

# *Autographa californica* Nuclear Polyhedrosis Virus DNA Polymerase: Measurements of Processivity and Strand Displacement

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**The DNA polymerase (DNAPol) of *Autographa californica* nuclear polyhedrosis virus was purified to homogeneity from recombinant baculovirus-infected cells. DNAPol was active in polymerase assays on singly primed M13 template, and full-length replicative form II product was synthesized at equimolar ratios of enzyme to template. The purified recombinant DNAPol was shown to be processive by template challenge assay. Furthermore, DNAPol was able to incorporate hundreds of nucleotides on an oligo(dT)-primed poly(dA) template with limiting amounts of polymerase. DNAPol has moderate strand displacement activity, as it was active on nicked and gapped templates, and displaced a primer in a replication-dependent manner. Addition of saturating amounts of LEF-3, the viral single-stranded DNA-binding protein (SSB), increased the innate strand displacement ability of DNAPol. However, when LEF-3 was added prior to the polymerase, it failed to stimulate DNAPol replication on a singly primed M13 template because the helix-destabilizing activity of LEF-3 caused the primer to dissociate from the template. *Escherichia coli* SSB efficiently substituted for LEF-3 in the replication of a nicked template, suggesting that specific protein-protein interactions were not required for strand displacement in this assay.**

*Autographa californica* nuclear polyhedrosis virus (AcNPV) is the best-understood member of the insect virus family *Baculoviridae*. It contains a double-stranded circular genome of 134 kb that may encode as many as 154 proteins. Transient expression assays have shown that six viral proteins (DNA polymerase [DNAPol], P143, IE-1, LEF-1, LEF-2, and LEF-3) are essential for DNA replication (15). The functions of only LEF-3 and IE-1 are known. LEF-3 is a single-stranded DNA (ssDNA) binding protein (SSB) (13), and IE-1 is a transactivator of early gene expression (10). IE-1 specifically binds to the conserved palindromes within regions known as *hrs* in the viral genome (8,9). These *hrs* function as enhancers and as origins of replication (10, 21). IE-1 is the major viral transactivator and is required for the expression of all delayed-early genes, including those encoding the other five DNA replication proteins. Thus, it is not clear whether the requirement for IE1 in transient replication reflects its known role in gene expression or a potential role in origin binding.

The functions of three of the remaining replication proteins have been predicted on the basis of amino acid homology, though biochemical assays have yet to confirm these predictions. AcNPV DNAPol has significant homology with the DNA polymerase B family, which includes eukaryotic DNA polymerases  $\alpha$  and  $\delta$  and the viral polymerases of adenoviruses, poxviruses, and bacteriophage T4 (24). The p143 (also called helicase) gene was originally identified as the site of a temperature-sensitive mutation with a DNA-negative phenotype (17). Analysis of the p143 protein revealed the presence of an ATP binding loop and additional motifs conserved among DNA helicases (17). Finally, several baculovirologists have speculated that LEF-1 is a primase, based on limited sequence similarity with the p48 subunit of DNA polymerase  $\alpha$ -primase (1, 4, 18).

Database searches for proteins homologous to LEF-2 have failed to yield any clues as to its function. However, functional predictions have been made based on yeast two-hybrid assays and glutathione *S*-transferase chromatographic assays which indicate that LEF-1 and LEF-2 interact (4). These results combined with the LEF-1-primase homology have led to speculations that LEF-2 is a primase-associated protein.

In other systems, essential proteins include helicase, primase, SSBs, DNA polymerase, and accessory proteins that impart high processivity to the DNA polymerase (16). Thus, we have good candidates for all of the usual essential functions except processivity factors. The processivity of a DNA polymerase is a relative measure of the number of nucleotides incorporated per binding event. In most cases, the processivity of a DNA polymerase is conferred by accessory factors that work by a clamp mechanism to stabilize the binding of the polymerase to the DNA, although some viruses, like phage  $\phi$ 29 and adenovirus (2, 5), encode enzymes that are inherently processive in the absence of additional factors. DNA polymerases with high processivity are required for leading-strand replication, while distributive enzymes are used for lagging-strand replication and DNA repair synthesis. Stable binding to the template is required for efficient leading-strand synthesis, but the polymerase must be able to repeatedly disengage from the template to accomplish lagging-strand synthesis. Modification by an accessory factor allows the processivity of a polymerase molecule to vary and thus meet the requirements of both leading- and lagging-strand DNA synthesis. Phage  $\phi$ 29 and adenovirus have linear genomes that are replicated symmetrically from each end by leading-strand synthesis. Therefore, a single processive enzyme is sufficient since these enzymes do not engage in lagging-strand synthesis.

We expected that baculovirus DNA replication would require one or more processivity factors. Indeed, AcNPV encodes a protein, called PCNA (proliferating nuclear cell antigen), with the potential to function as a processivity factor. This protein has 42% amino acid identity to mammalian PCNA, which is a processivity factor for DNA polymerase  $\delta$ .

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But PCNA was not identified by the transient replication assay, nor does it stimulate transient DNA replication (15), suggesting that it does not play a vital role in DNA replication. Furthermore, the gene encoding PCNA is not essential, although PCNA-null viruses have a delayed DNA replication phenotype (3). To further address the role of accessory proteins in baculovirus DNA replication, we overexpressed and purified AcNPV DNAPol and characterized its activity with respect to processivity and strand displacement. The purified enzyme was shown to possess polymerase activity on a singly-primed M13 template in amounts equimolar to template. AcNPV DNAPol was processive in the synthesis of poly(dA)-oligo(dT) templates and in template challenge assays. DNAPol was active on nicked and gapped templates and was shown to have strand displacement activity. This strand displacement activity was greatly increased by the addition of LEF-3.

#### MATERIALS AND METHODS

**Cells and virus.** *Spodoptera frugiperda* Sf9 cells were cultured in TNM-FH medium supplemented with 10% fetal calf serum. AcNPV strain E2 was propagated and maintained as previously described (23).

**Construction of a recombinant baculovirus expressing DNAPol.** Site-directed mutagenesis was performed on the genomic clone pBgIII-F (3) to insert a *Bam*HI site upstream of the DNAPol open reading frame, using a QuikChange mutagenesis kit (Stratagene) according to manufacturer's instructions. The resulting plasmid was digested with *Bam*HI and *Not*I, and the 3-kb fragment containing the DNAPol gene was ligated to pVL1393, also digested with *Bam*HI and *Not*I. The resulting transfer vector and RP6-S/C DNA, previously digested with *Bsu*36I, were cotransfected into Sf9 cells. Progeny virions were separated by plaque purification, and selected polyhedron-deficient plaques were further purified by plaque assay. Viral DNAs were screened by *Eco*RI digestion to verify that the selected plaques were double-crossover recombinants. A plaque isolate with the desired insertion was named AcDNAPol.

**Purification of DNAPol.** Sf9 cells ( $10^9$ ) were infected at a multiplicity of infection of 10 and harvested at 48-h postinfection. The cells were washed three times in cold phosphate-buffered saline and then resuspended in 1× PCV (packed-cell volume) of hypotonic buffer (20 mM HEPES [pH 7.5], 5 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1.0 mM dithiothreitol [DTT], 1 μg of leupeptin per ml, 1% aprotinin). After 10 min on ice, cells were Dounce homogenized and centrifuged at  $2,000 \times g$ . The cytoplasmic fraction was removed, and the pellet resuspended in 1× PCV of hypotonic buffer. An equal volume of hypotonic buffer plus 3.4 M NaCl was added, and the cell suspension was shaken gently for 1 h on ice. The nuclear extract was then centrifuged for 1 h at  $100,000 \times g$ , and the supernatant was dialyzed twice against buffer A (0.25 M KCl, 20 mM KH<sub>2</sub>PO<sub>4</sub> [pH 7.2], 1 mM EDTA, 10 mM β-mercaptoethanol).

The extract was applied to a 2-ml DE52 column previously equilibrated in buffer A and washed with 2 column volumes of buffer A. The flowthrough and wash fractions were combined and precipitated with 50% saturated ammonium sulfate overnight at 4°C. Following centrifugation at  $5,000 \times g$  for 20 min, the pellet was resuspended in buffer B (50 mM KCl, 20 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, 10 mM β-mercaptoethanol) and dialyzed against buffer B with three changes of 1 liter each. The sample was then loaded on a 5-ml heparin column (Bio-Rad) connected to a Pharmacia fast protein liquid chromatography system. The column was washed in buffer B and eluted with a 20-ml gradient from 0.05 to 0.5 M KCl. Peak fractions were chosen by analysis on a sodium dodecyl sulfate (SDS)-polyacrylamide gel, pooled, dialyzed against buffer B, and loaded onto a MonoQ HR 5/5 column (Pharmacia). The sample was eluted in a 20-ml gradient from 0.05 to 0.5 M KCl. Peak fractions were chosen by analysis on an SDS-polyacrylamide gel, pooled, dialyzed against buffer B, and loaded on a MonoS column. DNAPol was eluted with a linear salt gradient, dialyzed against buffer B, and loaded on a 1-ml ssDNA agarose column (Bethesda Research Laboratories). Peak fractions were eluted with a step gradient in 0.1 M increments from 0.1 to 0.5 M KCl in buffer B. DNAPol was shown to be purified to homogeneity by SDS-polyacrylamide gel electrophoresis (PAGE) and was dialyzed against buffer B plus 50% glycerol.

**DNA templates.** Singly primed single-stranded M13mp18 was made by annealing 25 pmol of M13(-20) primer to 2.5 pmol of ssDNA. The mixtures, containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl<sub>2</sub>, and 100 mM NaCl in 50 μl, were heated to 100°C and slowly cooled to room temperature. A gapped M13 template was made by annealing the 21-mer oligonucleotide (CCGCTACAATTCAC ACAAC) 270 nucleotides (nt) downstream from the first radiolabeled primer in 10:1 primer-to-template ratio. A second gapped template was constructed in the same manner; a 40-mer primer oligonucleotide (ATTTTAAATGCAATGCCT GAGTAATGTGTAGGTAAAGATT) was annealed 230 nt upstream of a radiolabeled 40-mer terminator oligonucleotide (AGGAAGATTGTATAAGCAAA TATTTAAATGTAAACGTTA) and then digested with *Dra*I. Poly(dA)<sub>2000-3000</sub> and oligo(dT)<sub>12-18</sub> (Pharmacia) were combined in a 1:1 molar ratio, heated to

65°C, and slowly cooled to room temperature. Where indicated, primers were radiolabeled with T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP. Unannealed primers were removed by Superdex G50 spun chromatography.

**DNA replication assay.** To investigate DNAPol activity on singly primed M13 or φX174 templates, 50-μl reactions were assembled as described by Tsurumi et al. (25). Reactions mixtures contained 20 fmol of template, 20 mM Tris-acetate (TrisAc; pH 7.3), 75 mM KAc, 5 mM MgAc, 1 mM DTT, 0.5 mM ATP, 60 μM dATP, dGTP, and dTTP, 20 μM dCTP, 2.5 μCi of [ $\alpha$ -<sup>32</sup>P]dCTP (800 Ci/mmol), 50 μg of bovine serum albumin (BSA), and purified DNA polymerase as indicated in the figure legends. After incubation at 37°C for 30 min, the reactions were stopped by the addition of an equal volume of a solution containing 1% SDS, 40 mM EDTA, and 60 μg of calf thymus DNA. The samples were subsequently extracted with phenol and precipitated with ethanol, and DNAs were separated by alkaline agarose gel electrophoresis (22).

**Measurements of processivity.** The template challenge assay was performed as previously described (7). Following ethanol precipitation, the pellets were resuspended in 20 μl of a solution containing 0.1 N NaOH, 5% glycerol, 1 mM EDTA, and 0.025% bromocresol green and loaded on an alkaline agarose gel (22). The dried gel was exposed to autoradiography film overnight.

DNAPol activity on a poly(dA)-oligo(dT) template was measured as previously described (19), with modifications. Reaction mixtures (25 μl) contained 336 fmol of poly(dA)<sub>2000-3000</sub> and oligo(dT)<sub>12-18</sub>, 20 mM TrisAc (pH 7.3), 75 mM KAc, 5 mM MgAc, 1 mM DTT, 0.5 mM ATP, 50 μg of BSA, 200 μM dTTP, and DNA polymerase as indicated in the figure legends. After incubation at 37°C for 5 min, reactions were terminated by addition of 10 μl of 1× Tris-borate-EDTA (TBE)-90% formamide-0.03% (wt/vol) bromophenol blue-0.03% (wt/vol) xylene cyanol; 5-ml aliquots were fractionated on 0.4-mm 7 M urea-12% polyacrylamide gels run in 1× TBE. Gels were dried and subjected to autoradiography.

**Strand displacement assays.** Strand-displacing DNA polymerase activity was measured by using a gapped M13 template according to the procedure of Hottinger et al. (14), with minor modifications. A 25-μl reaction mixture containing 20 fmol of template, 20 mM TrisAc (pH 7.3), 75 mM KAc, 5 mM MgAc, 1 mM DTT, 0.5 mM ATP, 60 mM dATP, dGTP, dTTP, and dCTP, 50 μg of BSA and 50 fmol of DNAPol was incubated at 37°C for 1 h. Reactions were stopped by the addition of an equal volume of a solution containing 1% SDS, 40 mM EDTA, and 60 μg of calf thymus DNA. Following ethanol precipitation, the pellets were resuspended in 20 μl of a solution containing 50 mM Tris-HCl (pH 7.5), 80% formamide, 20 mM EDTA, 0.03% (wt/vol) bromophenol blue, and 0.03% (wt/vol) xylene cyanol and run on a 6% acrylamide-TBE gel containing 7 M urea. Electrophoresis was performed at 200 V until the blue dye reached the bottom of the gel. The gel was dried and exposed to X-ray film overnight at -80°C.

Displacement of the single strand was directly quantitated by using a digested gapped template as described previously (11). After hybridization of the two oligonucleotides to the ssDNA template, the DNAs were digested with *Dra*I to yield a gapped template with oligonucleotides hybridized to the 5' and 3' ends. A 10-μl reaction mixture contained 20 fmol of template, 20 mM TrisAc (pH 7.3), 75 mM KAc, 5 mM MgAc, 1 mM DTT, 60 μM dATP, dGTP, dTTP, and dCTP, 50 μg of BSA, and DNA polymerase as indicated in the figure legends. Control reactions lacked dCTP and were used for background subtraction. After incubation at 37°C for 10 min, the reactions were stopped by the addition of 2 μl of 12% (wt/vol) sucrose-50 mM EDTA (pH 8)-1% SDS-0.03% (wt/vol) xylene cyanol. The reactions were electrophoresed on a 12% polyacrylamide gel in 0.5× TBE for 40 min at 200 V. The gel was fixed in 10% acetic acid-40% isopropanol and radioactivity was dried, and quantitated by PhosphorImager analysis.

Displacement of single strands was also verified by demonstrating that displaced primers were sensitive to single-strand-specific nuclease. The reaction consisted of the standard singly primed M13 synthetic assay as described above. After DNA synthesis was completed, the samples were extracted with phenol-chloroform and ethanol precipitated. The pellet was resuspended in mung bean nuclease buffer and incubated in the presence or absence of 1 U of mung bean nuclease. Following incubation for 30 min at 37°C, the reaction products were precipitated with ethanol and analyzed by alkaline agarose gel electrophoresis.

AcDNAPol activity was measured on a nicked template with and without the addition of SSBs as indicated in figure legends. Reaction mixtures (25 μl) contained 0.5 μg of nicked DNA, 20 mM TrisAc (pH 7.3), 75 mM KAc, 5 mM MgAc, 1 mM DTT, 0.5 mM ATP, 60 mM dATP, dGTP, and dTTP, 20 mM dCTP, 2.5 μCi of [ $\alpha$ -<sup>32</sup>P]dCTP (800 Ci/mmol), 50 μg of BSA, 16 ng of DNase I per ml, and Klenow or AcNPV DNA polymerase as indicated. After incubation for 1 h at 16°C, reactions were quenched with 1 μl of 0.5 M EDTA; 10 μl of each reaction mixture was spotted on glass filters and washed in trichloroacetic acid, and radioactivity was quantitated by Cerenkov counting (22).

#### RESULTS

**Overexpression and purification of the DNAPol.** The DNAPol gene was cloned into the transfer vector pVL1393 so that it was expressed under the control of the polyhedrin promoter. Recombinant virus was produced from cotransfection of pVL1393-dnapol and RP6-S/C viral DNA in Sf9 cells. One plaque, pu-

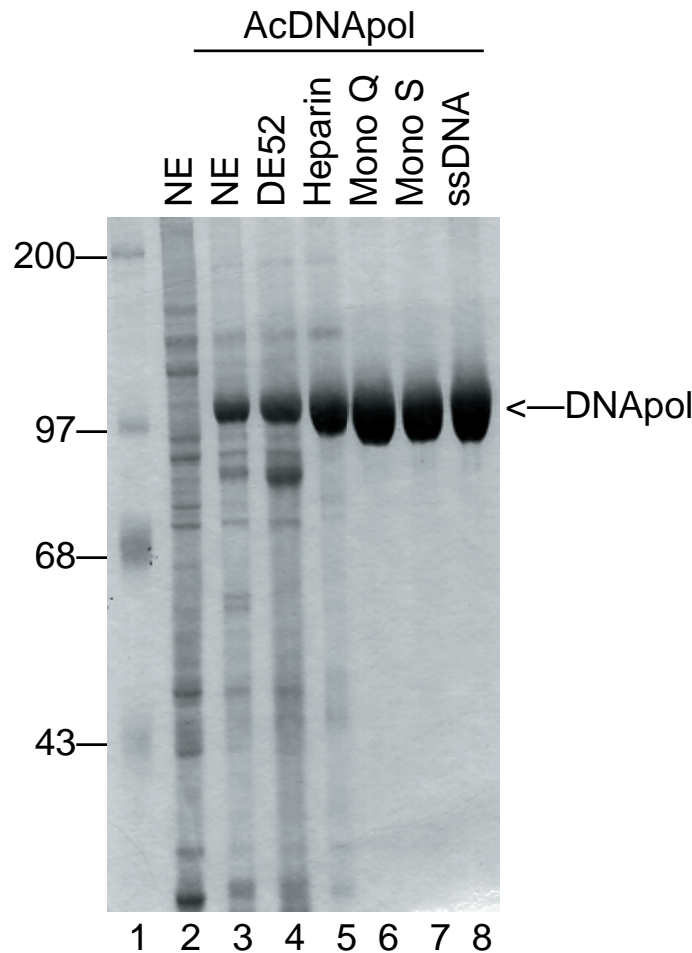


FIG. 1. Purification of DNAPol. Nuclear extracts (NE) prepared from AcDNApol-infected cells (lane 3) were subjected to chromatography on DE52 (lane 4), heparin (lane 5), MonoQ (lane 6), MonoS (lane 7), and ssDNA agarose (lane 8). Lanes 4 to 8 contain 10  $\mu$ g of protein from the peak fractions of each column. Lane 2 contains 10  $\mu$ g of crude nuclear extracts prepared from RP6-S/C-infected cells. The positions of protein molecular markers electrophoresed in lane 1 are shown in kilodaltons on the left; the position of DNAPol is indicated on the right. Samples were separated on SDS-8% polyacrylamide gels and stained with Coomassie blue.

rified and isolated, was shown to contain the correct insert by restriction enzyme analysis of extracted viral DNA (data not shown). The virus was amplified and named AcDNApol.

Nuclear extracts were prepared from cells infected with AcDNApol at 48 h postinfection. Comparison of the protein profiles with extracts prepared from the parental virus revealed strong overexpression of a protein with the expected molecular weight of DNAPol (Fig. 1; compare lanes 2 and 3). The nuclear extract was first passed over a DE52 column at 250 mM KCl to remove contaminating DNA. The DE52 flowthrough was precipitated with 50% ammonium sulfate to concentrate the protein. The pellet was dialyzed and loaded onto a heparin affinity column. The peak of DNAPol, as determined by SDS-PAGE analysis of column fractions, eluted between 0.35 and 0.49 M KCl. The peak heparin fractions were subsequently dialyzed and loaded onto a MonoQ HR5/5 anion-exchange column. DNAPol, which is positively charged at neutral pH, bound to the resin and was released at 0.13 M KCl. The MonoQ peak contained only minor amounts of contaminating low-molecular-weight proteins. The peak fraction from MonoQ was applied to a MonoS cation-exchange column. DNAPol bound to MonoS, presumably through affinity interactions, as the pI does not predict binding by ionic forces. DNAPol eluted from MonoS at 0.34 M KCl. The MonoS peak was essentially ho-

mogeneous, as judged by SDS-PAGE analysis. However, the MonoS peak was further fractionated on an ssDNA agarose column to ensure purity. DNAPol eluted from ssDNA in the 0.5 M KCl fraction. Analysis of 10  $\mu$ g of the peak fraction on a Coomassie blue-stained SDS-polyacrylamide gel revealed only a single protein band, indicating that DNAPol was purified to homogeneity (Fig. 1, lane 8).

**Activity of DNAPol on a singly primed ssDNA template.** Purified DNAPol was tested for its ability to extend a primer annealed to single-stranded M13 phage DNA (Fig. 2). Twenty femtomoles of singly primed M13 template was incubated with 20, 50, or 100 fmol of purified recombinant DNAPol in the presence of [ $\alpha$ - $^{32}$ P]dCTP. DNA synthesis was stopped after 30 min, and reaction products were fractionated on alkaline agarose gels. At an equimolar ratio of enzyme to template, full-length product (replicative form II [RFII]) was detected, as well as a shorter product of approximately 3.2 kb (Fig. 2, lane 2). Longer than full-length product was also visible, suggesting that the polymerase displaced the primer and some nascent DNA upon replicating the single-stranded region of the template. The 3.2-kb product likely represents pausing and/or dissociation of the enzyme at a region of secondary structure. The holoenzyme of Epstein-Barr virus DNA polymerase appears to pause in the same place (25) on the singly-primed M13 template.

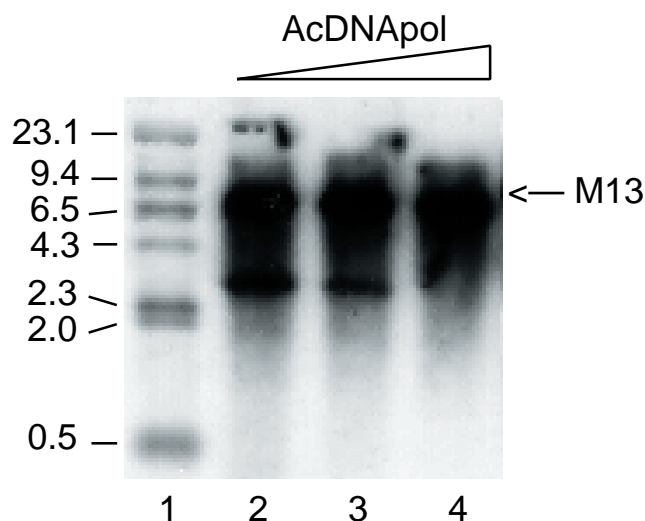


FIG. 2. Replication of singly primed M13 DNA by AcNPV DNAPol. Purified DNAPol (20, 50, or 100 fmol; lanes 2 to 4) was incubated with 20 fmol of singly primed M13 DNA. Reaction products were denatured and separated on a 0.8% alkaline agarose gel. Lane 1 contains  $^{32}\text{S}$ -labeled *Hind*III-digested  $\lambda$  DNA. Sizes of the molecular markers are shown in kilobases on the left; the position of full-length M13 DNA (7.2 kb) is indicated on the right.

Still, the fact that full-length product was observed, even at the lowest ratio of enzyme to template, suggests that DNAPol can unwind regions of secondary structure. Excess enzyme to template increases the amount of full-length product, presumably by increasing the likelihood of reassociation of the enzyme to the partially extended primer.

With increasing amounts of enzyme (Fig. 2, lanes 2 to 4), the overall amount of incorporation was not increased, although proportionally larger amounts of substrate were converted to RFII. The fact that incorporation did not increase from equimolar to fivefold excess of enzyme to template indicates that all of the templates were actively engaged in DNA replication when the enzyme was equimolar to template. Therefore, the polymerase activity was not significantly influenced by minor contaminating proteins in the preparation.

**Processivity of DNAPol on poly(dA)-oligo(dT).** We first assayed the processivity of DNAPol on a homopolymeric template lacking secondary structure. Incorporation on a poly(dA)-oligo(dT) primer-template was assayed at a range of enzyme concentrations. The product produced at an equimolar ratio of enzyme to template is shown to demonstrate the length of product obtainable when enough enzyme is available to extend the primer template multiple times. At very low ratios of enzyme to template, most primers are not extended at all; extended primers are the result of a single round of processive DNA synthesis.

As shown in Fig. 3, we compared the processivity of AcNPV DNAPol (lanes 15 to 22) with that of the Klenow fragment of *Escherichia coli* DNA polymerase (lanes 1 to 7) and bacteriophage T4 DNA polymerase (lanes 8 to 14) on a poly(dA)-oligo(dT) template. With Klenow enzyme, a distributive DNA polymerase, the average length of product increased as a function of the enzyme concentration. At the lowest enzyme-to-template ratio, most primers were extended by only 1 to 4 nt (lane 1). With increasing amounts of enzyme (lanes 2 to 4), the average length of the extended products increased proportionately, indicating that each primer was extended by repeated rounds of synthesis. At the higher concentrations of enzyme, all of the products were full length (lanes 5 to 7). With T4 DNA polymerase, a processive enzyme, very long products were de-

tected at all but the lowest concentration of enzyme (lanes 8 to 14). The amount of product increased as a function of input enzyme, but the average length of the products did not change significantly with increasing amounts of enzyme.

The distribution of products synthesized by AcNPV DNAPol was similar to that of T4 DNA polymerase (Fig. 3, lanes 15 to 21). At low ratios of enzyme to template (lanes 15 to 17), there is an excess of unreacted primers, which verifies that the extended primers were limited to one round of processive synthesis. The amount of product is low, since only a few primers were extended, and therefore full-length products are not evident in the figure, although they were visible on the original autoradiograph and in duplicate experiments (data not shown). In lane 18, products in the size range of 2,000 to 3,000 nt can be seen, while at an equivalent amount of Klenow enzyme (lane 3), the average length of product was only 16 nt. This result indicates that once initiated, each polymerase molecule incorporated nucleotides onto the primer-template until the end of the substrate was reached.

**Template challenge assay.** We then examined the processivity of AcNPV DNAPol by using a template challenge assay (Fig. 4). This is a more stringent test of processivity because it uses a longer substrate. However, the results are complicated by secondary structure elements, which often cause pausing and dissociation. In this experiment, DNAPol was allowed to assemble on a singly primed circular template, either M13 or  $\phi\text{X174}$ , in the presence of dATP, dGTP, and dTTP. The enzyme was denied dCTP, thus preventing elongation. A fivefold molar excess of a second singly primed circular template was then added along with dCTP. Aliquots were removed at subsequent time points to monitor DNA synthesis on the challenge template.

Under these conditions, a processive polymerase would completely replicate the first template before extending the challenge template. However, a distributive enzyme would preferentially replicate the challenge template, as it would dissociate from the first template after adding a few nucleotides. The enzyme would then have a greater likelihood of reassembling on the challenge template because it was present in excess.

As shown in Fig. 4 (lanes 3 to 8), singly primed M13 was first challenged by the addition of a fivefold molar excess of singly primed  $\phi\text{X174}$ . A band corresponding to the 3.2-kb M13 pause product was detected 3 min after the addition of dCTP and the challenge template. The amount of this product was increased after 5 min and then remained at constant levels throughout the time course. Full-length M13 RF was detected at 5 min, and M13 RFII continued to accumulate through 30 min. Synthesis of  $\phi\text{X174}$  RFII was not detected until 30 min after the addition of the challenge template, even though it is 1 kb smaller. This indicates that most of the DNAPol molecules that were initially bound to M13 completed one round of synthesis before shifting to the challenge template.

A similar result was obtained with DNAPol loaded onto the  $\phi\text{X174}$  template and challenged with M13. Full-length  $\phi\text{X174}$  was detected 3 min after the addition of dCTP, while M13 was not detected until 20 min after addition of the challenge template. The results of this assay indicate that DNAPol is a processive enzyme.

**Strand displacement activity of DNAPol.** In the replication assay performed on singly primed M13, product longer than 7,250 nt (the expected size of a linear M13 product) was detected. To produce a product longer than the template on singly primed circular DNA, DNA polymerase must be able to displace the primer and some of the newly synthesized DNA after completion of one round of replication. Therefore, this result suggests that AcNPV DNAPol is capable of strand displacement.

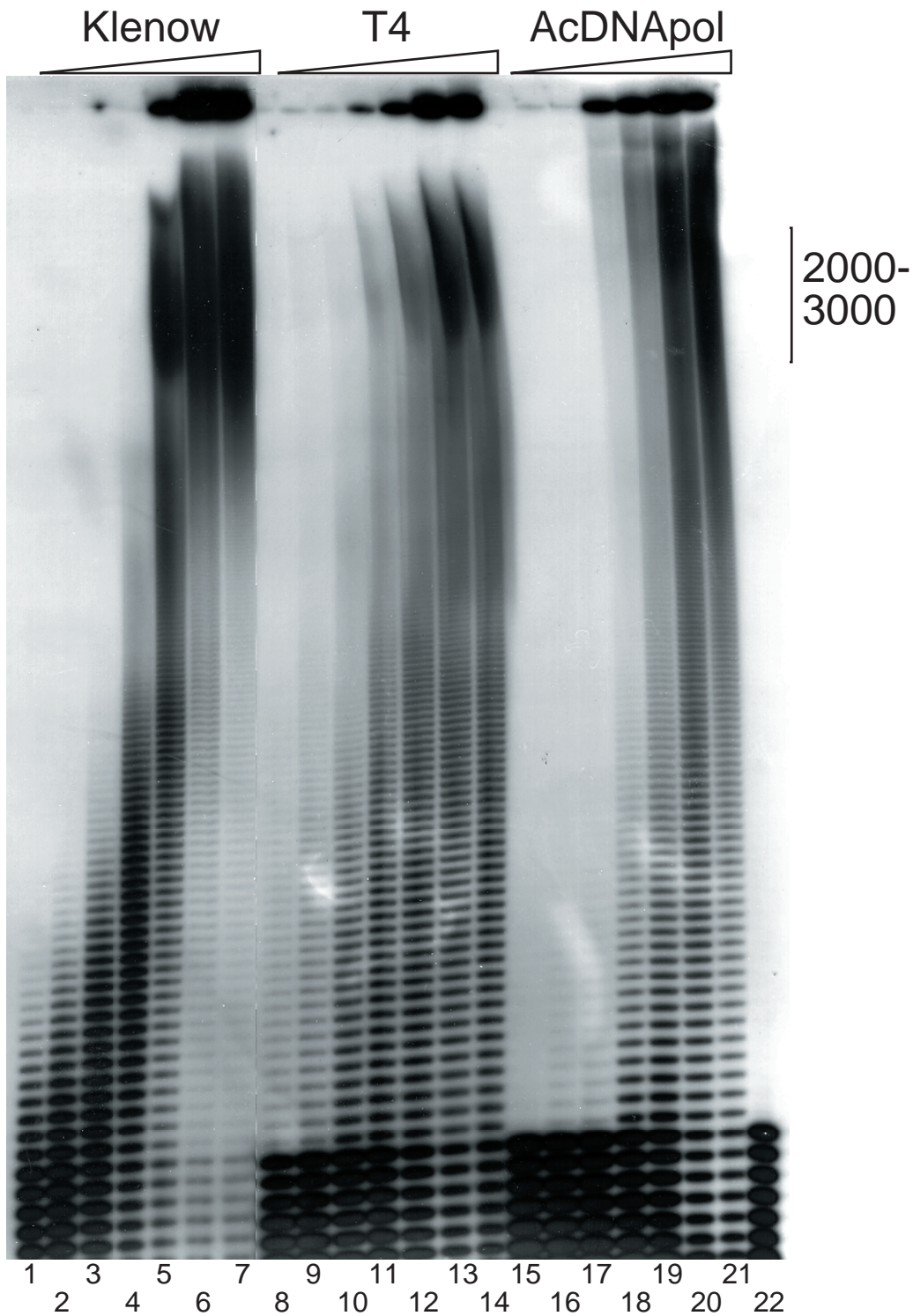


FIG. 3. Processivity of DNA polymerases on poly(dA)-oligo(dT). Reaction mixtures contained 336 fmol of poly(dA)-oligo(dT) template and 5.25, 10.5, 21, 42, 84, 168, 336 fmol of each polymerase. Klenow fragment was used in the reaction mixtures in lanes 1 to 7; those in lanes 8 to 14 contain T4 DNA polymerase; those in lanes 15 to 21 contain AcNPV DNApol. A control reaction with no enzyme is shown in lane 22. Reactions were electrophoresed on a 7 M urea-12% polyacrylamide gel. Sizes are indicated in nucleotides.

To test this hypothesis, replication products from singly primed M13 assays were treated with mung bean nuclease, which is a single-strand-specific nuclease (Fig. 5). If ssDNA were produced as a result of the polymerase displacing the primer and newly synthesized DNA, the larger product should be sensitive

to mung bean nuclease whereas full-length DNA should be double stranded and, therefore, resistant. As shown in Fig. 5, DNAs treated with mung bean nuclease did not exceed 7,250 nt in length, while the untreated products were longer. This result suggests that AcNPV DNApol is capable of strand displacement.

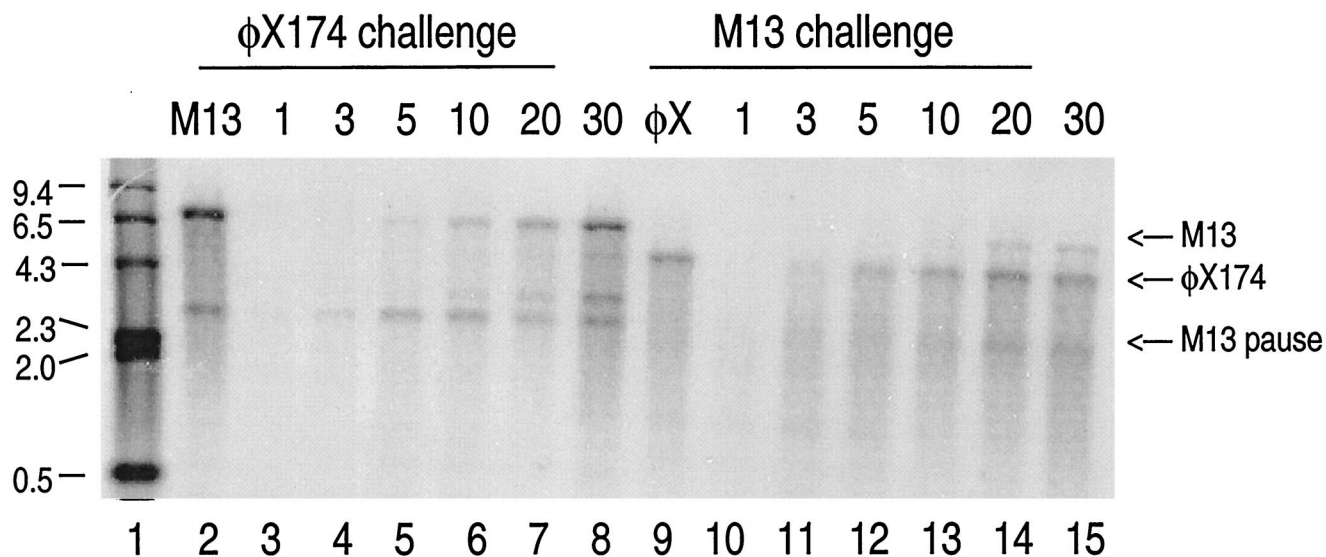


FIG. 4. Template challenge assay: demonstration of the processive nature of AcNPV DNApol on singly primed M13 and  $\phi$ X174 templates. Reaction mixtures of 50  $\mu$ l contained 20 fmol of purified recombinant AcNPV DNApol and 20 fmol of each template. Lane 2 contains singly primed M13 template alone without  $\phi$ X174 challenge template; lanes 3 to 7 contain reactions in which the polymerase was allowed to assemble on the M13 template before addition of the  $\phi$ X174 challenge template and dCTP. Aliquots were removed at the times (minutes) indicated at the top. Lane 8 contains  $\phi$ X174 template alone; lanes 9 to 13 contain reaction mixtures in which the polymerase was allowed to assemble on the  $\phi$ X174 template before addition of excess M13 challenge template and dCTP. Aliquots were removed at the times indicated at the top. DNA products were fractionated on a 0.8% alkaline agarose gel. Sizes are indicated in kilobases.

Replication activity on a gapped template provided another test of strand displacement activity (Fig. 6A). In this assay, a second (unlabeled) primer was annealed 270 nt downstream from the first (radiolabeled) primer. DNA polymerase should extend both primers at the same rate, but only replication products made by extension of the radiolabeled primer can be detected after denaturation of the DNAs.

T4 DNA polymerase, which lacks strand displacement activity, added only 270 nt to the radiolabeled primer (Fig. 6B, lane 2), since further elongation was blocked by the terminator primer. The Klenow fragment of *E. coli* DNA polymerase, which has strand-displacing activity, was able to remove the elongated terminator primer and replicated the entire M13 circle from the radiolabeled primer (Fig. 6B, lane 3). The migration of fully replicated M13 product is shown in lane 1 of Fig. 6B. This reaction contained AcNPV DNApol in the absence of a terminator primer, so there was no block to prevent DNApol from synthesizing the entire M13 circle. With AcNPV DNApol and a terminator primer, the radiolabeled primer was extended more than 270 nt, but little full-length product was detected (Fig. 6B, lane 4). Therefore, AcNPV DNApol appears to have moderate strand displacement ability, less than that of the Klenow fragment but more than that of T4 DNA polymerase.

An additional assay using a different gapped template with a radiolabeled terminator (Fig. 7A) was performed to verify strand displacement. In this experiment, the terminator primer is radiolabeled, and synthetic displacement of the terminator due to extension of the unlabeled primer was quantitated. As shown in Fig. 7B, AcDNApol was able to displace the radiolabeled terminator primer. Displacement was proportional to the concentration of input enzyme. Release of the terminator oligonucleotide was dependent on the addition of dCTP, indicating synthetic displacement.

**Strand displacement in the presence of LEF-3.** We tested whether LEF-3, the viral SSB (13), increased the strand displacement activity of DNApol. In the experiment shown in Fig. 7B, LEF-3 was added to the gapped template after the

DNApol. This was done to prevent displacement of the primer by LEF-3, which like most SSBs is capable of helix destabilization (data not shown). Addition of DNApol prior to LEF-3 allowed DNApol to bind to the 3' end of the primer and begin elongation, thereby stabilizing the primer-template junction. In reactions containing the gapped template with a radiolabeled primer, more full-length product was synthesized in the presence than in the absence of LEF-3 (Fig. 7B, lane 5). Therefore, it is unlikely that the increased ability of the DNApol to pass

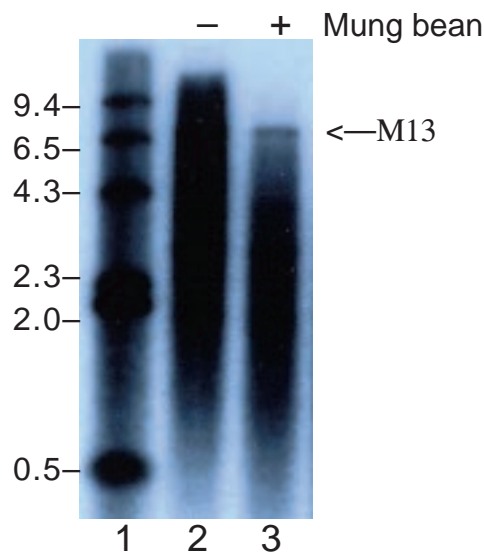


FIG. 5. Mung bean nuclease sensitivity of AcDNApol replication products. Standard singly primed M13 assays were extracted with phenol and precipitated with ethanol. DNA was resuspended in mung bean nuclease buffer and incubated in the presence (lane 3) or absence (lane 2) of mung bean nuclease. Reaction products were analyzed by 0.8% alkaline agarose gel electrophoresis. The migration of molecular markers is shown in lane 1, and the relevant sizes are indicated in kilobases on the left. The migration of full-length M13 DNA is shown on the right.

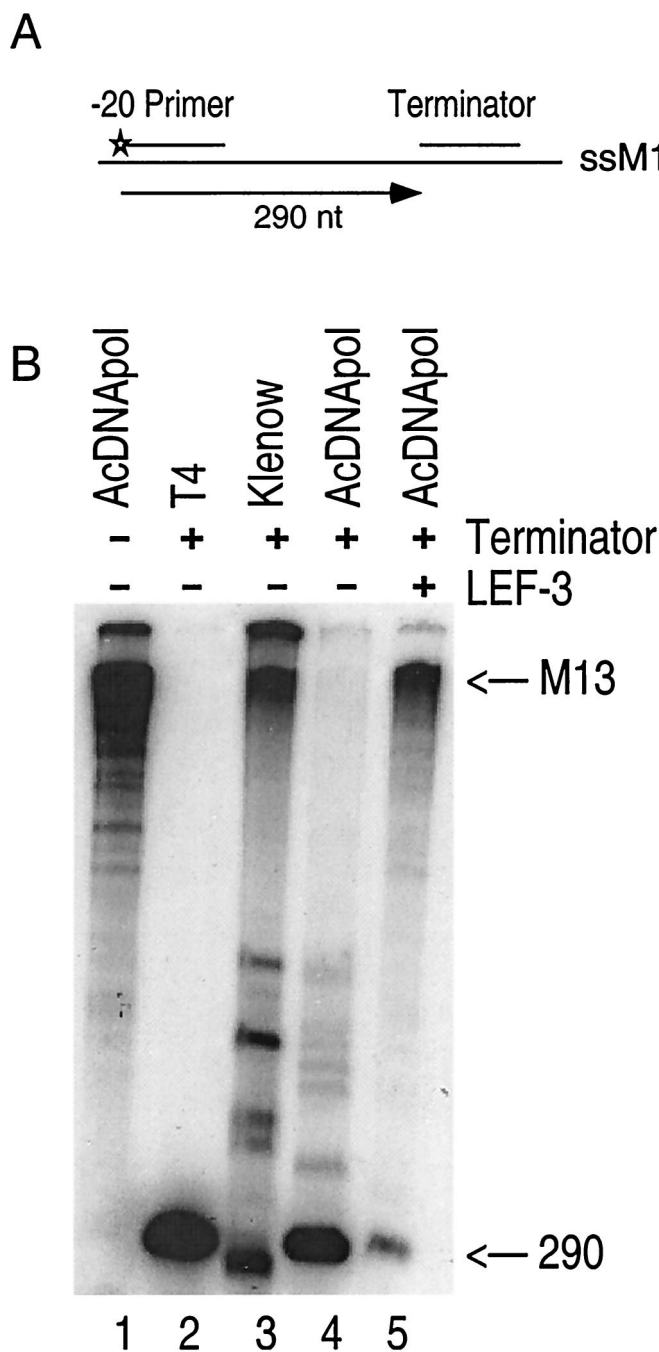


FIG. 6. Strand displacement assay on a gapped DNA template; (B) strand displacement activity of AcNPV DNApol with and without LEF-3 on a gapped DNA substrate. Reaction mixtures contained 20 fmol of DNA template and 50 fmol of the indicated DNA polymerase. Lane 1, AcNPV DNApol incubated with singly primed M13 template; lane 2, gapped M13 template with T4 DNA polymerase; lane 3, gapped M13 template incubated with Klenow fragment; lane 4, gapped M13 template incubated with AcNPV DNApol; lane 5, gapped M13 template incubated with AcNPV DNApol and 10 pmol of LEF-3. 290, 290-nt product.

the terminator in the presence of LEF-3 is due to the removal of the terminator by LEF-3 prior to elongation. Furthermore, the total amounts of product synthesized were equivalent in lanes 4 and 5, suggesting that the LEF-3 did not destabilize the radiolabeled primer, which has a much lower melting temperature than the terminator primer. Rather, the most likely explanation for this result is that LEF-3 stimulated strand displacement by binding to the 5' end of the terminator primer as the polymerase began to displace it.

**Stimulation of replication by LEF-3 is affected by order of addition.** The stimulatory effect of LEF-3 was also evident in the replication of a singly primed M13 template. In this experiment, we showed that the order of addition of LEF-3 and DNApol was important to the observed stimulation of DNA replication by LEF-3. Three reactions were set up simultaneously: one in which LEF-3 was incubated with primed template for 5 min on ice in the absence of DNApol, one in which DNApol was added first and allowed to incubate on ice 5 min

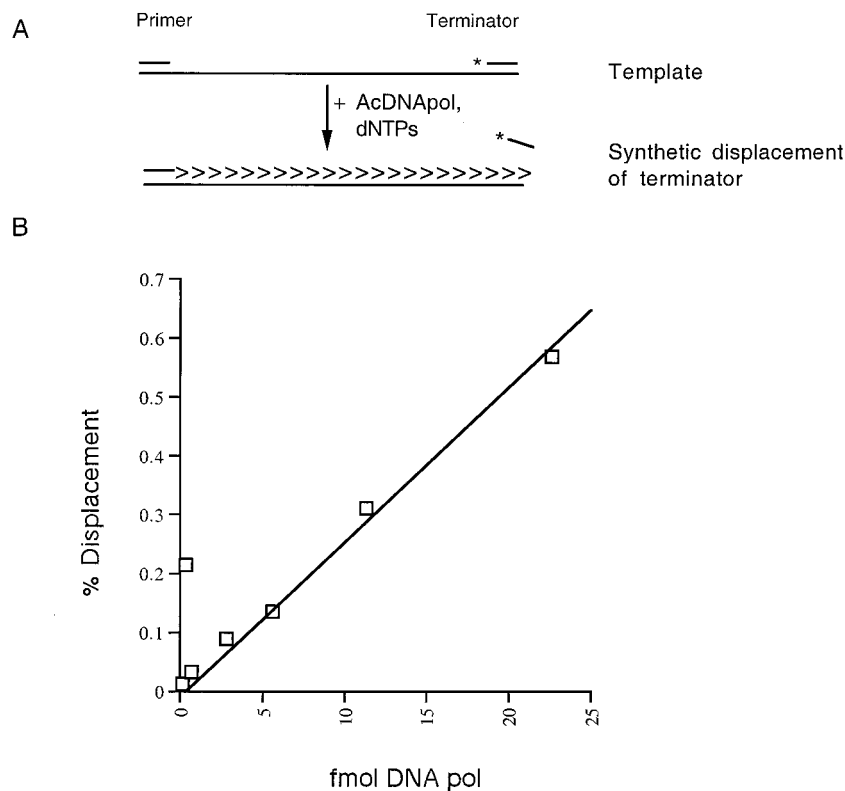


FIG. 7. Replication-dependent displacement of a radiolabeled primer. (A) Schematic of the gapped DNA substrate and assay. dNTPs, deoxynucleoside triphosphates. (B) Gapped template was incubated with indicated amounts of AcNPV DNApol. Samples were electrophoresed on a 12% polyacrylamide gel to separate the displaced oligonucleotide from the template. The amount of displacement primer was quantitated by PhosphorImager analysis. Control reactions were run in the absence of dCTP, and the values shown for synthetic displacement were adjusted for background radioactivity in the absence of dCTP.

before the addition of LEF-3, and a control reaction containing DNApol alone. Aliquots were removed from each tube at 1, 3, 5, 10, 20, and 30 min after the addition of radiolabel and analyzed by alkaline agarose gel electrophoresis to determine the extent of DNA synthesis under the different sets of conditions (Fig. 8).

When saturating amounts of LEF-3 were added before DNApol, no products were synthesized (Fig. 8, lanes 1, 4, 7, 10, 13, and 16), probably because the helix-destabilizing ability of LEF-3 removed the primer during the 5-min preincubation before the addition of DNApol. Addition of saturating amounts of LEF-3 to a singly primed M13 template with DNApol already bound to the primer-template junction initially decreased the rate of synthesis. After 3 min of incubation, the average size of products produced in the presence of SSB was approximately 2.5 kb. After 3 min of synthesis in the absence of SSB, most of the polymerase molecules were paused at the hairpin 3.0 kb from the primer, although longer reaction products, up to 4.0 kb in length, were evident (Fig. 8; compare lanes 5 and 6). After 5 min of incubation, some full-length molecules were detected in reactions lacking SSB, although most of the polymerases were still paused at 3.0 kb. In the presence of SSB, no full-length products were detected at this time; the average length of product was 4.0 kb, and there was also no evidence of pausing. The 3-kb product was eliminated by the addition of LEF-3, which indicates that SSB removed the secondary structure that would otherwise cause the polymerase to pause (Fig. 8; compare lanes 8 and 9). After 30 min of synthesis, longer than full-length products were detected in both reactions. However, these products were both longer and more abundant in

the reactions containing LEF-3 than those without LEF-3 (Fig. 8; compare lanes 17 and 18). The results of this experiment indicates that LEF-3 stimulates replication by improving the ability of the polymerase to strand displace.

**LEF-3 and *E. coli* SSB stimulate AcNPV DNApol activity on a nicked template.** The ability of DNApol to extend a 3' terminus at a nick in a double-stranded template was tested in the presence or absence of an SSB (Fig. 9). In the absence of an SSB, approximately half as much dCTP was incorporated in reactions containing AcNPV DNApol as in reactions containing the Klenow fragment of *E. coli* DNA polymerase. This is further evidence of the ability of AcNPV DNA polymerase to accomplish moderate strand displacement. The level of incorporation was greatly increased by the addition of LEF-3 in both the AcNPV DNApol and Klenow reactions (Fig. 9A). *E. coli* SSB also stimulated the strand displacement ability of DNApol, although it was less efficient than LEF-3 at equivalent molar amounts. As expected the addition of *E. coli* SSB to the reactions containing the Klenow fragment of *E. coli* DNA polymerase increased the extent of synthesis, and LEF-3 efficiently substituted for *E. coli* SSB in reactions containing the Klenow fragment.

## DISCUSSION

A recombinant virus, named AcDNApol, which overexpressed the DNApol gene under the control of the polyhedrin promoter, was constructed in order to efficiently express and purify DNApol. Comparison of nuclear extracts prepared from insect cells infected with AcDNApol or the parental virus re-



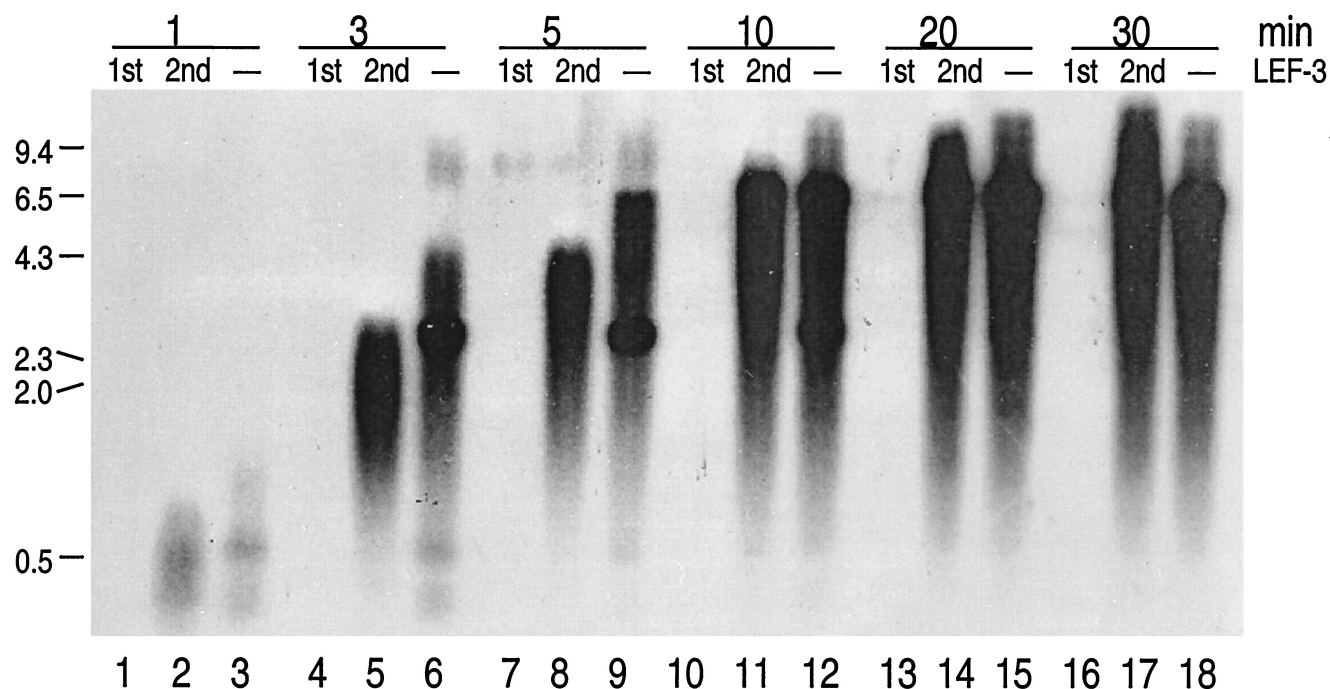


FIG. 8. LEF-3 stimulates replication in a manner that depends on order of addition. The products fractionated in lanes 1, 4, 7, 10, 13, and 16 were taken from a reaction mixture in which 45 pmol of LEF-3 was added first, followed by incubation for 5 min on ice, addition of 300 fmol of DNAPol, and incubation at 37°C; 300 fmol of DNAPol was added first to the reaction mixture distributed in lanes 2, 5, 8, 11, 14, and 17; after 5 min on ice, 45 pmol of LEF-3 was added; 300 fmol DNAPol only was added to the reaction mixture allocated to lanes 3, 6, 9, 12, 15, and 18. Aliquots of 25  $\mu$ l from each of the three reaction mixtures were removed 1, 3, 5, 10, 20, and 30 min after incubation at 37°C. Sizes are indicated in kilobases.

vealed that a single protein with an apparent molecular weight of 110,000 was strongly overexpressed in the cells infected with the recombinant virus. Production of a protein in this size range agrees well with the predicted molecular weight of 114,000 for the AcNPV DNAPol gene product (24).

DNAPol was purified to homogeneity starting from nuclear extracts prepared from Sf9 cells infected with AcNPV. SDS-PAGE analysis of 10  $\mu$ g of protein from the final column revealed a single band of protein with no contaminating peptides. Homogeneous preparations of DNAPol had the ability to extend oligonucleotide primers annealed to native templates. Singly primed M13 was converted to RFII even at equimolar amounts of enzyme to template. A predominant pause site was detected at equimolar ratios of enzyme to template, and addition of excess enzyme increased the amount of full-length product. Although the pattern of synthesis was affected by the addition of excess enzyme, the total amount of synthesis did not increase. This result indicates that all of the template molecules were actively engaged in DNA synthesis at equimolar ratios of enzyme to template. This observation argues strongly that the polymerase activity was not influenced by minor contaminants that were not detectable by SDS-PAGE.

The processivity of DNAPol was examined to characterize the viral enzyme and as an aid in identification of other factors essential for replication. DNAPol was shown to be a processive enzyme by template challenge assays and by its ability to synthesize very long products on a poly(dA)-oligo(dT) template with limiting amounts of enzyme.

Despite the level of processivity exhibited by DNAPol, it seems unlikely that baculoviruses do not encode one or more processivity factors. Most of the large complex DNA viruses encode DNA polymerase and associated accessory factors needed for highly processive synthesis (16). The exceptions to this are the linear DNA viruses that initiate DNA replication by

protein priming, such as adenovirus and phage  $\phi$ 29. Viral DNA replication is initiated at each 3' end and proceeds symmetrically by leading strand displacement synthesis only. Thus, these DNA polymerases need function only as highly processive enzymes. Baculoviruses which have circular genomes probably replicate either by a bidirectional or rolling-circle mode and thus most likely have a mechanism to coordinate leading- and lagging-strand replication. We have also purified DNA polymerase expressed from its own promoter and extracted from infected cells at 18 h postinfection, a time during which DNA replication is ongoing (12). The processivity of this enzyme is nearly identical to that of the overexpressed enzyme reported here, indicating that our results reflect the true nature of AcNPV DNAPol.

Several researchers have predicted that the AcNPV protein PCNA is a processivity factor because it has 42% amino acid sequence identity with mammalian PCNAs (20). This hypothesis is supported by *in vivo* experiments showing that disruption of the PCNA gene results in a delayed DNA replication and late gene expression phenotype (3, 20). PCNA, however, is not essential for DNA replication by transient expression assays, nor does addition of PCNA stimulate the efficiency of transient plasmid replication (15). These apparently contradictory results may be explained by our data showing that DNAPol has a high intrinsic processivity. Thus, accessory factors may not be required for replication of small plasmids, although they may be needed for replication of the large viral genome.

We have shown that AcNPV DNAPol has modest strand displacement ability. Full-length M13 template was synthesized on a gapped template. This required the polymerase to displace a 21-nt primer and the nucleotides added to it by DNAPol. This should be approximately 290 nt if both primers are extended at the same rate, since DNAPol extending from

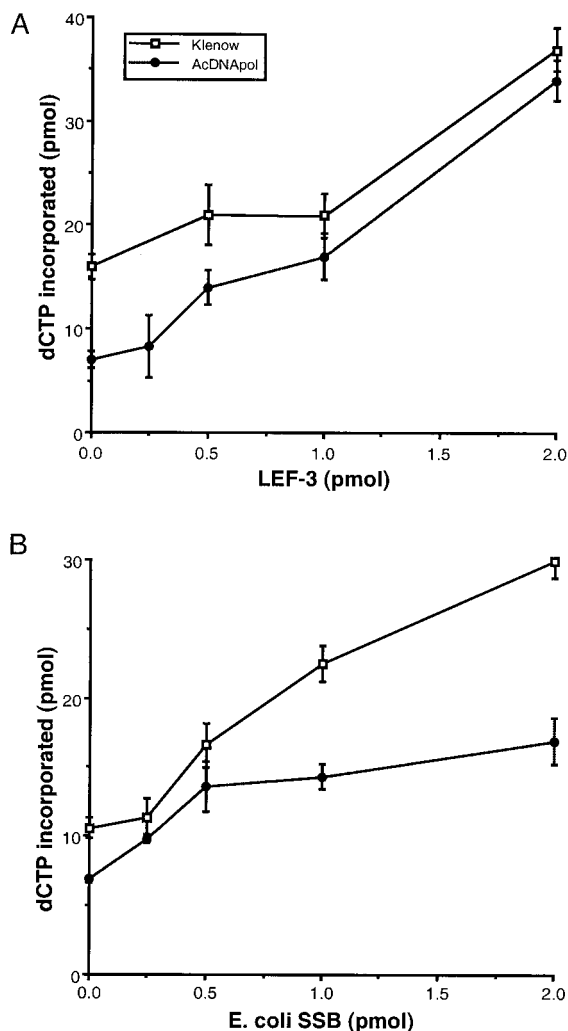


FIG. 9. Strand displacement on nicked template. (A) Strand displacement in the presence of LEF-3. DNAPol (3.8 pmol) or Klenow fragment (0.5 U) was incubated with 1 pmol of nicked DNA template, and then the indicated amount of LEF-3 was added. (B) DNAPol (3.8 pmol) or Klenow fragment (0.5 U) was added to each reaction, and then the indicated amount of *E. coli* SSB was added. Each point represents the average of three separate experiments.

the first radiolabeled primer must incorporate 270 nt before the polymerase reaches the second primer. DNAPol also incorporated nucleotides onto a nicked template, a further indication of strand displacement activity. Finally, DNAPol was able to displace a 30-nt primer in a replication-dependent manner, providing direct proof of strand displacement.

LEF-3 (the baculovirus SSB) stimulated viral replication on three different templates: singly primed M13, gapped M13, and nicked double-stranded DNA templates. On singly primed M13 templates, LEF-3 appeared to remove a secondary structural element that caused the DNAPol to pause and occasionally dissociate in the absence of additional factors. It also aided in strand displacement as seen by the longer than full-length products synthesized in reactions containing saturating amounts of LEF-3 compared to reactions without LEF-3. Addition of LEF-3 to DNAPol reactions on a gapped M13 template significantly increased the ability of DNAPol to displace a terminator primer. Finally, on a nicked template, addition of increasing amounts of LEF-3 produced a corresponding linear increase in the incorporation of nucleotides into DNA.

The effect of *E. coli* SSB on AcNPV DNAPol activity on a nicked template was examined to determine if DNAPol could utilize a heterologous SSB. *E. coli* SSB was able to substitute for LEF-3 even though it does not share any sequence homology to LEF-3 and is very different in structure and size. This finding suggests that a specific interaction between LEF-3 and DNAPol is not required in this assay. LEF-3 also stimulated the activity of the Klenow fragment *E. coli* DNA polymerase I on a nicked template, although Klenow enzyme has a higher intrinsic strand displacement ability than AcNPV DNAPol.

The order of addition of LEF-3 and DNAPol in replication assays on a singly primed M13 template was shown to be important. When LEF-3 was added prior to DNAPol, very little product was synthesized, presumably because of the helix-destabilizing activity of LEF-3, resulting in dissociation of the primer from the template. When DNAPol was added to the reaction and allowed to incubate on ice 5 min before the addition of LEF-3, equivalent amounts of product were synthesized as in the absence of LEF-3. This result suggests that binding of DNAPol to the primer-template junction stabilized the primer and prevented helix destabilization due to binding of LEF-3. Although equivalent amounts of DNA were synthesized in the presence and absence of LEF-3, the patterns of products differed remarkably. First, in the presence of LEF-3, the strong pause at 3 kb was eliminated, suggesting that LEF-3 removed the secondary structure that caused the pausing of DNAPol. Second, longer than full-length products were made in the presence of LEF-3, presumably due to displacement of the primer after completion of one round of synthesis and continued synthesis of DNA displacing the newly replicated strand.

Our results showed that *E. coli* SSB could substitute for LEF-3 in the nicked template assay. However, transient replication assays indicate that LEF-3 is specifically required for replication of an origin-containing plasmid. Furthermore, the fact that baculoviruses encode an SSB suggests that the host enzyme or another heterologous enzyme cannot substitute *in vivo*. Alternatively, LEF-3 may be required for a function that is not directly tied to DNA replication. A recent report by Wu and Carstens (26) shows that LEF-3 is required for nuclear targeting of P143; that task alone may provide sufficient reason for the virus to encode LEF-3.

In this report we have shown that AcNPV encodes a moderately processive DNA polymerase with strand displacement ability. LEF-3, the viral SSB, stimulates viral replication by improving the strand displacement ability of DNAPol and by eliminating secondary structure from the template. This information may help to reveal the mechanism of AcNPV DNA replication and the functions of other gene products shown to be essential for replication.

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