It has been reported previously that UL42 stimulates the activity of Pol on activated DNA (13). We addressed this question by purifying Pol and UL42 free of each other by using recombinant baculovirus-infected insect cells as a source of each subunit and comparing the activity of the isolated subunits, alone and in combination, to the activity of the purified native enzyme. In contrast to a previous report (13), in our experiments UL42 had no effect on the activity of Pol when activated DNA was used as primer-template. We did find, however, that the presence of UL42 had a profound effect on the ability of the DNA polymerase to copy long single-stranded DNA templates, such as singly primed M13 DNA. In the absence of UL42, the isolated Pol subunit synthesized primarily short DNA chains; in the presence of UL42, the DNA polymerase synthesized long DNA chains equal in size to the template. Substrate challenge experiments clearly showed that the increase in the size of the products in the presence of UL42 was due, at least in part, to an increase in processivity of the enzyme. Preliminary experiments have suggested that this increase in processivity is accompanied by an increase in the affinity of the enzyme for the ends of duplex DNA (J. Gottlieb and M. Challberg, unpublished data). While differences in processivity per se would not be expected to have an effect on the activity of a DNA polymerase on the short single-stranded DNA regions that are thought to be the predominant template in activated DNA, it is possible that under relatively dilute assay conditions an increase in the affinity for primer termini brought about by UL42 would have led to the stimulation of activity observed by other investigators. We have not systematically tried to reproduce such conditions. It has also been reported (31), in contrast to the results reported here, that the purified catalytic subunit of HSV DNA polymerase carries out highly processive DNA synthesis in the absence of other subunits. The reason for this discrepancy is not known with certainty, although the most likely explanation is that the preparation of HSV DNA polymerase used by O'Donnell et al. (31) contained at least some UL42. In that study, the DNA polymerase was purified from HSV-infected cells. Since the UL42 polypeptide was not a recognized component of the viral DNA polymerase at that time, no specific immunological assays were used to test for its presence.

The UL42 protein was originally identified as an abundant, infected-cell-specific protein with high affinity for double-stranded DNA. The relationship between the in vitro DNA-binding activity and the function of UL42 has not yet been explored. As mentioned, we have shown that the presence of UL42 in the native DNA polymerase is associated with an increase in the affinity of the enzyme for the ends of double-stranded DNA (J. Gottlieb and M. Challberg, unpub-

lished data). It is tempting to speculate that this increased affinity for termini is related to the 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 dissociates from the template after each cycle of catalysis. An analogous role has been proposed for other DNA polymerase accessory subunits, such as the T4 gene 45 and 44/62 proteins (23) and the thioredoxin subunit of T7 DNA polymerase (17, 18). Since we have shown that it is possible to reconstitute processive DNA synthesis by using isolated subunits produced independently in a heterologous expression system, it should now be possible to test this model in more detail.

HSV DNA polymerase and DNA polymerase δ. Sequence comparison studies have shown that the Pol subunit of HSV DNA polymerase is a member of the eucaryotic DNA polymerase α family (29). More recent studies have indicated, however, that HSV Pol is more closely related to DNA polymerase δ than to Pol α itself (3). The studies reported here suggest that the similarities between Pol δ and the HSV DNA polymerase extend beyond primary amino acid sequence alone. Structurally, the two enzymes are at least superficially alike. Both comprise two tightly associated subunits: a large, catalytic subunit and a smaller subunit of about 50 kDa (2, 21). It will be of some interest to determine whether UL42 shares any amino acid sequences with the small subunit of Pol δ when the gene encoding that polypeptide is cloned and sequenced. The catalytic properties of the HSV DNA polymerase also appear similar to those of Pol δ. In contrast to Pol α, both enzymes have an intrinsic 3′ to 5′ exonuclease (21) and both carry out highly processive DNA synthesis (2, 47). In the case of Pol δ, however, processive synthesis requires the participation of another protein, PCNA (2, 47). In preliminary experiments, we have not found any effect of purified PCNA on the activity of the HSV Pol subunit, either in the presence or absence of UL42 (J. Gottlieb, unpublished data), nor have we detected any amino acid sequence homology between UL42 and PCNA. More-detailed structural studies of the two enzymes will be required to establish the basis for the similarities and differences between these two enzymes.

Pol-UL42 and the replication fork. There is a dichotomous requirement for processivity during the synthesis of the two daughter strands at a replication fork. Synthesis of the leading strand is expected to be highly processive, whereas synthesis of the lagging strand is expected to be less processive, since DNA polymerase must dissociate from the 3′ end of the growing chain at the completion of synthesis of each Okazaki fragment. These two different levels of processivity are met in a number of ways in various DNA replication systems that have been studied to date. For example, the eucaryotic chromosomal replication apparatus uses two different DNA polymerases: leading-strand synthesis is carried out by DNA polymerase δ, and lagging-strand synthesis is accomplished by DNA polymerase α (38, 50). In other systems, the same DNA polymerase is used, but its properties are modulated by interaction with other protein factors which differ on the two sides of the fork. The finding that the processivity of the HSV DNA polymerase catalytic subunit is altered by the presence of UL42 protein suggests a simple model for the HSV DNA replication fork in which the Pol-UL42 complex carries out leading-strand synthesis and the Pol subunit alone is responsible for lagging-strand synthesis. Although our data do not bear directly on this question, we have shown that there is a molar excess of UL42 relative to Pol in infected cells, and that virtually all of the HSV DNA polymerase present in extracts of infected cells can be purified in the form of a Pol-UL42 complex. We therefore favor a more complex model in which UL42 is a component of the DNA polymerase on both sides of the fork, and processivity is modulated by interaction with other replication proteins. Alternatively, our data do not rule out the possibility that there are two different forms of the Pol-UL42 complex which differ in the degree of processivity. The fact that UL42 has been shown to be a phosphoprotein (26) suggests one possible means by which the properties of the polymerase could be altered. The data reported in this paper represent a starting point for developing strategies to address these issues.

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