The DNA polymerase encoded by herpes simplex virus 1 consists of a single polypeptide of M, 136,000 that has both DNA polymerase and 3'-5' exonuclease activities; it lacks a 5'-3' exonuclease. The herpes polymerase is exceptionally slow in extending a synthetic DNA primer annealed to circular single-stranded DNA (turnover number ~0.25 nucleotide). Nevertheless, it is highly processive because of its extremely tight binding to a primer terminus (K < 1 nM). The single-stranded DNA-binding protein from Escherichia coli greatly stimulates the rate (turnover number ~4.5 nucleotides) by facilitating the efficient binding to and extension of the DNA primers. Synchronous replication by the polymerase of primed single-stranded DNA circles coated with the single-stranded DNA-binding protein proceeds to the last nucleotide of available 5.4-kilobase template without dissociation, despite the 20-30 min required to replicate the circle. Upon completion of synthesis, the polymerase is slow in cycling to other primed single-stranded DNA circles. ATP (or dATP) is not required to initiate or sustain highly processive synthesis. The 3'-5' exonuclease associated with the herpes DNA polymerase binds a 3' terminus tightly (K < 50 nM) and is as sensitive as the polymerase activity to inhibition by phosphonoacetic acid (K, ~ 4 μM), suggesting close communication between the polymerase and exonuclease sites.

The replication of a duplex DNA chromosome requires the concerted action of several proteins that are thought to assemble into a multiprotein complex (1). To understand the molecular mechanism by which a eukaryotic chromosome is replicated, we have chosen to study herpes simplex virus 1 (HSV-1). The HSV-1 genome which is a linear duplex approximately 150 kb in length encodes many of the enzymes required for its replication, including a DNA polymerase and a single-stranded DNA-binding protein (2). Partial purification of the herpes DNA polymerase has shown it to be an approximately 150-kDa polypeptide (3) in good agreement with the molecular mass of 136 kDa predicted from the nucleotide sequence of the gene (4, 5). As a first step in our analysis of HSV-1 DNA replication, we have purified the herpes-induced DNA polymerase to homogeneity and examined the dynamics of its replication of ssDNA templates.

A second viral encoded protein known to be essential for HSV-1 DNA replication, ICPS (6-8), binds ssDNA tightly and cooperatively (9) and is therefore analogous to the procaryotic single-stranded DNA-binding proteins typified by Escherichia coli SSB and T4 gene 32 protein (1). The interaction of ICPS with the DNA polymerase in the presence of single- and double-stranded DNA templates is the subject of the accompanying paper (10).

EXPERIMENTAL PROCEDURES

Materials—Unlabeled and labeled nucleotides were purchased from Pharmacia P-L Biochemicals and Amersham Corp., respectively. dAMP-PNP was a gift from Dr. B. Alberts (University of California, San Francisco). dATP and M13GoriI viral DNAs were prepared as described (11); all viral DNA concentrations are expressed as DNA molecules and were calculated using an A260 of 1 as equivalent to 36 μg/ml. (dA)40 and (dT)17 were purchased from Pharmacia P-L Biochemicals. Calf thymus DNA, purchased from Sigma, was activated as described (12). SSB (4 × 10⁶ units/mg) (13) was a gift from Dr. D. Soltis (this department). DNA polymerase III holoenzyme fraction V (7 × 10⁶ units/mg) was prepared as described (14). E. coli DNA ligase was prepared as described (15). T4 DNA polymerase and T4 polynucleotide kinase were obtained from the United States Biochemical Co. Phosphonoacetic acid was obtained from Sigma. Bio-Gel A-1.5m and protein molecular weight markers were obtained from Bio-Rad. Polyethyleneimine-cellulose sheets (Polygram MN300) were obtained from Brinkmann Instruments; Centricon 10 was from Amicon.

Buffers—Buffer A was 20 mM Tris-Cl (pH 7.5), 6 mM MgCl₂, 4% glycerol, 0.1 mM EDTA, 40 μg/ml bovine serum albumin, 5 mM dithiothreitol. Buffer B was 20 mM Hepes/Na⁺ (pH 7.5), 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% (w/v) glycerol. Buffer C was 50 mM Tris-Cl (pH 7.5), 150 mM (NH₄)₂SO₄, 0.5 mM dithiothreitol, 0.1 mM EDTA.

Cells and Viruses—RD305 (16), a thymidine kinase-deficient mutant of HSV-1 [F], was used to infect roller-bottle cultures of Vero cells using a multiplicity of infection of 5-10 plaque-forming units/cell.

Purification of HSV-1 DNA Polymerase—The herpes-induced DNA polymerase was purified by a modification of the method of Powell and Purifoy (3). The steps in the purification up to chromatography on phosphocellulose have been described (17). Briefly, nuclei extraction of 50 roller bottles of infected cells (35 g, wet weight) and extracted with 1.7 M NaCl. The DNA was removed by ultracentrifugation at 100,000 × g, and the supernatant (200 ml) was dialyzed for 6 h against two changes (2 liters each) of buffer B and loaded onto a phosphocellulose column (16-ml bed volume) equilibrated with buffer B. The phosphocellulose column was eluted with a linear gradient from 0.1 to 0.6 M NaCl in a total volume of 200 ml of buffer B. The DNA polymerase activity eluted at approximately 0.3 M
Purification of HSV-1 DNA polymerase from HSV-1-infected Vero cells

| Fraction | Total protein (mg) | Total activity* (units/mg) | Specific activity (units × 10^{-12}) | Overall yield (%) | Purification fold |
|----------|-------------------|---------------------------|--------------------------------------|------------------|------------------|
| Ia. Cytoplasm | 267 | 1.9 | 0.79 | 12 |
| Ib. Nuclear extract | 123 | 13.0 | 10.5 | 82 |
| Before dialysis | 60 | 14.0 | 23 | 88 |
| After dialysis | 7.9 | 4.8 | 61 | 30 |
| II. Phosphocellulose | 0.30 | 3.7 | 123 | 23 |
| III. DNA-cellulose | 0.12 | 2.6 | 220 | 16 |
| IV. Glycerol gradient | 583 |

* One unit is equal to 1 pmol of DNA synthesis in 10 min at 37 °C.

RESULTS

HSV-1 DNA Polymerase Is Stimulated by E. coli SSB—The HSV-1 DNA polymerase was only minimally active with circular φX ssDNA (5.4 kb) primed with a synthetic 15-mer (Fig. 1). Activity was, however, stimulated more than 20-fold upon coating the primed ssDNA with E. coli SSB (Fig. 1). The herpes DNA polymerase was also stimulated (1.5-5-fold) by 250 mM NaCl (data not shown), the extent of stimulation depending upon the relative amounts of DNA polymerase and DNA in the assay.

The influence of ionic strength on polymerase activity when the herpes DNA polymerase was in molar excess over singly primed ssDNA circles is shown in Fig. 2. DNA polymerase activity was stimulated 3-fold by 150 mM NaCl at early times.
of SSb, the herpes DNA polymerase was maximally active on SSb-coated ssDNA at an ionic strength of approximately 70 mM (data not shown).

**HSV-1 DNA Polymerase Is Highly Processive**—DNA synthesis by the herpes DNA polymerase with SSb-coated ssDNA as template reached a plateau value after 20–30 min even though all of the available DNA had not been replicated (Fig. 1). Moreover, the extent of DNA synthesis was proportional to the amount of DNA polymerase added (data not shown). The limited DNA synthesis suggests a highly processive mode of nucleotide polymerization wherein each polymerase molecule completely extends a DNA primer around the ssDNA circle without dissociation and is slow in cycling to another primed template.

Analysis of replication products from singly primed SSb-coated ssDNA by native agarose gel electrophoresis supports the highly processive mode of nucleotide polymerization (Fig. 3B). During the time in which full-length products (RF II) were formed, most of the primed ssDNA remained unchanged (detected by UV-induced ethidium bromide fluorescence). The 20 min required for the complete replication of a φX ssDNA molecule (5.4 kb) yields an average turnover number of 4.5 nucleotides/s/polymerase molecule. The lack of significant radioactivity in the region of the gel between the primed ssDNA and RF II product after 30 min indicates that cycling of the polymerase to other primed ssDNA molecules is slow. That the remaining primed circles were effective templates was demonstrated by their replication upon further addition of DNA polymerase (not shown).

The herpes DNA polymerase was also highly processive in the absence of SSb. An agarose gel analysis of the replication products formed with singly primed φX ssDNA showed that most of the DNA templates had not reacted; nevertheless, discrete, partially replicated species and some fully replicated RF II molecules were evident (Fig. 3A).

The processivity of the HSV-1 DNA polymerase was demonstrated in a second type of experiment diagramed in Fig. 4A. The DNA polymerase was preincubated with an 18-fold excess of singly primed φX ssDNA coated with SSb so that each polymerase molecule was bound to a primer terminus. dCTP and dGTP (the 3′-terminal nucleotides of the primer and the first 4 nucleotides needed for synthesis) were present during the preincubation to protect the primer from removal of the terminal nucleotides by the 3′→5′ exonuclease activity of the polymerase (see below). After a short preincubation period, an excess (2-fold over φX ssDNA circles) of challenge DNA, i.e. singly primed, M13Gori1 ssDNA (8.6 kb), was added. After further preincubation for either 5 s or 3 min, replication was initiated by the addition of dATP and [α-32P] dTTP; after 6 min, further incorporation of radioactivity was prevented by addition of excess unlabeled dTTP. Incubation for an additional 40 min ensured complete replication of templates to which a polymerase molecule was bound at the time unlabeled dTTP was added. Analysis of the replication products by agarose gel electrophoresis is shown in Fig. 4B. Essentially all the label was incorporated into the φX ssDNA template following the 5-s preincubation period. Hence, the herpes DNA polymerase remained bound to the φX DNA during synthesis. After a 3-min preincubation period, most of the radioactivity was associated with the φX ssDNA, showing only minimal transfer of the herpes polymerase to the challenging M13Gori1 DNA before initiation of synthesis. A control reaction in which the polymerase was added to a mixture of φX and M13Gori1 ssDNAs showed about twice as much incorporation of labeled nucleotide into the M13Gori1 ssDNA as into the φX ssDNA (Fig. 4B, third lane), consistent
ATP (or dATP) hydrolysis, there was no effect of ATP or dATP, and only a portion of the reaction mixture was removed after 10 min, leaving a nick that can be sealed by DNA ligase.

There is evidence that the herpes DNA polymerase is dimeric, whose tight association is initiated by the addition of the dNTPs. As shown in Fig. 3B, the replication intermediates persisted throughout the 30-min period of replication. Quantitation of radioactivity of excised gel slices showed that after 30 min, the 32P at the position of the completed RF II circles was approximately 75% that of the primer before the beginning of replication (0 min); 19% of the remaining 32P was present in the smear below the RF II products.

The reaction of Fig. 5 was initiated by adding DNA polymerase to a solution containing the primed DNA and all four dNTPs; hence, the replication intermediates appeared as a smear. A discrete band of replication intermediates (as in Fig. 3) is observed only when the polymerase is preincubated for a brief period with the primed DNA and synchronous replication is initiated by addition of the dNTPs.

**HSV-1 DNA Polymerase and 3'-5' Exonuclease Are Present within the Same Polypeptide**—The HSV-1 DNA polymerase and its associated 3'-5' exonuclease co-sediment perfectly in a 10–30% glycerol gradient at the position of a 158-kDa marker protein (Fig. 6A), consistent with previous reports (24, 25). Moreover, as shown in Fig. 6B, the herpes DNA polymerase is nearly homogenous (>90%) as judged by Coomassie Blue staining of the gradient fractions following SDS-polyacrylamide gel electrophoresis. The DNA polymerase and 3'-5' exonuclease active sites would therefore appear to reside within the same polypeptide chain. The herpes polymerase showed no detectable primase activity (data not shown).

**3'-5' Exonuclease Has Proofreading Activity**—An earlier report demonstrated that the 3'-5' exonuclease associated with the herpes DNA polymerase has proofreading activity (26). We have examined the proofreading capacity of the 3'-5' exonuclease associated with the herpes DNA polymerase.
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**HSV-1 DNA Polymerase**

**FIG. 5.** Complete replication of primed φX ssDNA circles by herpes DNA polymerase which lacks 5’→3’ exonuclease activity. A, scheme for detecting 5’→3’ exonuclease activity and complete replication of ssDNA. The 5’ end-labeled 15-mer primer annealed to φX ssDNA was prepared as described under “Experimental Procedures.” A slight excess of herpes DNA polymerase (1000 fmol) was added to initiate replication of the singly primed φX ssDNA (1.3 µg, 750 fmol as circles) in 150 µl of buffer A containing 13 µg of SSB, 0.5 mM ATP, 60 µM dCTP, dGTP, and dATP, and 20 µM [3H]dTTP (2500 cpm/fmol). After 10 min, 50 µl was removed and incubated with NAD+ (0.1 mM final concentration) and 0.3 pg of E. coli ligase. Samples (12.5 µl) were removed at the times indicated and quenched with SDS/EDTA; the DNA products were analyzed by neutral agarose gel electrophoresis; and DNA synthesis was quantitated as described under “Experimental Procedures.” B, autoradiogram of 0.8% neutral agarose gel electrophoresis of the products of replication. Eight to tenth lanes, the time noted is after the addition of ligase; Arrows mark the positions of RF II, RF I, and ssDNA standards detected by UV-induced ethidium bromide (EtBr) fluorescence.

5’ exonuclease using defined synthetic DNA hairpin templates (57-mers) having either a paired or unpaired labeled 3’ terminus (diagramed in Fig. 7A). In the presence of the 4 dNTPs, the 3’→5’ exonuclease completely removed the unpaired 3’-terminal nucleotide (Fig. 7B). In contrast, the paired 3’ terminus was stable to the 3’→5’ exonuclease in the presence of dNTPs (Fig. 7B), due presumably to the addition of nucleotides to the paired 3’ terminus by the polymerase. The 3’→5’ exonuclease hydrolyzed an unpaired 3’-terminal nucleotide at just over twice the rate at which the paired 3’-terminal nucleotide was hydrolyzed (Fig. 7C). The rates of hydrolysis of paired and unpaired 3’-terminal nucleotides did not change over the range 1 µM to 50 nM template, setting an upper limit of the Km for hydrolysis at 50 nM (Table II). Since substrate was present at saturating concentrations, apparent turnover numbers for removal of paired and unpaired 3’-terminal nucleotides could be calculated from the observed rates of hydrolysis (Table II). Use of Mn+2 in place of Mg+2 had no effect on the rate of hydrolysis of the paired 3’ terminus but stimulated removal of the unpaired 3’ terminus 1.5-fold (Table II). The effect of the herpes-encoded ssDNA-binding protein, ICP8, on the 3’→5’ exonuclease activity (Table II) is considered more fully in the accompanying report (10).

During DNA synthesis with the homopolymer template, (dA)1000·(dT)1000, a significant amount of dTMP was generated, approximately 10% of the level incorporated into the homopolymer (compare Fig. 7, B and C). However, in the absence of a primer-template, dTMP was not produced (data not shown). The dTMP is most likely formed upon hydrolysis of paired 3’ termini during DNA synthesis as observed previously for the herpes polymerase (25) and for other DNA polymerases that contain 3’→5’ exonuclease activity (1).

**Phosphonoacetic Acid Inhibits DNA Polymerase and 3’→5’ Exonuclease to Equal Extents during DNA Synthesis—** Phosphonoacetic acid inhibits both the polymerase and 3’→5’ exonuclease activities of the herpes DNA polymerase with a Ki value of approximately 4 µM (Fig. 8, A and B). In the absence of dNTPs, the Ki for phosphonoacetic acid inhibition of the 3’→5’ exonuclease on the hairpin template with either paired or unpaired 3’ termini is 50 µM (Table II), the same as the Km for hydrolysis of both paired and unpaired 3’-terminal nucleotides (see above).

**DISCUSSION**

Despite its slow rate of DNA synthesis, the HSV-1-induced DNA polymerase is strikingly processive. Once bound to its primer-template, the herpes polymerase does not dissociate during the approximately 20 min required to fully replicate a 5.4-kb φX ssDNA circle. This highly processive mode of nucleotide polymerization may be of importance in replicating the 150-kb viral chromosome and, even more important, in the synthesis of multiple copies of the genome in rolling circle DNA replication (2). Although it is not known whether Oka-zaki fragments are intermediates in replication of the herpes chromosome, the high processivity, replication to a nick sealable by DNA ligase, and lack of 5’→3’ exonuclease activity are clearly desirable features in the synthesis of discontinuous DNA fragments.

The rate of fork movement during replication of pseudorabies virus, a member of the herpes virus family, is approximately 50 nucleotides/s at 37°C (26), similar to that of eukaryotic chromosomes (27, 28). One might therefore anticipate a turnover number of at least 30–40 nucleotides/s for the herpes DNA polymerase at 30°C. The apparent turnover number of 0.25 nucleotide/s with singly primed φX ssDNA circles is far too low to sustain productive herpes virus infection (assuming 10,000 copies/cell in 10 h). However, the 20-fold stimulation of the herpes DNA polymerase upon coating the ssDNA with E. coli SSB approaches the rate in vivo. HSV-1 may therefore encode a functional analogue of E. coli SSB. Like E. coli SSB, the herpes-induced ICP8 binds tightly and cooperatively to ssDNA (9), is essential for ongoing DNA replication (8), and is present at stoichiometric levels (29). However, despite its similarity to the E. coli SSB, binding of ICP8 to ssDNA completely inhibits the replication of singly primed φX ssDNA by herpes polymerase (10). In contrast, ICP8 markedly stimulates synthesis by the polymerase on duplex DNA templates (10).

E. coli DNA polymerase III holoenzyme hydrolyzes the β,γ-phosphodiester bond of ATP (or dATP) to initiate highly processive DNA synthesis (22). In contrast, the monomeric herpes DNA polymerase does not require ATP or dATP. Thus, a complex subunit structure and hydrolysis of the terminal phosphate of ATP or dATP are not essential for highly processive DNA synthesis.

The response of the herpes DNA polymerase to ionic strength is complex. At a molar excess of DNA polymerase to the terminal phosphate of ATP or dATP, the value of approximately 4 µM (Fig. 8, A and B). In the absence of dNTPs, the Ki for phosphonoacetic acid inhibition of the 3’→5’ exonuclease on the hairpin template with either paired or unpaired 3’ termini is 50 µM (Table II), the same as the Km for hydrolysis of both paired and unpaired 3’-terminal nucleotides (see above).
FIG. 6. Glycerol gradient sedimentation analysis of herpes DNA polymerase. A, glycerol gradient sedimentation of herpes DNA polymerase is described under “Experimental Procedures.” DNA polymerase (Pol, O) was assayed using activated calf thymus DNA, and the 3'→5' exonuclease (Exo, Δ) was assayed using the synthetic 57-mer hairpin DNA with a labeled unpaired 3' terminus as described under “Experimental Procedures.” Position of protein standards in a parallel glycerol gradient are marked by brackets. B, SDS-polyacrylamide gel electrophoresis of glycerol gradient peak fractions. The tick marks correspond to the migration of molecular mass standards in the same gel.

FIG. 7. Comparison of paired and unpaired 3' termini as substrates for the 3'→5' exonuclease of herpes DNA polymerase. A, diagram of synthetic DNA hairpin substrates labeled at the 3' terminus with either a paired deoxyadenylate or unpaired deoxythymidylate residue; B, plots of 3'→5' exonuclease activity on the 3'-paired and -unpaired hairpin templates in the presence of dNTPs; C, plots of 3'→5' exonuclease activity on 3'-paired and -unpaired hairpin templates in the absence of dNTPs. Reactions were as described under “Experimental Procedures.”

| 3' termini | Turnover number (nucleotides/s) | 30 °C, no addition | 37 °C, no addition | 30 °C, ICP8 added | 30 °C, Mn2+ | Kₐ, 3' termini (µM) | K, PAA (µM) |
|------------|-------------------------------|-------------------|-------------------|----------------|----------|----------------|-------------|
| Paired     |                               | 0.066             | 0.073             | 0.178          | 0.066    | <0.05          | 50          |
| Unpaired   |                               | 0.16              | 0.23              | 0.046          | 0.24     | <0.05          | 50          |

*PAA, phosphonoacetic acid.

primer termini, the polymerase is stimulated at early times of synthesis by an ionic strength of 100 mM; however, it is inhibited at later times under these conditions. This behavior may be explained by salt stimulation of a rate-limiting step in polymerase catalysis while enzyme is bound to DNA, which is offset by an increase in the stability of DNA sequence-specific pause sites at salt concentrations above 100 mM (e.g. hairpin structures). However, at substoichiometric ratios of polymerase to primer termini, polymerase activity is stimulated up to an ionic strength of 250 mM. Under these conditions, the polymerase can extend a primer to a sequence-specific pause site and then dissociate from the terminus to gain access to an unused primer terminus. Maximum activity of herpes DNA polymerase on SSB-coated singly primed ssDNA circles at moderate ionic strength (60–70 mM) may result from the combined effect of SSB-induced removal of sequence-specific barriers and an intrinsic stimulation of nucleotide polymerization at this ionic strength.

The exceptionally low Kₐ value of the 3'→5' exonuclease for a primer terminus may underlie the only 2-fold difference in rates of removal of paired and unpaired 3' termini. The 3'→5' exonuclease of the DNA polymerase isolated from HSV-2-infected cells has been reported to remove a 3' unpaired terminus six times faster than a 3'-paired terminus in the absence of dNTPs. The basis for the discrepancy between that result and the one presented here is unclear, but may reside in the different assay conditions and/or source of enzyme.

J. Abbotts, Y. Nishiyama, S. Yoshida, and L. A. Loeb, unpublished observations.
It is not surprising that the 3′→5′ exonuclease and polymerase activities share the same polypeptide chain. Other polymerases with associated exonuclease activity have both active sites on a single polypeptide (1). However, the roughly equal sensitivity of the herpes polymerase and 3′→5′ exonuclease activities to inhibition by phosphonoacetic acid during DNA synthesis was unexpected. In the case of the E. coli DNA polymerase I large fragment (31), crystallographic analysis has demonstrated that the two active sites are physically separated from each other by approximately 25 Å (32). Possibly, phosphonoacetic acid binds to one active site and thereby prevents switching of the primer terminus to the other site.

The herpes polymerase differs in many important respects from DNA polymerase α (Table III). These differences may reflect differences in the complexity of replicating host chromosomes organized into a complex nucleosomal structure as compared with the relatively simple 150-kb HSV-1 genome. In both cases, however, the DNA polymerase is very likely associated with accessory replication proteins that could very significantly alter their catalytic properties.

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| TABLE III |
| Comparison of HSV-1 DNA polymerase with DNA polymerase α |

|       | HSV-1 DNA polymerase | DNA polymerase α |
|-------|----------------------|------------------|
| M, 10^6 | 136,000              | 280,000          |
| No. of subunits | 1                  | 4                |
| Primase | No                    | Yes              |
| 3′→5′ exonuclease | No                | Yes              |
| 5′→3′ exonuclease | No                | No               |
| Rate of polymerization (nucleotides/minute)/enzyme | 4.5              | 2                |
| Processivity | >5000               | 15               |
| Effect of 0.2 M NaCl | Stimulates         | Inhibits         |
| Inhibition by PAA | Yes                | No               |

*DNA polymerase from Drosophila embryos (33).  
1Singly primed ex ssDNA coated with E. coli SSB as template.  
2Activated calf thymus DNA template.  
3PAA, phosphonoacetic acid.

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