Enzymatic activities of overexpressed herpes simplex virus DNA polymerase purified from recombinant baculovirus-infected insect cells

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ABSTRACT

Biochemical characterization of the herpes simplex virus (HSV) DNA polymerase, a model DNA polymerase and an important target for antiviral drugs, has been limited by a lack of pure enzyme in sufficient quantity. To overcome this limitation, the HSV DNA polymerase gene was introduced into the baculovirus, *Autographa californica* nuclear polyhedrosis virus, under the control of the polyhedrin promoter to give rise to a recombinant baculovirus, BP58. BP58-infected *Spodoptera frugiperda* insect cells expressed a polypeptide that was indistinguishable from authentic polymerase by several immunological and biochemical properties, at levels approximately ten-fold higher per infected cell than found in HSV-infected Vero cells. The DNA polymerase was purified to apparent homogeneity from BP58-infected insect cells. Using activated DNA as primer-template, the purified enzyme exhibited specific activity similar to that of enzyme isolated from HSV-infected Vero cells, indicating that additional polymerase-associated proteins from HSV-infected cells are not critical for activity with this primer-template. 3'-5' exonuclease activity co-purified with the BP58-expressed HSV DNA polymerase, demonstrating that this activity is intrinsic to the polymerase polypeptide. The purified enzyme also exhibited RNase H activity. The recombinant baculovirus should permit detailed biochemical and biophysical studies of this enzyme.

INTRODUCTION

The herpes simplex virus (HSV) DNA polymerase (Pol) is essential for HSV replication (1,2) and serves as the target for a number of antiviral drugs including the most widely used agent, acyclovir (3,4). HSV Pol belongs to a family of DNA

polymerases that include mammalian DNA polymerase α , yeast DNA polymerases I and III, and the polymerases encoded by numerous other animal and bacterial viruses (5,6). The HSV enzyme provides a model system for the study of this family of polymerases in that it is amenable to genetic, pharmacological, and biochemical approaches. For example, a combined genetic and pharmacological approach has permitted identification of amino acids in the HSV Pol involved in recognition of drugs that mimic deoxynucleoside triphosphate (dNTP) and pyrophosphate, leading to the proposal that two regions of sequence similarity shared among diverse DNA polymerases interact directly with these substrates (7).

Although there have been many biochemical studies of the HSV Pol, it has been difficult to answer detailed questions regarding its mechanisms of catalysis and inhibition by antiviral drugs, and the relationships of these mechanisms to enzyme structure. This has been due in part to the low levels of Pol in HSV-infected mammalian cells, which result from inefficient translation of *pol* mRNA (8, D.R. Yager, A.I. Marcy, and D.M. Coen, manuscript submitted for publication), that prevent the isolation of Pol in 'substrate' quantities (9). Additionally, it has been difficult to purify Pol away from other cellular and viral proteins, especially the HSV-encoded double stranded DNA binding protein, which is a product of the UL42 gene (10–14). This has made it difficult to determine whether activities associated with HSV Pol are intrinsic to the polypeptide product of the HSV *pol* gene (UL30) or, if they are intrinsic, whether they require other proteins for stimulation. These activities include polymerase activity on various primer-templates, 3'-5' exonuclease (12, 15–18), and a recently described 5'-3' exonuclease with RNase H activity (19).

To permit detailed biochemical studies of HSV Pol, we have introduced the *pol* open reading frame into a baculovirus expression system. The recombinant baculovirus expresses abundant quantities of HSV Pol in insect cells, permitting purification of the enzyme to apparent homogeneity, and assessment of various enzyme activities of the Pol polypeptide.

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MATERIALS AND METHODS

Cells and viruses

Detailed procedures for cell culture of *Spodoptera frugiperda* (Sf9) cells and the propagation of wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and recombinant baculoviruses have been previously described (20). AcMNPV/UL5-II is a polyhedrin-negative recombinant baculovirus, which was derived during efforts to express the HSV UL5 gene. It expresses no detectable foreign protein (P. Olivo and M. Challberg, unpublished results) and thus serves as a control virus. Procedures for culture of Vero cells and propagation of HSV-1 wild type strain KOS have been described (21).

Construction of baculovirus recombinant BP58

The vector pAc373 contains the baculovirus polyhedrin promoter with a unique BamHI site 50 base pairs (bp) downstream of the polyhedrin mRNA cap site (22). Plasmid pDP3, derived from pDP1 (A.I. Marcy, D.R. Yager, and D.M. Coen, manuscript submitted) contains a unique BclI site 11 bp upstream of the ATG that starts the major HSV *pol* open reading frame and a unique XbaI site approximately 150 bp downstream of the TAG that terminates the reading frame. Its derivation was essentially identical to that of pDP4, which is described elsewhere (Marcy et al., submitted). pDP3 was digested with XbaI, treated with the Klenow fragment of DNA polymerase I to fill in overhanging ends, ligated to BclI linkers, digested with BclI, and then treated again with ligase to form plasmid pDP3B. The 3.8 kbp BclI fragment was removed from pDP3B and ligated into BamHI-digested pAc373 DNA to obtain pAcDP (Fig. 1). Infectious AcMNPV DNA was cotransfected with pAcDP DNA using method II described by Summers and Smith (20). Viral plaques with an altered plaque morphology were picked and infected cells assayed by dot-blot DNA hybridization (20) for the presence of *pol* sequences. Hybridizing plaques were then screened by Southern blot hybridization to determine if the baculovirus recombinants contained the expected chimeric polyhedrin-*pol* gene. One recombinant, BP58, was selected for further study.

Removal of Pol activity with antisera

Extracts from cells infected with either BP58 or wild-type AcMNPV were prepared 4 days after infection using a modification of the procedure described by Wang and Kelly (23). Briefly, infected cells were scraped, pelleted, and resuspended at a concentration of 10^7 cells/ml in a buffer containing 50 mM Tris, pH 7.5, 10 mM dithiothreitol (DTT), 1 mM EDTA, 10 mM NaHSO₃, 2 μ g/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10% glycerol. The cells were lysed by sonication using a cup-horn sonicator, and then cleared by centrifugation at $100,000\times g$ for 1 hr at 4°C. Five μ l of supernatant was mixed with an equal volume of either preimmune rabbit sera or varying amounts of immune rabbit antisera [an equal mixture of antisera PP5 and BGG4, both of which are specific for HSV Pol (D.R. Yager et al., submitted)]. The total volume of serum was kept constant by addition of preimmune rabbit serum. The samples were incubated at 0°C overnight, and then combined with 20 μ l of a 25% slurry of protein A-sepharose (Sigma). After a 30 min. incubation at 4°C, the protein A-sepharose was pelleted by centrifugation and the supernatants were assayed for DNA polymerase activity as described (24).

Purification of HSV Pol from BP58-infected insect cells

Pol was purified from BP58-infected insect cells using a modification of the method of O'Donnell et al. (25). Briefly, 50 225 cm² flasks of confluent Sf9 cells were infected with BP58 at a multiplicity of infection of 10.54 hrs. after infection, the cells were shaken off the flasks, washed with serum-free Grace's medium, and resuspended in hypotonic lysis buffer (Buffer A: 20 mM HEPES, pH 7.6, 0.5 mM DTT, 10 mM NaHSO₃, 0.5 mM PMSF, 2 μ g/ml leupeptin). After ten minutes on ice, the cells were lysed using a Dounce homogenizer. Nuclei were collected by low speed centrifugation (10 min. at $1200\times g$), the supernatant (cytoplasmic fraction) was removed and retained for immunoblot analysis, and the nuclei were repelleted at $12,000\times g$ for 30 min. The pellet was resuspended in buffer A and lysed by the addition of an equal volume of buffer A containing 3.4 M NaCl. The viscous extract was centrifuged at $100,000\times g$ for 1 hr. The resulting supernatant was dialyzed twice against buffer A containing 10% glycerol and 0.5 mM EDTA (buffer C). The precipitate that formed during dialysis was removed by centrifugation, and the supernatant fraction was applied to a phosphocellulose column (15 ml bed volume). The column was eluted with a linear gradient from 0 to 1 M NaCl in buffer C in a total volume of 200 ml. Fractions containing both immunoreactivity to anti- HSV Pol antisera (26) and DNA polymerase activity (measured as described below) were pooled, dialyzed against buffer C, and applied to a 3 ml single-stranded DNA agarose column (Bethesda Research Laboratories) equilibrated in buffer C. The column was eluted with a linear gradient from 0 to 1 M NaCl in buffer C in a total volume of 30 ml. Fractions containing DNA polymerase activity were pooled, dialyzed against buffer C, and concentrated to 400 μ l in a Centricon 30 ultrafiltration device (Amicon). The concentrated fractions were loaded onto 10.6 ml 15–35% glycerol gradients in buffer C and centrifuged in an SW 40Ti rotor at 40,000 RPM for 44 hrs. at 4°C. After centrifugation, 0.3 ml fractions were collected from the bottoms of the tubes.

Other enzymes

E. coli DNA polymerase I and Klenow fragment of DNA polymerase I were purchased from Bethesda Research Laboratories. E. coli RNase H was purchased from Pharmacia. HSV DNA polymerase from HSV-infected Vero cells was isolated by the method of O'Donnell et al. (25).

Measurement of DNA polymerase activity

DNA polymerase activity was assayed in a reaction (25 μ l) containing 2.5 μ g of activated calf thymus DNA (Sigma), 50 μ M each dATP, dGTP, and dTTP, and 5 μ M (α -³²P)-dCTP (Amersham, 5–10 μ Ci/nmol), 150 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 5% glycerol, 5 mM DTT, and 40 μ g/ml bovine serum albumin. The reactions were initiated by addition of aliquots of column or gradient fractions. After a 30 min. incubation at 37°C, the reactions were terminated by the addition of 0.2 ml of 0.1 M sodium pyrophosphate/10 mM EDTA and 0.5 ml of cold 10% trichloroacetic acid (TCA). Radiolabeled acid-insoluble material was collected by filtration on glass fiber filters, which were washed extensively with 5% TCA, rinsed with ethanol, and dried. Filter-bound radioactivity was determined by scintillation counting. One unit of HSV Pol incorporated one picomole of nucleotide into acid-insoluble material in 30 min. at 37°C.

Fig. 1. Construction of pAcDP. Plasmids pAc373 (20), pDP3B, and pAcDP are depicted in the upper left portion of the figure. Bacterial vector sequences are indicated by heavy stippling, HSV *pol* sequences by light stippling, the polyhedrin (PH) gene is indicated by hatch marks and the polyhedrin promoter is filled-in. The ATG initiation and TGA termination codons of the major *pol* open reading frame are shown. The 3.8 kbp BclI fragment containing the *pol* open reading frame was ligated into BamHI-cleaved pAc373 to yield pAcDP, which is depicted at the bottom of the figure.

Measurement of 3'-5' exonuclease activity

3'-5' exonuclease activity was assayed in a reaction (50 μ l) containing the same components as the DNA polymerase assay except that it lacked deoxynucleotides and the activated DNA was replaced with 1 μ g of DNA labeled at its 3' end with 32 P (0.1 μ Ci/ μ g), prepared by digestion of pUC18 with TaqI and treatment with the Klenow fragment of *E. coli* DNA polymerase I in the presence of (α - 32 P)-dCTP. The reactions were initiated by addition of 1 μ l aliquots of gradient fractions and incubated for 30 min. at 37°C. Each reaction was terminated with 0.2 ml of 0.1M sodium pyrophosphate, 10 mM EDTA containing 10 μ g/ml of calf thymus DNA as carrier, followed by 0.5 ml of 10% TCA. After 10 min. on ice, the mixtures were centrifuged for 30 min. at 10,000 $\times$ g and the radioactivity in 0.5 ml of each supernatant was determined by scintillation counting.

RNase H assay

RNase H activity was assayed in a reaction (25 μ l) containing 48 mM Tris-HCl, pH 8.6, 1 mM MgCl₂, and an RNA-DNA hybrid substrate prepared by annealing 3 H-polyadenylic acid (> 15 Ci/mmol, Amersham) with an excess of polydT. Each reaction contained 36 pmol (in terms of nucleotides) of 3 H-polyadenylic acid in the RNA-DNA hybrid substrate. Activity against single-stranded RNA was assayed in parallel in reactions that were identical except that mock-annealed 3 H-polyadenylic

acid was used instead of the RNA-DNA hybrid. The reactions were initiated by addition of enzyme and incubated for 30 min. at 37°. Each reaction was terminated by addition of 50 μ g calf thymus DNA, 50 μ l of 0.1 M sodium pyrophosphate, 10 mM EDTA, and 100 μ l 10% TCA. After 30 min. on ice, the mixtures were centrifuged for 10 min. at 10,000 $\times$ g and the radioactivity in 100 μ l of each supernatant was determined by scintillation counting.

Protein analysis

Protein concentrations were determined using a kit (Bio-Rad) according to the manufacturer's instructions using bovine serum albumin as a standard. Sodium-dodecyl-sulfate (SDS) polyacrylamide gel electrophoresis was performed essentially as described by Laemmli (27).

Immunoblot analysis

Following SDS polyacrylamide gel electrophoresis, proteins were transferred to an Immobilon membrane (Millipore) by electroblotting for 2 hrs at 40 volts in Tris/glycine buffer, pH 8.3 containing 15% methanol and 0.1% SDS. Following incubation with phosphate buffered saline, 0.3% Tween 20 (PBS/Tween) for 30 min. at room temperature, the membrane was incubated for 2 hrs. at room temperature with anti-HSV Pol rabbit antisera (26). The membrane was washed three times with PBS/Tween and incubated with 125 I-labeled protein A (Amersham) for 30 min. at room temperature, washed five times with PBS/Tween, air dried, and exposed to Kodak XAR-5 film with an intensifying screen at -70°. Similar results were obtained when immunoblot analysis was performed as described by Yager et al. (submitted) (unpublished results).

RESULTS

Construction of a baculovirus recombinant containing the HSV *pol* open reading frame

To facilitate cloning of the HSV-1 strain KOS *pol* gene into the baculovirus polyhedrin gene at a BamHI site (the *pol* open reading frame contains two BamHI sites) and to remove sequences from the *pol* gene that might decrease its expression, we introduced a BclI restriction site 11 bp upstream of the first ATG in the major open reading frame of the *pol* gene (Marcy et al., manuscript in preparation). Additionally, we added a BclI restriction site linker about 90 bp downstream of the *pol* mRNA polyadenylation site (8,28). These manipulations generated plasmid pDP3, which is depicted in Fig. 1. pDP3 was digested with BclI, which generates 5' overhanging ends compatible with Bam HI ends, and the *pol* open reading frame was inserted into BamHI-cleaved pAc373 (22), resulting in a plasmid, pAcDP, with the *pol* open reading frame properly oriented downstream of the baculovirus polyhedrin promoter (Fig. 1).

pAcDP was co-transfected into Sf9 cells with infectious AcMNPV DNA. Polyhedrin-negative viruses arising from the transfection were identified by altered plaque morphology and were tested for the presence of HSV *pol* sequences by dot blot DNA hybridization. Positive viruses were then tested by Southern blotting to confirm the presence of the full length *pol* open reading frame in the proper orientation. One recombinant, BP58, was selected for further study.

Fig. 2. SDS polyacrylamide gel analysis of extracts of BP58-infected Sf9 cells. Cleared whole-cell extracts prepared as described in Materials and Methods under 'Removal of Pol activity with antisera' were prepared from Sf9 cells that were mock-infected (lane 1), infected with wild-type baculovirus for 24 hrs (lane 2), infected with wild-type baculovirus for 72 hrs (lane 3), or infected with BP58 for 72 hrs. (lane 4). The material from approximately 1×10^5 cells was loaded onto each lane. After electrophoresis, the gel was stained with Coomassie Blue. The numbers to the right of the figure refer to the molecular weights in kilodaltons of protein markers which were electrophoresed on the same gel. The arrow indicates the position of the new species in the BP58-infected cell extract.

Expression of HSV Pol polypeptide in BP58-infected insect cells

We first asked whether BP58 expressed a novel polypeptide upon infection of Sf9 cells. Figure 2 shows a Coomassie Blue-stained SDS-polyacrylamide gel of extracts of Sf9 cells either mock-infected (lane 1), infected with wild-type AcMNPV and harvested at different times post-infection (lanes 2 and 3) or infected with BP58 (lane 4). A new polypeptide species, with an apparent molecular weight of 140 kilodaltons was abundant in the BP58-infected cell extract. We estimate that approximately 1 microgram of this species was produced in 10^6 BP58-infected cells.

To determine the relationship of this polypeptide species to HSV Pol, proteins from BP58-infected Sf9 cells were transferred to membrane filters and reacted with antisera directed against HSV Pol (Fig. 3). The antisera detected a major immunoreactive species in nuclear and cytoplasmic fractions of about 140 kilodaltons (Fig. 3 lanes 2 and 3), which co-migrated both with the new Coomassie-blue stained species and with a species from HSV-infected Vero cells that has been identified as the authentic HSV Pol polypeptide (26, Yager et al, submitted) (Fig. 3, lane 1). The antisera also detected small amounts of more rapidly migrating species (lanes 1 and 2), which appear to be due to limited proteolysis since their abundance increases in the absence of certain protease inhibitors (unpublished results). No immunoreactive material was identified in extracts of Sf9 cells infected with a control recombinant baculovirus,

Fig. 3. Immunoblot analysis of extracts of BP58-infected Sf9 cells. Nuclear and cytoplasmic extracts were prepared as described by O'Donnell et al. (16) and submitted to SDS polyacrylamide gel electrophoresis and immunoblot analysis as described in Materials and Methods. Lanes: (1) nuclear extract of HSV-infected Vero cells; (2) nuclear extract of BP58-infected Sf9 cells; (3) cytoplasmic extract of BP58-infected Sf9 cells; (4) nuclear extract of AcMNPV/UL5-II-infected Sf9 cells; (5) cytoplasmic extract of AcMNPV/UL5-II-infected Sf9 cells. The numbers to the left of the figure refer to the size in kilodaltons of ^{14}C -labeled protein markers run on the same gel.

TABLE 1. Polymerase activity in extracts of Sf9 cells.

Sf9 cells infected with:	Polymerase activity (Cpm incorporated $\times 10^{-3}$)	
	No $(\text{NH}_4)_2\text{SO}_4$	150 mM $(\text{NH}_4)_2\text{SO}_4$
Mock	109	12
Wild type AcMNPV	142	81
BP58	688	681

Equal amounts of the extracts in terms of cell equivalents (polypeptide profiles are displayed in Fig. 2, lanes 1,3, and 4) were assayed for DNA polymerase activity.

AcMNPV/UL5-II, which does not encode HSV Pol (Fig. 3, lanes 4 and 5) or with wild-type baculovirus (not shown). When the numbers of cell equivalents in each sample are taken into account (10^5 Sf9 cell equivalents or 10^6 Vero cell equivalents per lane), the data in Fig. 3 indicate that there is approximately ten times more HSV Pol polypeptide expressed per BP-58 infected Sf9 cell than per HSV-infected Vero cell. Similar values were obtained in comparing whole cell lysates (unpublished results).

Polymerase activity associated with HSV Pol in extracts of BP58-infected insect cells

To determine whether the HSV Pol polypeptide expressed in BP58-infected Sf9 cells was associated with DNA polymerase activity, we tested the whole cell extracts prepared 4 days after infection (Fig. 2, lanes 1,3, and 4) and examined them for DNA polymerase activity on activated DNA templates in the presence or absence of 150 mM ammonium sulfate, which is optimal for HSV Pol activity (18 and unpublished results). Extracts of

Fig. 4. Removal of Pol activity with antisera specific for HSV Pol. Whole cell extracts of wild-type baculovirus-infected Sf9 cells (open circles) or BP58-infected Sf9 cells (closed circles) were reacted with the indicated volume of an equal mixture of PP5 and BGG4 antisera, which are specific for HSV Pol (Yager, Marcy, Coen, submitted) and protein-A sepharose as described in Materials and Methods. The DNA polymerase activity remaining in the supernatant fraction is indicated.

BP58-infected cells exhibited levels of polymerase activity approximately 5-fold greater than those found in either mock-infected or wild-type AcMNPV infected cells (Table 1) and this activity was salt-resistant (Table 1). Equivalent amounts of cell protein were present in each extract (Fig. 2). About half of this activity could be removed from the extracts by treatment with antisera specific for HSV Pol and protein A-sepharose, which had no effect on activity from wild-type AcMNPV-infected insect cells (Fig. 4). It is likely that the remaining activity is due to other polymerase activities in the extract and to incomplete removal of HSV DNA polymerase as the antisera used do not neutralize viral polymerase from HSV-infected cells completely (Yager et al., submitted).

Purification of HSV Pol from BP58-infected insect cells

The DNA polymerase activity associated with the HSV Pol polypeptide in BP58-infected Sf9 cells was purified by a modification of the procedure developed by O'Donnell et al. (25) for isolation of HSV Pol from HSV-infected mammalian cells. At each step, fractions were tested both for DNA polymerase activity and for polypeptide species reacting with anti-HSV Pol antisera on immunoblots. The first chromatographic step of the purification was a phosphocellulose column. Two peaks of DNA polymerase activity were resolved by phosphocellulose chromatography of nuclear extracts of BP58-infected cells, one at about 0.3 M NaCl and a larger peak at about 0.6 M NaCl (Fig. 5A). The latter peak corresponded to the major peak of activity that eluted when nuclear extracts of cells infected with a control baculovirus recombinant, which does not encode HSV Pol, were chromatographed on phosphocellulose (Fig. 5B). This

Fig. 5. Phosphocellulose chromatography of polymerase activity from recombinant baculovirus-infected Sf9 cells. Nuclear extracts from BP58-infected Sf9 cells were subjected to phosphocellulose chromatography. Fractions were submitted to immunoblot analysis of SDS polyacrylamide gels using anti-HSV Pol antisera (A, top; only the HSV Pol portion of the blot is shown) and, in parallel, (A, bottom) were assayed for DNA polymerase activity (open squares). Nuclear extracts from AcMNPV/UL5-II-infected Sf9 cells were subjected to phosphocellulose chromatography (B). Fractions were assayed for DNA polymerase activity (open squares). In A, bottom, and B, NaCl concentration is indicated by the diagonal lines.

activity is presumed to be baculovirus DNA polymerase, which has been reported to elute from phosphocellulose at relatively high salt concentrations (23,29). (The amount of this activity present at 54 hours post-infection relative to HSV Pol is greater than that seen in Table 1 where the extracts were prepared at 96 hours, most probably due both to baculovirus DNA polymerase activity peaking early in infection while the HSV DNA polymerase activity peaks much later and to the fact that the experiment in Table 1 assessed whole cell extracts while the material in Fig. 5 was derived from nuclear extracts.)

The polymerase activity that eluted at about 0.3 M NaCl was found in extracts of BP58-infected cells (Fig. 5A), but not in extracts of the control virus-infected cells (Fig. 5B). Only this peak of activity was associated with the 140 kilodalton polypeptide species that reacts with anti-HSV Pol antisera (Fig. 5A, top). Its chromatographic behavior on phosphocellulose (elution at about 0.3 M NaCl) was similar to that of HSV DNA polymerase from HSV-infected cells (25; Olivo and Challberg, unpublished results).

Peak fractions that contained polymerase activity and material reactive with anti-HSV Pol antisera were then subjected to single-stranded DNA-agarose chromatography (Fig. 6). A single peak

Fig. 6. Single-stranded DNA agarose chromatography of HSV Pol from BP58-infected Sf9 cells. Peak fractions containing DNA polymerase activity and immunoreactive material from the phosphocellulose column shown in Fig. 5A bottom were pooled, dialyzed, and chromatographed on single-stranded DNA agarose as described in Materials and Methods. Fractions were assayed for DNA polymerase activity (open squares). NaCl concentration is indicated by the diagonal line.

of DNA polymerase activity eluting at about 0.25 M NaCl was detected. This activity was associated with a peak of immunoreactive material (not shown) and represented a eight-to nine-fold purification with little loss of activity (Table 2). Its chromatographic behavior on single-stranded DNA agarose was similar to that of HSV DNA polymerase from HSV-infected cells (25; Olivo and Challberg, unpublished results). The fractions containing activity were pooled and applied to a glycerol gradient. DNA polymerase activity sedimented as a single peak, with a sedimentation coefficient consistent with a spherical species of about 140 kilodaltons (Fig. 7A). The activity co-purified with material that reacted with anti-HSV Pol antisera (Fig. 7B). This step, which gave only a slight increase in specific activity (Table 2), nevertheless removed a number of minor contaminants to yield a single Coomassie blue-stained species of 140 kilodaltons on SDS-polyacrylamide gel electrophoresis (Fig. 7C). Thus, the HSV Pol produced in BP58-infected cells was purified to apparent homogeneity.

Specific activity on activated DNA of polymerase from BP58-infected insect cells

The activity of the purified DNA polymerase from BP58-infected insect cells was compared with enzyme isolated from HSV-infected cells using activated DNA as primer-template. The enzyme activities were normalized to the amounts of immunoreactive 140 kilodalton species determined from densitometry of immunoblots. The specific activities of the two enzyme preparations were indistinguishable (approximately 200,000 units/mg).

3'-5' exonuclease and RNase H activities of HSV Pol purified from BP58-infected insect cells

HSV Pol isolated from HSV-infected mammalian cells is associated with a 3'-5' exonuclease activity (12,17,25). Fractions of the glycerol gradient used to purify the Pol from BP58-infected insect cells were tested for 3'-5' exonuclease activity. The activity

TABLE 2. Purification of DNA polymerase from BP58-infected Sf9 cells

Step	Total Protein (mg)	Total Activity ^a (units $\times 10^{-4}$)	Specific Activity (units/mg $\times 10^{-2}$)
Nuclear extract	50	ND ^b	ND
Phosphocellulose	5.2	14	270
SS DNA-agarose	0.37	8.5	2300
Glycerol gradient	0.10	2.4	2400

^a One unit equals the incorporation of one picomole of nucleotide into acid-insoluble material in 30 min. at 37° C.

^b Not done. It was not possible to determine accurately what percentage of total DNA polymerase activity in this fraction was due to the HSV DNA polymerase.

co-sedimented with the polymerase activity and the immunoreactive 140 kilodalton polypeptide (Fig. 7A,B). The specific activity of purified enzyme in the exonuclease assay used was 50 nmol nucleotide released/hr/mg.

Recently, preparations of HSV Pol isolated from HSV-infected mammalian cells have been shown to contain an RNase H activity, at least part of which co-migrates with the 140 kilodalton Pol polypeptide on activity gels (19). We therefore tested the HSV Pol purified from BP58-infected insect cells for this activity using poly rA and poly rA/poly dT substrates (Table 3). *E. coli* RNase H, *E. coli* DNA polymerase I (Pol I), and the large fragment of Pol I (Klenow fragment) were also tested, primarily to evaluate the substrates. As expected, *E. coli* RNase H degraded the RNA portion of the RNA/DNA hybrid, but showed little activity on the single-stranded substrate. Pol I, on the other hand, degraded both substrates, but was more active on the duplex substrate. Klenow fragment, which lacks 5'-3' exonuclease activity, showed little or no activity on either substrate, consistent with the idea that the RNase H activity of Pol I is related to its 5'-3' exonuclease activity (30). The HSV Pol purified from BP58-infected insect cells exhibited RNase H activity similar to Pol I in that it digested both substrates, but was more active against duplex molecules.

DISCUSSION

Authenticity of overexpressed HSV Pol

We describe here the overexpression of a polypeptide from a recombinant baculovirus that contains the major long open reading frame from the HSV *pol* gene. This polypeptide is indistinguishable from authentic HSV Pol in its migration on SDS polyacrylamide gels and its reaction with antisera specific for HSV Pol (Figs. 2 and 3). Its DNA polymerase activity is very similar that of authentic HSV Pol in its salt resistance (Table 1), removal by antisera specific for HSV Pol (Fig. 4), specific activity on activated DNA, 3'-5' exonuclease activity (Fig. 7) and RNase H activity (Table 3). The overexpressed enzyme exhibits chromatographic properties (Figs. 5–7) that are similar to those for enzyme from HSV-infected mammalian cells purified under identical conditions (Olivo and Challberg, unpublished results). The authenticity of the overexpressed HSV Pol is in accord with the biological activity of plasmid pDP1 (Marcy et al., submitted) from which the recombinant baculovirus was derived.

Two other reports have described the expression of active HSV Pol in heterologous systems (31,32). Among the differences between the present study and the two previous reports are that in this study HSV Pol was purified to apparent homogeneity, the purified enzyme was compared with Pol from HSV-infected cells for its specific activity on activated DNA, the purified enzyme was examined for 3'-5' exonuclease and RNase H activities, and much higher levels of expression were obtained.

Fig. 7. Glycerol gradient sedimentation of HSV Pol from BP58-infected Sf9 cells. Active fractions from the single-stranded DNA-agarose column were pooled, dialyzed, concentrated, and sedimented in a 15–35% glycerol gradient as described in Materials and Methods. Fractions were assayed for DNA polymerase and 3'-5' exonuclease activities and for material reactive with anti-HSV Pol antisera. A. Profile of DNA polymerase (open squares) and 3'-5' exonuclease (closed diamonds) activities. Arrows show the positions of size markers (catalase, 220 kilodaltons; bovine serum albumin, 68 kilodaltons) sedimented in a parallel gradient. B. Immunoblot analysis of an SDS polyacrylamide gel of the indicated fractions from the gradient in A performed as in Fig. 3. C. SDS-polyacrylamide gel electrophoresis analysis of an aliquot of the pooled active fractions. Following electrophoresis, the gel was stained with Coomassie Blue.

Specific activity of HSV Pol using activated DNA as primer-template

It has been very difficult, if not impossible, to purify HSV Pol from HSV-infected mammalian cells without contamination with other proteins, especially the double-stranded DNA binding protein that is the product of the HSV UL42 gene (10–14,19). Indeed, it has been suggested that the UL42 gene product and the single-stranded DNA binding protein, ICP8, stimulate or

otherwise function as accessory proteins for HSV Pol (11,14,16,33).

Previous studies using heterologous expression systems (31,32) have allowed the conclusion that no other viral proteins are essential for HSV Pol activity on activated DNA templates, but have not ruled out the involvement of cellular proteins or stimulatory effects of viral proteins.

The data reported here indicate that HSV Pol does not require any cellular proteins for its activity. Moreover, because the

TABLE 3. RNase H activity

Enzyme	% Substrate Rendered ³ H-poly rA/poly dT	Acid-soluble ^a ³ H-poly rA
BP58-expressed pol, 1 unit	39	6
BP58-expressed pol, 5 units	94	76
<i>E. coli</i> DNA polymerase I, 5 units	100	72
<i>E. coli</i> RNase H, 0.5 units	70	6
Klenow fragment, 5 units	9	1

^a The amount of radioactivity that was acid-soluble after digestion with the indicated enzyme less the amount that was acid-soluble after mock-digestion (8% for the hybrid, 14% for the poly rA) divided by the total amount of radioactivity in the reaction less the amount that was acid-soluble after mock-digestion.

specific activity of the purified HSV Pol on activated calf thymus DNA was very similar to that of HSV Pol isolated from HSV-infected mammalian cells, it appears that any stimulatory activity on this primer-template of proteins associated with polymerase isolated from HSV-infected cells, such as the UL42 gene product, is slight. Perhaps, certain HSV proteins can greatly stimulate HSV pol on other primer-template combinations or certain preparations of activated DNA. The purification of HSV Pol to apparent homogeneity (Fig. 7C) should be useful for investigations of interactions with other proteins.

3'-5' exonuclease activity is intrinsic to the HSV Pol polypeptide

Several lines of evidence have suggested that the 3'-5' exonuclease activity associated with HSV Pol is intrinsic to the HSV Pol polypeptide (12,15-17,34). However, because previous experiments were performed with polymerase preparations that almost certainly contained additional polypeptides, these data could be explained by the presence of a separate polypeptide that carries the exonuclease activity and is closely associated with polymerase. The co-purification of 3'-5' exonuclease activity and apparently homogeneous HSV Pol polypeptide from cells lacking other HSV proteins (Fig. 7) permits us to conclude that the 3'-5' exonuclease is intrinsic to the HSV Pol polypeptide, as is true with many other DNA polymerases.

Based on sequence comparisons of the polymerases encoded by HSV, T4, and other members of their family with *E. coli* polymerase I, Reha-Krantz (35) has proposed that a region of sequence similarity, termed region IV by Wong et al. (36), is involved in 3'-5' exonuclease activity. The availability of the baculovirus expression system and site-directed mutagenesis methods should facilitate testing this hypothesis for HSV Pol.

RNase H activity associated with the HSV Pol polypeptide

Crute and Lehman (19) have detected RNase H activity in preparations of HSV Pol isolated from HSV-infected mammalian cells. The RNase H activity co-migrated with the approximately 140 kilodalton Pol polypeptide on activity gels, providing crucial evidence that this activity is intrinsic to the Pol polypeptide. Our finding of this activity in apparently homogeneous purified Pol from BP58-infected insect cells (Table 3) provides complementary evidence, confirming their finding.

Crute and Lehman have also observed that the RNase H activity associated with HSV DNA polymerase is a 5'-3' exonuclease activity that is also active against double-stranded DNA, as is the case with *E. coli* Pol I (19). Consistent with this observation, the BP58-expressed HSV Pol exhibited an RNase H activity that is similar to Pol I in substrate specificity. L. Reha-Krantz

(personal communication) has found a region of sequence similarity among *E. coli* RNase H, the N-terminal 5'-3' exonuclease domain of *E. coli* DNA polymerase I that harbors its RNase H activity, and N-terminal regions of both T4 DNA polymerase and HSV DNA polymerase. Intragenic complementation suggestive of multiple polymerase functions have been observed in both the T4 and HSV systems, in which mutations located in the N-terminal regions of the polymerase can complement mutations that are more C-terminal and affect polymerase activity (21,37; Marcy et al., submitted; Reha-Krantz, personal communication). All of these observations taken together engender the hypothesis that, like *E. coli* DNA polymerase I, HSV DNA polymerase contains a 5'-3' exonuclease/RNase H activity in a discrete N-terminal domain. It should be possible to test this hypothesis.

Levels of expression of HSV pol in recombinant baculovirus-infected cells

The best understood DNA polymerase is *E. coli* DNA polymerase I. Progress on this enzyme accelerated greatly following the successful overexpression of its large (Klenow) fragment, which retains polymerase and 3'-5' exonuclease activity (reviewed in 38). The availability of large quantities of pure Klenow fragment has greatly facilitated biophysical studies of enzyme structure and substrate conformation (39-41) and detailed biochemical studies of polymerase and exonuclease mechanisms (42,43). The levels of expression of HSV Pol that we have obtained make it possible to contemplate analogous experiments with both wild type and mutant viral enzymes.

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