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Purification and Properties of the Deoxyribonucleic Acid Polymerase Induced by Vaccinia Virus*

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The vaccinia virus-induced DNA polymerase has been purified about 500-fold from a cytoplasmic extract of vaccinia-infected HeLa cells. Analysis of the purified fraction by sodium dodecyl sulfate-polyacrylamide gel electrophoresis reveals a single polypeptide of 110,000 daltons, which is greater than 95% pure. This polypeptide co-sediments with polymerase activity through a glycerol gradient. The sedimentation coefficient of the enzyme is 6.3 S, and its Stokes radius is 4.6 nm. The molecular weight of the native enzyme derived from these values is 115,000. Vaccinia polymerase is therefore a single large polypeptide of 110,000 to 115,000 daltons.

The purified fraction has no significant endonuclease activity, but a strong exonuclease activity co-purifies with polymerase activity through every step in the isolation. The polymerase and exonuclease activities are inactivated at 45°C at the same rate. It is likely, therefore, that both activities are catalyzed by the same polypeptide. The exonuclease hydrolyzes DNA predominantly in the 3′ → 5′ direction, to produce 5′ mononucleotides. The exonuclease degrades single-stranded DNA more rapidly than duplex DNA, and the rate of digestion of both single-stranded and double-stranded DNA increases as the size of the substrate decreases. Single-stranded circular DNA is a potent inhibitor of the exonuclease activity, but duplex circular DNA has no significant effect on its activity.

Vaccinia virus, a member of the Poxvirus family, contains a linear, double-stranded DNA genome of about 130 million daltons (1). When vaccinia infects a susceptible cell, its DNA is replicated and progeny virions are assembled within the cytoplasmic compartment of its host. Infection is accompanied by an increase in several enzymatic activities which may be involved in DNA replication. These include DNA ligase (2), thymidine kinase (3), several deoxyribonucleases (4, 5), and DNA polymerase (6). It is likely, though not proven, that these induced activities and other essential replication functions are specified by the viral genome, since DNA replication occurs in the cytoplasm, where host replicative enzymes may not normally be found.

We are currently investigating the enzymatic mechanisms responsible for vaccinia virus DNA replication. As a first step toward reconstructing viral replication *in vitro*, we have purified the vaccinia-induced DNA polymerase. Some information about this polymerase was already known. It was first detected in infected cell extracts by Jungwirth and Joklik (6). Magee and Miller subsequently showed that the induced polymerase is immunologically distinct from the major host DNA polymerase (7), and Berns *et al.* (8) and Citarella *et al.* (9) separated it from cellular polymerases by chromatography on DEAE-cellulose.

In this communication we report the purification to near homogeneity and the characterization of the vaccinia-induced DNA polymerase from infected HeLa cells. As previously suggested, the viral polymerase is associated with an exonuclease (9), and we shall also describe some of the properties of this activity.

A preliminary report has been published previously (10).

EXPERIMENTAL PROCEDURES

Materials

Cells and Virus

HeLa cells (strain S3, a gift of Dr. Bernard Moss of the National Institutes of Health) were maintained in suspension culture at a density of 1 to 10 × 10^5 cells/ml. The growth medium was Eagle's minimal essential medium for suspension culture (11) (Microbiological Associates) supplemented with penicillin (100 units/ml), streptomycin sulfate (0.1 mg/ml), and 5% horse serum (previously heated to 56°C for 30 min).

Vaccinia virus (strain WR, also a gift of Dr. Bernard Moss) was propagated as follows: HeLa cells, grown to a density of 5 to 8 × 10^5 cells/ml, were collected by centrifugation (about 2000 × *g* for 5 min at room temperature) in sterile glass bottles. They were resuspended at a density of 2 × 10^7 cells/ml in fresh medium, and virus stock (preincubated in an equal volume of 0.25% trypsin at 37°C for 30 min to disrupt clumps of virions) was added at a multiplicity of 1 plaque-forming unit/cell. After gentle stirring at 37°C for 30 min, the infected cells were diluted with fresh medium to a density of 5 × 10^5 cells/ml and were incubated at 37°C for 48 to 72 h. The cells were then centrifuged as described above. The cell pellet was resuspended at a density of 3 × 10^7 cells/ml (based on the number of cells present at the time of infection) in Eagle's minimal essential medium (for monolayer cultures)[1] containing no serum. The cells were lysed by three cycles of freezing and thawing. This crude virus stock was stored at −20°C until use. The titre, determined on BSC-1 cells (12), was normally about 6 to 8 × 10^9 plaque-forming units/ml.

Infected cells for DNA polymerase isolation were prepared by a method similar to that of Berns *et al.* (8). Cells (5 to 8 × 10^5 cells/ml) were centrifuged (5 min at 2000 × *g* at room temperature), and the

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[1] Virus stock prepared by resuspending the infected cells in Eagle's minimal essential medium for suspension cultures had exactly the same titre, but produced very asynchronous infections. This is probably due to the difference in concentration of Mg^{2+} and Ca^{2+} in the two different media, but we have not investigated the point systematically.

supernatant medium was saved for subsequent redilution of the cells. The cell pellet was resuspended in fresh medium at a density of 2×10^7 cells/ml. Trypsinized virus stock was added at a multiplicity of 5 to 10 plaque-forming units/cell. The cells were incubated at 37°C for 30 min and then diluted back to their original density with the old medium. Following incubation for 5½ h at 37°C, cells were harvested by centrifugation for 10 min at 3000 rpm in a Sorvall GSA rotor. They were washed once in 0.32 M sucrose, 1 mM potassium phosphate, pH 7.6, 2 mM $MgCl_2$. The pellet was frozen at −20°C and used within 1 month.

Enzymes and Proteins

E. coli RNA polymerase was a generous gift of Dr. Lawrence Loeb of the University of Washington. Rabbit muscle lactate dehydrogenase was from Sigma Chemical Co. and was assayed by the method described in the Worthington catalog. Crystalline bovine serum albumin was from Nutritional Biochemicals. Bacterial alkaline phosphatase was from Worthington Biochemicals and was repurified on DEAE-cellulose as described by Weiss *et al.* (13). Polynucleotide kinase was from P-L Biochemicals. T4 DNA polymerase was prepared by the method of Goulian *et al.* (14). Snake venom 5′ nucleotidase was from Sigma Chemical Co.

Nucleotides and Nucleic Acids

Unlabeled deoxynucleoside triphosphates were from Schwarz/Mann. [α-^{32}P]deoxythymidine triphosphate was either synthesized (15) or purchased from New England Nuclear. [γ-^{32}P]ATP was synthesized by the method of Maxam and Gilbert (16). The template-primer for DNA polymerase assays was salmon DNA (Type III from Sigma Chemical Co.) which was activated by incubating at 4°C for 48 h in the presence of 5 mM $MgCl_2$ (17). T7 [^{32}P]DNA and T7 [^{3}H]DNA were prepared as described previously (15). ϕX174 DNA, ϕX174 [^{3}H]DNA, and isolated restriction fragments of ϕX174 RF [^{3}H]DNA were prepared as described in the following paper (18). SV40 DNA was a gift of Dr. David Shortle, The Johns Hopkins University. *E. coli* [^{3}H]DNA was a gift of Dr. Lawrence Grossman, The Johns Hopkins University. 3′ terminally labeled [^{32}P]DNA was prepared by incubating the DNA with T4 DNA polymerase in the presence of a single deoxynucleoside[α-^{32}P]triphosphate (15). 5′ terminally labeled [^{32}P]DNA was prepared by first dephosphorylating the DNA with bacterial alkaline phosphatase (13), and then rephosphorylating using T4 polynucleotide kinase and [γ-^{32}P]ATP (13, 16).

Other Materials

DEAE-cellulose was Whatman DE52. Phosphocellulose was Whatman P11. DNA agarose (from Dr. Michael Mann, The Johns Hopkins University) was single-stranded calf thymus DNA covalently linked to agarose beads (19). Pyran Sepharose (20) was the gift of Dr. Jack Chirikjian of Georgetown University. Hydroxylapatite was from Bio-Rad. Benzamidine and diisopropylfluorophosphate were from Sigma Chemical Co. All other chemicals were the highest grade commercially available.

Methods

Assays

The standard assay solution for vaccinia polymerase (0.2 ml) contained 400 μg/ml of albumin,[2] 50 mM potassium phosphate, pH 7.6, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 15 μg of activated salmon DNA, 100 μM each dATP, dCTP, and dGTP, 100 μM [α-^{32}P]dTTP (2 to 8×10^4 cpm/nmol), and 0.05 to 0.5 unit of polymerase. After 30 min at 37°C, the reaction was terminated by the addition of 0.2 ml of 0.2 M sodium pyrophosphate followed by 1.0 ml of ice-cold 1.0 M perchloric acid. Acid-precipitable material was collected on a Whatman GF/C filter. The filter was washed with about 50 ml of 1.0 M HCl, 0.1 M sodium pyrophosphate, rinsed with 10 ml of ethanol, and, after drying, counted in a toluene-based scintillation fluid. A unit of polymerase activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of total nucleotide into an acid-precipitable form in 30 min at 37°C. In some assays the polymerase was diluted into a solution containing 0.1 M potassium phosphate, pH 7.5, 10% glycerol, 100 μg/ml of albumin and 0.1 mM dithiothreitol.

The standard solution for nuclease assays (50 μl) contained 400 μg/ml of albumin, 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 μg of sonicated, heat-denatured T7 [^{32}P]DNA (1 to 5×10^3 cpm/nmol of nucleotide), and 0.02 to 0.2 unit of nuclease. After 30 min at 37°C, the reaction was stopped by the addition of 5 μl of 0.5 M EDTA and chilling to 0°C. Acid-soluble nucleotide in the reaction mixture was measured in one of two ways:

Method 1—A 20-μl aliquot of the reaction was spotted onto a polyethyleneimine-cellulose thin layer plate (Brinkmann). The chromatogram was developed with 2.0 M HCl, following which the origin and the solvent front of the chromatogram were cut out, placed in 20-ml glass scintillation vials, covered with 1.0 ml of 0.1 M HCl, and autoclaved for 20 min. After cooling, 9.0 ml of Hydromix scintillation fluid (Yorktown) was added, and the samples were counted in a scintillation counter. Autoclaving in acid releases all nucleotides and polynucleotides from the polyethyleneimine-cellulose and gives uniform counting efficiency for both the acid-soluble and acid-insoluble components of the reaction.

Method 2—After the reaction was stopped with EDTA, 5 μl of a solution of sonicated calf thymus DNA (3 mg/ml) was added, followed by 100 μl of 1.0 M perchloric acid. After 10 minutes at 0°C, the sample was centrifuged for 2.5 min in a Beckman microfuge. Supernatant fluid (100 μl) was carefully removed and counted with Hydromix in a scintillation counter.

A unit of exonuclease activity is defined as the amount catalyzing the conversion of 1 nmol of nucleotide to an acid-soluble form in 30 min at 37°C.

Polyacrylamide Gel Electrophoresis

Electrophoretic analysis of proteins was carried out using the SDS/Tris/glycine-polyacrylamide gel system of Laemmli (21). The stacking gel was polymerized from a solution containing 5% acrylamide and 0.13% bisacrylamide, and the running gel from a solution containing 7.5% acrylamide and 0.2% bisacrylamide. Cylindrical gels had dimensions of 0.3 $cm^2 \times 12$ cm, and slab gels measured 30 cm × 15 cm × 1.5 mm. The protein sample (5 to 50 μg) was precipitated by the addition of cold trichloroacetic acid (final concentration, 10%) and was collected by centrifugation for 5 min in a Beckman microfuge. The pellet was washed with ether, dried in a stream of N_2, redissolved in 20 μl of 5% SDS, 10 mM Tris-HCl, pH 7.0, 1 mM EDTA, 2.5% 2-mercaptoethanol, and heated at 100°C for 1 min. Immediately prior to electrophoresis, 6 μl of 30% glycerol, 0.3% bromphenol blue was added, and the sample was layered onto the top of the stacking gel. Electrophoresis was at 2.5 to 3.5 V/cm for 12 to 13 h at room temperature. Gels were stained with Coomassie blue (22). For photography the stained gel was sandwiched between two clean glass plates and photographed with Polaroid type 55 film with illumination from below.

Measurement of Hydrodynamic Properties

Sedimentation Coefficient—The sedimentation coefficient of vaccinia polymerase was determined by centrifugation through a sucrose density gradient (23). The sample (0.15 ml), containing vaccinia DNA polymerase (50 units), albumin (500 μg), and lactate dehydrogenase (10 units), was layered onto a 5.0-ml linear sucrose gradient (5 to 20%) containing 0.1 M potassium phosphate, pH 7.5, and 4 mM 2-mercaptoethanol. Centrifugation was at 50,000 rpm for 16 h at 4°C in a Spinco SW 50.1 rotor. Fractions (0.17 ml) were collected from the bottom of the tube. DNA polymerase and lactate dehydrogenase were monitored by enzymatic assay, and albumin was monitored by its absorbance at 280 nm. Recovery of polymerase activity was 30 to 40%.

Stokes Radius—The Stokes radius was measured by gel filtration (24). A column of Sephadex G-150 (0.5 $cm^2 \times 100$ cm) was equilibrated with 0.1 M potassium phosphate, pH 7.5, 5% glycerol, 4 mM 2-mercaptoethanol. A sample containing DNA polymerase (250 units), lactate dehydrogenase (30 units), albumin (1.5 mg), and [^{32}P]orthophosphate (4×10^4 cpm) was dialyzed against the column buffer and applied to the column in a volume of 0.4 ml. Fractions of about 0.3 ml were collected. Proteins were assayed as described in the previous paragraph, and [^{32}P]orthophosphate was assayed by counting Cerenkov radiation in a scintillation counter. The excluded volume of the column was measured in a separate experiment with Blue Dextran.

Other Methods

Protein concentration was measured by the procedure of Lowry *et al.* (25) with albumin as standard. Digestion with 5′ nucleotidase was according to Deutscher and Kornberg (26).

[2] The abbreviations used are: albumin, bovine serum albumin; SDS, sodium dodecyl sulfate.

RESULTS

Purification

The results of the purification are summarized in Table I. All operations were carried out at 0–4°C unless otherwise noted, and each step was begun immediately after the preceding step was concluded.

Step I: Preparation of Cytoplasmic Extract—Vaccinia-infected HeLa cells (27 g frozen in 3-g aliquots) were thawed, and each 3-g aliquot was resuspended in 30 ml of 10 mM NaCl, 2 mM Tris-HCl, pH 7.6, 0.1 mM benzamidine. After 10 min at 0°C, the cells were disrupted with 10 strokes of a Dounce homogenizer fitted with a tight pestle. After the lysates were pooled (total volume, 270 ml), nuclei, mitochondria, and unbroken cells were removed by centrifugation for 20 min at 15,000 × *g*. The supernatant fluid (235 ml) was diluted with 35 ml of glycerol and treated with 2.3 ml of 0.5 M diisopropylfluorophosphate in isopropyl alcohol. This solution (Fraction I) was incubated for 1 h at 0°C.

Step II: DEAE-cellulose Chromatography—Fraction I was applied to a column of DE52 (16 cm^2 × 10 cm) equilibrated with a buffer composed of 20 mM potassium phosphate, pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, and 0.1 mM benzamidine. The column was washed with 400 ml of buffer, and polymerase was eluted by a linear gradient (1.4 liters) of 0.02 to 0.3 M potassium phosphate, pH 7.5, containing 10% glycerol, 0.1 mM benzamidine, and 5 mM 2-mercaptoethanol. Fractions eluting between 0.06 and 0.12 M potassium phosphate were pooled. The pooled fractions (Fraction II) contained about 50% of the applied DNA polymerase activity. Fractions eluting from 0.12 to 0.2 M potassium phosphate contained another 25% of the applied polymerase activity, but were discarded because at least half of this activity is host DNA polymerase α, as shown by Citarella *et al.* (9).

Step III: DNA-Agarose Chromatography—A column of DNA agarose (3 cm^2 × 5.5 cm) was washed with a buffer containing 75 mM potassium phosphate, pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM benzamidine. Fraction II (310 ml) was diluted with 30 ml of 10 mM potassium phosphate, pH 7.5, 5 mM 2-mercaptoethanol, 30% glycerol. This amount of diluent was chosen so that the diluted solution had the same conductivity as the column buffer. EDTA (0.5 M) was then added to a final concentration of 1 mM, and the solution was applied to the DNA-agarose column. The column was washed with 60 ml of buffer, and enzyme was eluted with a linear gradient (200 ml) of 0.075 to 0.5 M potassium phosphate, pH 7.5, containing 10% glycerol, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM benzamidine. Fractions eluting from 0.2 to 0.3 M potassium phosphate (50 ml), containing about 25% of the applied activity, were pooled. This material is Fraction III.

Step IV: Phosphocellulose Chromatography—A P-11 column (3 cm^2 × 4 cm) was washed with a buffer containing 0.075 M potassium phosphate, pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 0.1 mM benzamidine. Fraction III (50 ml) was diluted with 85 ml of 10 mM potassium phosphate, pH 7.5, 15% glycerol, 5 mM 2-mercaptoethanol, 0.1 mM benzamidine. The sample was then applied to the column, and the column was washed with 100 ml of buffer. Enzyme was eluted with a linear gradient (150 ml) of 0.075 to 0.5 M potassium phosphate, pH 7.5, containing 10% glycerol, 5 mM 2-mercaptoethanol, and 0.1 mM benzamidine. Polymerase activity eluted at about 0.2 M potassium phosphate, and the pooled fractions (Fraction IV, 20 ml) contained about 60% of the applied polymerase activity.

Step V: Hydroxylapatite Chromatography—Fraction IV was dialyzed overnight against 25 mM potassium phosphate, pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, 0.1 mM benzamidine. The sample was then loaded onto a hydroxylapatite column (0.5 cm^2 × 3 cm) that had been equilibrated with the same buffer, except that it contained 5%, instead of 10%, glycerol. The column was washed with 10 ml of buffer, and the enzyme was eluted with a linear gradient (30 ml) of 0.025 to 0.4 M potassium phosphate, pH 7.5, containing 5% glycerol, 5 mM 2-mercaptoethanol, 0.1 mM benzamidine. The enzyme eluted at about 0.1 M potassium phosphate, and the peak fractions (Fraction V, 5 ml) contained about 70% of the applied activity.

Step VI: Glycerol Gradient Sedimentation—Equal portions of Fraction V were layered onto three linear glycerol gradients (10 to 30%, 12 ml), containing 0.1 M potassium phosphate, pH 7.5, and 0.2 mM dithiothreitol. The gradients were centrifuged in a Beckman SW 41 rotor at 40,000 rpm for 24 h. Polymerase activity sedimented about one-half of the way down the tube. Fractions containing polymerase activity (1.8 ml from each gradient) were pooled and dialyzed against 50% glycerol, 0.1 M potassium phosphate, pH 7.5, 0.2 mM dithiothreitol. The final volume was 2.5 ml, and the polymerase concentration was 700 units/ml. The solution was stored at −20°C. This preparation (Fraction VI) was used in all subsequent experiments unless otherwise indicated.

TABLE I

Purification of vaccinia virus DNA polymerase

The purification is from 27 g of vaccinia-infected HeLa cells, prepared as described under "Methods." The final yield is about 100 μg of enzyme.

Fraction	Activity	Protein	Specific activity
	units × 10^{-3}	*mg*	*units/mg*
I. Extract[a]	55	1,495	36
II. DEAE-cellulose[a]	28	268	104
III. DNA-agarose	6.6	10.9	610
IV. Phosphocellulose	3.9	1.3	2,800
V. Hydroxylapatite	2.8	0.52	5,400
VI. Glycerol gradient	1.7	0.089[b]	19,000

[a] Activity in Fractions I and II includes both vaccinia and host polymerases.

[b] To conserve purified enzyme, the protein concentration in this fraction was determined by a scan of a stained SDS-polyacrylamide gel (Fig. 2) with bovine serum albumin as standard.

Stability of Enzyme

Polymerase activity is stable in the cytoplasmic extract for at least 24 h at 0°C. After subsequent steps in purification, the activity of the enzyme is stable at 0°C for at least 12 h in the presence of potassium phosphate, pH 7.5, in concentrations of 0.1 M or greater. Fraction IV and Fraction VI are stable for at least 9 months when stored at −20°C in 50% glycerol, 0.1 mM dithiothreitol, 0.1 M potassium phosphate, pH 7.5.

Initial attempts at purification were done in the absence of any protease inhibitors. Under these conditions, Fraction IV, which is 15 to 20% pure, was subject to proteolysis even when stored at −20°C. Proteolysis was detected by the appearance, after several weeks of storage, of multiple species of polymerase activity which were separable by Pyran Sepharose chromatography (20). Moreover, when a sample of stored Fraction IV was electrophoresed on a SDS-polyacrylamide gel, the major polypeptide band, at 110,000 daltons, was visibly smeared, and in some gels the smear could be resolved into multiple bands. To avoid proteolysis, the extract was treated with diisopropylfluorophosphate, and benzamidine (27) was included in all column buffers. Using this protocol, we have never detected proteolysis of either the partially pure or most pure fractions of polymerase.

Enzyme Purity

The specific activity of Fraction VI was about 500 times that of the cytoplasmic extract (Table I). The enzyme appears to be nearly homogeneous, since when a sample of Fraction VI was electrophoresed on a polyacrylamide gel in the presence of SDS, at least 95% of the protein migrated as a single band (Fig. 1). To learn whether this major component is actually the polymerase, samples from each fraction of a glycerol gradient were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). The major polypeptide co-sedimented with DNA polymerase activity, indicating that the activity probably resides in this polypeptide.

Molecular Weight

The sedimentation coefficient and the Stokes radius of native vaccinia DNA polymerase were determined as described under "Methods." The $s_{20,w}$ is 6.3 S and the Stokes radius is 4.6 nm. The molecular weight calculated from these values is 115,000. The polypeptide molecular weight was determined by electrophoresis on a SDS-polyacrylamide gel (Fig. 2). The single major polypeptide of Fracton VI has a molecular weight of 110,000, in close agreement with the molecular weight of the native enzyme. We therefore conclude that vaccinia polymerase is a single polypeptide with a molecular weight of 110,000 to 115,000.

No evidence for aggregation of the enzyme was obtained by sucrose gradient sedimentation when the potassium phosphate concentration in the gradient was varied from 0.05 to 0.5 M. However, during electrophoresis on a nondenaturing polyacrylamide gel (28), all of the protein (and activity) precipitated at the top of the gel under the low ionic strength conditions employed.

Requirements for Polymerase Activity

Vaccinia polymerase requires a divalent cation and is maximally active in 5 mM $MgCl_2$. The enzyme is 50% as active in 50 μM $MnCl_2$, the optimum Mn^{2+} concentration. The enzyme activity is maximal in potassium phosphate buffer between pH 8.0 and 9.2, 50% maximal at pH 7.0, and 75% maximal at pH 10.1. Polymerase activity is about 10 times greater in 50 mM potassium phosphate as in 50 mM Tris-HCl at the same pH. The polymerase activity is 50% inhibited by 200 mM NaCl or 2% ethanol. It requires a sulfhydryl reagent (0.1 mM dithiothreitol or 2 mM 2-mercaptoethanol) for maximal activity and is completely inhibited by 10 mM *N*-ethylmaleimide or 30 μM *p*-mercuribenzoate (in the absence of sulfhydryl reagent). The effect of deoxynucleoside triphosphate concentration was measured by varying the concentration of all four simultaneously in the standard assay mixture; the apparent K_m is about 15 μM.

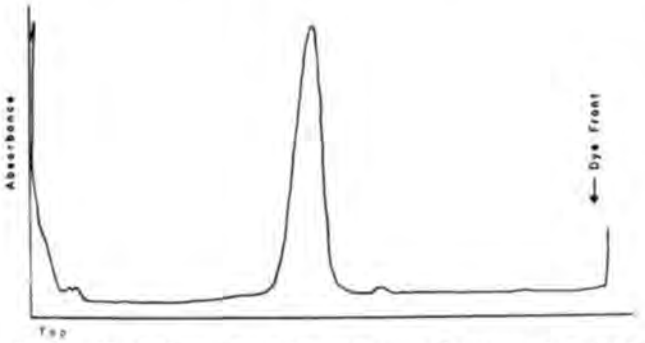

FIG. 1. **SDS-polyacrylamide gel electrophoresis of vaccinia DNA polymerase.** A sample (14 μg of protein) of Fraction VI was electrophoresed on a cylindrical SDS-polyacrylamide gel as described under "Methods." After staining, the gel was scanned in a Gilford spectrophotometer at a wavelength setting of 540 nm. The absorbance spike at the origin is an optical artifact from the edge of the gel.

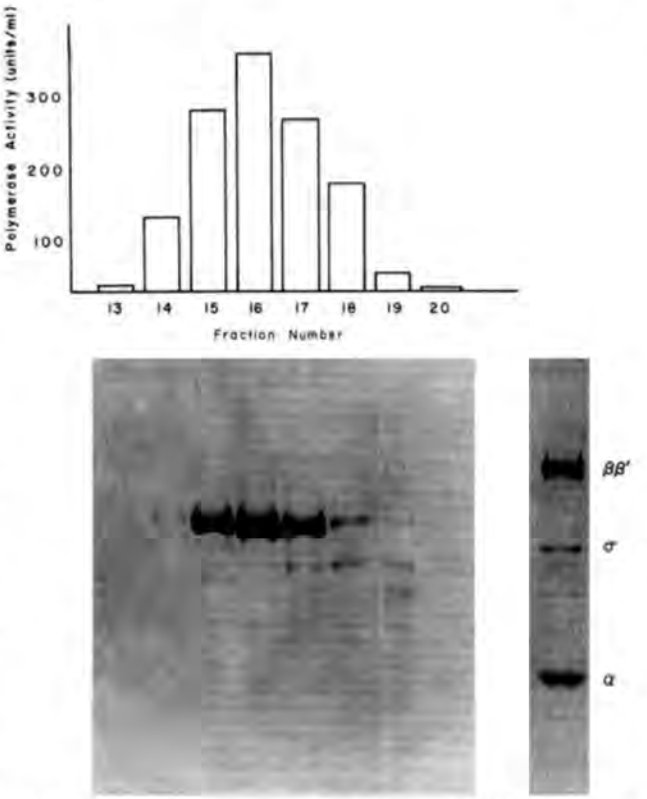

FIG. 2. **Co-sedimentation of protein and DNA polymerase activity.** A sample of Fraction V (600 units in 1.2 ml) was sedimented through a glycerol gradient as described in Step VI of the purification. A total of 31 fractions were collected from the bottom of the tube. A portion of each fraction was analyzed for DNA polymerase activity as described under "Methods." The protein remaining in each fraction was electrophoresed on a SDS-polyacrylamide slab gel. Only those fractions with detectable DNA polymerase activity are shown. Bands designated ββ', σ, and α are subunits of *E. coli* RNA polymerase which were run on the same gel as size markers.

The enzyme has maximal activity on activated DNA. It has relatively low activity on φX174 DNA annealed to a strand of a single restriction enzyme fragment of φX174 RF (see following paper (18)). It has no detectable activity on either unprimed φX174 viral DNA or covalently closed φX174 RF I DNA. The enzyme will add a maximum of one to two nucleotides for each nick introduced into φX174 RF DNA with pancreatic DNase I. We conclude, therefore, that vaccinia polymerase will neither nick-translate nor strand-displace.

Intrinsic Exonuclease Activity

Citarella *et al.* (9) reported that a nuclease activity persisted in their partially purified preparations of vaccinia DNA polymerase, and we found this activity in Fraction VI as well. The nuclease activity is very strong. One unit of polymerase, which will catalyze the synthesis of 1 nmol of DNA, will catalyze the degradation of about 2.5 nmol of DNA in the exonuclease assay (in the absence of triphosphates). To define whether this nuclease is an integral activity of the polymerase, we have assayed all fractions in the purification for exonuclease activity. Polymerase and exonuclease activity co-elute from DNA agarose, phosphocellulose, and hydroxylapatite (data not shown). In addition, the two activities co-sediment in a sucrose density gradient (Fig. 3*A*) and elute in parallel during chromatography on Pyran Sepharose (Fig. 3*B*). The

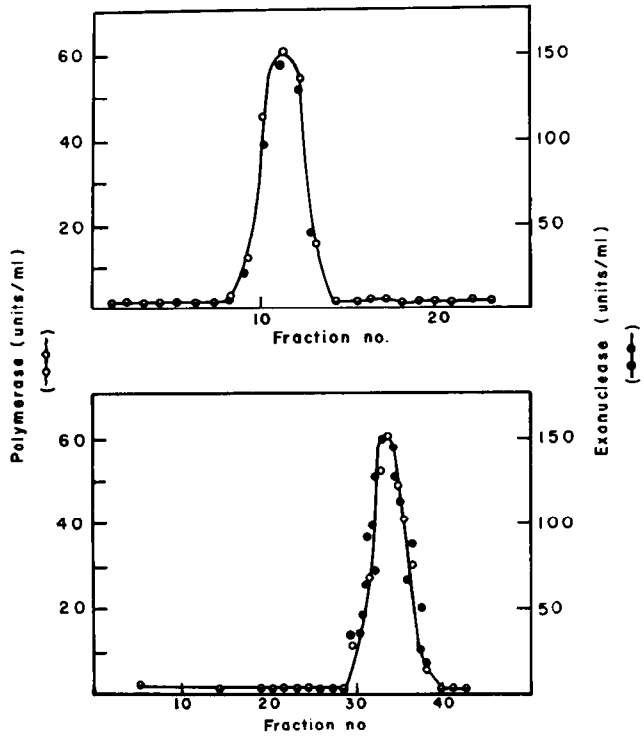

FIG. 3. **Association of polymerase and exonuclease activity.** Fraction IV (200 units) was analyzed by sedimentation through a sucrose gradient (*A*) as described under "Methods" or by chromatography on Pyran Sepharose (20). Polymerase activity (●) and exonuclease activity (○) were assayed as described under "Methods." Recovery of polymerase activity was 50% in (*A*) and 80% in (*B*). The Pyran Sepharose column (0.5 cm^2 × 3 cm) was equilibrated with 0.1 M potassium phosphate, pH 7.6, 10% glycerol, 5 mM 2-mercaptoethanol, 0.5 mM EDTA. Enzyme was loaded on the column, and after washing with 10 ml of column buffer, the enzyme activity was eluted with a gradient (20 ml) of 0.1 to 0.5 M potassium phosphate, pH 7.6, containing 10% glycerol, 5 mM 2-mercaptoethanol, and 0.5 mM EDTA. Fractions (0.5 ml) were collected and assayed for DNA polymerase and nuclease activity (Method 1).

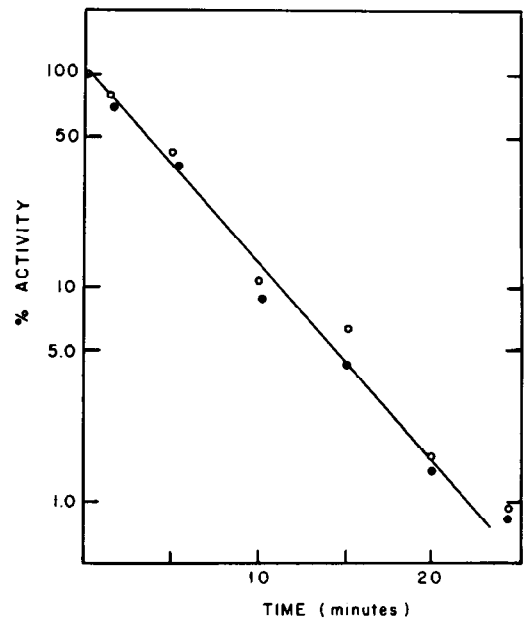

FIG. 4. **Heat inactivation of polymerase and exonuclease.** Vaccinia DNA polymerase (40 units; Fraction IV) in 0.1 M potassium phosphate, pH 7.5, 10% glycerol, 100 μg/ml of albumin, 0.1 mM dithiothreitol was incubated at 45°C. At the indicated times, aliquots were withdrawn and assayed for either polymerase (○——○) or exonuclease (●——●) activity (Method 1).

ratio of the two activities is the same after either fractionation procedure. Finally, as shown in Fig. 4, polymerase and exonuclease activities decay at the same rate when incubated at elevated temperature. These data provide strong evidence that both the DNA polymerase and the exonuclease activities are catalyzed by the same protein.

To test whether the intrinsic nuclease is an endonuclease, covalently closed φX174 RF I [^{3}H]DNA was incubated with the polymerase and then analyzed by alkaline sucrose gradient sedimentation. A single scission per molecule converts the DNA from a fast sedimenting form (53 S) to a more slowly sedimenting form (16 to 18 S). Both native and irreversibly denatured φX174 DNA (29) were used as substrates. After incubating 0.5 μg of either substrate for 60 min with 2.5 units of polymerase, no endonucleolytic activity was observed. Assuming that one nick in 10% of the molecules could be detected in this assay, the upper limit of endonuclease activity is 1 internal phosphodiester bond broken by an amount of enzyme capable of incorporating 10^5 nucleotides.

These experiments indicate that the intrinsic nuclease of vaccinia polymerase is an exonuclease. To identify the product of the exonuclease, we digested T7 [^{32}P]DNA to completion (>95% acid-soluble) and treated the product with snake venom 5′ nucleotidase. The 5′ nucleotidase converted greater than 95% of the ^{32}P-labeled acid-soluble product from a form adsorbable to Norit to a form not adsorbable to Norit. The major products of the nuclease are thus 5′ mononucleotides, in agreement with the findings of Citarella *et al.* (9). We have not ruled out the possibility that oligonucleotides are formed early in the reaction and are eventually hydrolyzed to mononucleotides.

Requirements of the Exonuclease Activity

The exonuclease activity requires a divalent cation and is completely inactive in the presence of 1 mM EDTA. The optimum concentration of $MgCl_2$ is between 8 and 16 mM, and activity falls to 50% at 2 and 20 mM. $MnCl_2$ can substitute for $MgCl_2$; the exonuclease is twice as active in the presence of 50 μM $MnCl_2$, the optimum Mn^{2+} concentration, as it is in 10 mM $MgCl_2$. The pH optimum, between pH 8.0 and 9.2, is similar to that for polymerase activity. Like the polymerase, the exonuclease is inhibited by salt; its activity is reduced by 50% when 50 mM NaCl is added to the standard reaction which contains 50 mM Tris-HCl. Whereas the polymerase activity is about 10 times greater in 50 mM potassium phosphate than in 50 mM Tris-HCl, the exonuclease activity is twice as great in the Tris-HCl buffer.

The activity of the exonuclease on duplex DNA is inhibited 80% by a mixture of all four deoxynucleoside triphosphates (20 μM each). Hydrolysis of single-stranded DNA is less sensitive to deoxynucleoside triphosphates; the four triphosphates (100 μM each) lower the activity by 50%.

Polarity of Hydrolysis

To determine whether the intrinsic exonuclease attacks the 3′ or the 5′ terminus, we measured the relative rates of release of ^{32}P- and ^{3}H-nucleotides from a restriction fragment of φX174 RF DNA which was uniformly labeled with ^{3}H and labeled at either its 3′ or 5′ terminus with ^{32}P. The data in Fig. 5 show that the polarity of degradation is 3′ to 5′. All of the 3′ terminal ^{32}P-nucleotide was removed before any significant release of ^{3}H-nucleotides was detected, and only 30% of the 5′ terminal nucleotides were removed when 70 to 80% of the ^{3}H-substrate had been degraded. These data indicate that 3′ → 5′ hydrolysis proceeds at a rate at least several hundred times

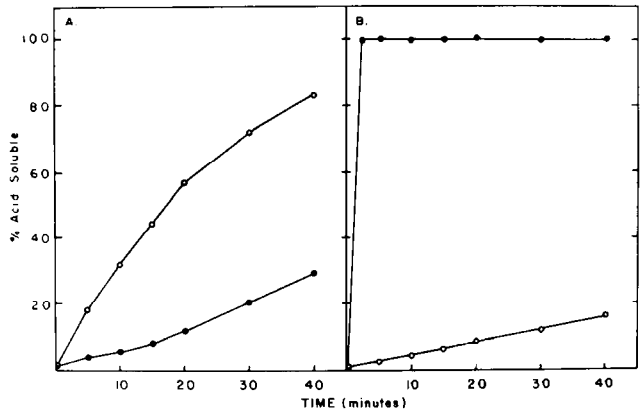

FIG. 5. **Polarity of intrinsic exonuclease activity.** ϕX174 ^{3}H-restriction fragment *Hae* III-5 (310 base pairs) was labeled with ^{32}P at either its 3′ or 5′ termini as described under "Methods." 3′ [^{32}P]DNA (*Panel B*) was incubated with 0.3 unit of vaccinia polymerase and 5′ [^{32}P]DNA (*Panel A*) was incubated with 2 units of vaccinia polymerase in a volume of 0.2 ml under standard exonuclease assay conditions at 37°C. Aliquots of 20 μl were withdrawn and analyzed for both acid-soluble and acid-insoluble radioactivity by Method 2 as described under "Methods." The radioactivity released from the 5′ termini was in the form of a 5′ mononucleotide, as shown by its Norit-adsorbability and sensitivity to 5′ nucleotidase. The *open circles* represent the ^{3}H uniform label and the *closed circles* represent the ^{32}P-terminal label

greater than 5′ → 3′ hydrolysis. To further establish whether the slow release of ^{32}P from the 5′ terminus (Fig. 5*A*) was a contaminant or an intrinsic activity of the polymerase, we assayed independently for 3′ → 5′ and 5′ → 3′ exonuclease activity during the purification procedure. The assay procedure used was the same as that in Fig. 5, except that the terminally labeled DNA was T7 DNA. The 3′ → 5′ exonuclease activity co-purified with polymerase activity during every step in the purification. The 5′ → 3′ exonuclease activity, on the other hand, differed from the polymerase in its elution from phosphocellulose and had a slightly different sedimentation rate in a sucrose density gradient (6.5 S compared to 6.3 S). The 5′ → 3′ exonuclease also decayed at a 50% slower rate than the polymerase when incubated at 45°C. Based on these findings and the fact that the rate of 5′ → 3′ hydrolysis is low compared to that in the 3′ → 5′ direction, we feel that the weak 5′ → 3′ exonuclease activity present in Fraction VI is probably a contaminant. Further interpretation of these data is presented under "Discussion."

Exonuclease Activity on Various DNA Substrates

Table II shows the relative rates of exonucleolytic activity on various DNA substrates. The exonuclease is more active on single-stranded DNA than on duplex DNA. The rate of hydrolysis on alkali-denatured sonicated *E. coli* [^{3}H]DNA is 13 times that on the same substrate before denaturation, and the rate on the alkali-denatured ϕX174 *Hae* III-5 restriction fragment is 7 times that of the rate on the same DNA prior to denaturation.

The rate of the exonuclease activity is also dependent on the size of the DNA substrate (Table II). For example, the rate of hydrolysis of alkali-denatured ϕX174 *Hae* III-5 restriction fragment (310 nucleotides in length) is 25-fold greater than that of alkali-denatured T7 DNA (about 40,000 nucleotides). The exonuclease also hydrolyzes short duplex chains faster than long duplex DNA molecules, although the dependence on size is much less severe for double-stranded DNA (compare the rate on native and denatured ϕX174 *Hae* III-5 and T7 DNA).

Similar effects of chain length on reaction rate have been

TABLE II

Exonuclease activity on various DNA substrates

The DNAs, all uniformly labeled with [^{3}H]thymidine, were incubated with 0.7 unit of vaccinia polymerase for 30 min at 37°C under standard exonuclease assay conditions. Acid-soluble radioactivity was determined by Method 2. The amount of DNA in the assays was: *Hae* III-5, 0.5 μg; *Hae* III-1, 0.3 μg; T7, 0.2 μg; sonicated *E. coli* DNA, 0.75 μg. In each case, the amount of DNA was determined in a separate experiment to be saturating in the assay. Therefore, the effect of fragment size on exonuclease activity is not dependent on the amount of termini present.

DNA	Size	Nucleotide hydrolyzed/30′
	base pairs	*pmol*
ϕX174 *Hae* III-5	310	147
ϕX174 *Hae* III-5, alkali-denatured		1,061
ϕX174 *Hae* III-1	1,353	105
ϕX174 *Hae* III-1, alkali-denatured		294
T7	40,000	32
T7 alkali-denatured		41
Sonicated *E. coli* DNA		120
Sonicated *E. coli* DNA, alkali-denatured		1,500

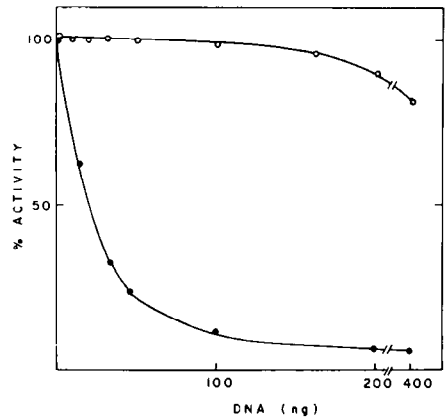

FIG. 6. **Effect of added circular DNA on exonuclease activity.** Native (○——○) or heat-denatured (●——●) ϕX174 ^{3}H-restriction fragment *Hae* III-5 (0.1 μg) was incubated with 1 unit of vaccinia polymerase in the presence of the indicated amount of unlabeled ϕX174 phage DNA (●——●) or Form I SV40 DNA (○——○) under standard exonuclease assay conditions at 37°C for 30 min. Acid-soluble radioactivity was determined by Method 1 as described under "Methods."

observed with other exonucleases (for examples, see Refs. 30–33). Huang and Lehman (31) have suggested as an explanation for this phenomenon that internal sequences, which are more abundant in long DNA molecules, may provide nonproductive binding sites for the enzyme. To test this possibility, we controlled the ratio of internal sequences to termini by adding increasing amounts of unlabeled circular DNA to a fixed amount of labeled linear substrate. The data in Fig. 6 show the effect of increasing concentrations of single-stranded ϕX174 DNA on the rate of hydrolysis of alkali-denatured ϕX174 *Hae* III-5 restriction fragment, and the effect of covalently closed duplex SV40 DNA on the rate of hydrolysis of the duplex substrate. Circular single-stranded DNA is a good inhibitor of the exonuclease, as one would predict from the model of Huang and Lehman (31). Half-maximal incubation is observed when the amount of single-stranded circular DNA is about one-fifth that of the labeled linear DNA. In contrast, circular duplex DNA has little, if any, effect on hydrolysis of linear duplex DNA. A 6-fold excess of unlabeled SV40 DNA inhibited the rate of hydrolysis of the linear substrate by less than 20%. Even this low level of inhibition can probably be

attributed to contamination of the Form I SV40 by adventitiously nicked molecules, which provide unlabeled substrate for the exonuclease.

DISCUSSION

Previous reports have shown that vaccinia infection causes induction of a new DNA polymerase (6-9). This induced enzyme can be chromatographically separated from the major host DNA polymerase (8, 9). It is more sensitive than polymerase β and less sensitive than polymerase α to the sulfhydryl blocking reagent *p*-mercuribenzoate. Its apparent size, 110,000 daltons, is also intermediate between the sizes of polymerases α and β (34). Unlike polymerase γ, the vaccinia polymerase is not able to copy a ribonucleotide template (9, 34). In contrast to many eukaryotic DNA polymerases, an exonuclease activity persists in the partially purified preparations of the vaccinia enzyme (9). The enzyme was presumed to be virally coded because it is immunologically distinct from the host enzymes (7) and because the vaccinia genome is large enough (120 million daltons) to encode several hundred proteins (1).

In this paper we describe a more extensive purification of the vaccinia DNA polymerase. The most pure fraction (Fraction VI) has a specific activity 500-fold higher than a cytoplasmic extract of infected cells, and is obtained in about 3% overall yield. When electrophoresed on an SDS-polyacrylamide gel, Fraction VI shows a single major polypeptide band, comprising about 95% of the Coomassie blue-stainable protein. The sedimentation rate of the polypeptide is the same as that of DNA polymerase activity on a glycerol gradient, providing evidence that the major protein in the preparation is the polymerase. The molecular weight of the protein, as estimated by SDS-polyacrylamide gel electrophoresis, is 110,000. This value is close to that of the native enzyme, calculated from its sedimentation coefficient and Stokes radius. Therefore, like many other DNA polymerases, the vaccinia polymerase consists of a single, large, polypeptide chain. The molecular weight value of 110,000 is in excellent agreement with that determined by Citarella *et al.* (9) using gel filtration.

Since a purification of only 500-fold yields a homogeneous enzyme, it is clear that there is an exceptionally large quantity of polymerase in the vaccinia-infected cell. From the specific activity and molecular weight of the purified polymerase, and from the activity per cell in the crude extract, we calculate that the cytoplasm of each infected HeLa cell contains more than 10^6 molecules of the viral polymerase. This high concentration may reflect the fact that, in comparison with other animal viruses, the replication of vaccinia DNA occurs very rapidly over a short period of time. Over 80% of the viral DNA replication occurs between 3 and 5 h after infection. This observation suggests that vaccinia-infected HeLa cells will be a relatively rich source of other replication enzymes.

As previously reported by Citarella *et al.* (9), a very strong exonuclease activity is found associated with the polymerase through all stages of purification. It is likely that the exonuclease, which degrades DNA 3′ → 5′, is an intrinsic activity of the enzyme molecule. The polymerase and exonuclease elute together from DNA agarose, phosphocellulose, hydroxylapatite, and Pyran Sepharose, and they sediment at the same rate through sucrose or glycerol gradients. Both activities decay at the same rate when incubated at elevated temperature. Intrinsic 3′ → 5′ exonucleases are absent in most eukaryotic DNA polymerases (34), but they are common in prokaryotic polymerases as well as in the polymerase induced by Herpes simplex virus (35). In some prokaryotic polymerases, such as *E. coli* DNA polymerase I and the DNA polymerase induced by phage T4, the 3′ → 5′ exonuclease exercises a proofreading function by removing nonbasepaired nucleotides from the primer terminus (36, 37). The exonuclease of the T4 enzyme is more active on single-stranded DNA than duplex DNA, as would be expected for an enzyme which preferred unpaired nucleotides. There is no available information as to whether the 3′ → 5′ exonuclease of the vaccinia enzyme is responsible for proofreading. However, it does have a 7- to 10-fold preference for single-stranded DNA.

In our purest preparations we find a very low, but detectable, 5′ → 3′ exonuclease activity. This activity is most likely a contaminant, since most of it elutes from phosphocellulose differently than the polymerase. However, we cannot rule out the possibility that within the cell the vaccinia polymerase has both a 3′ → 5′ and a 5′ → 3′ exonuclease activity, similar to *E. coli* DNA polymerase I. Despite the presence of protease inhibitors during cell lysis and during the purification, this activity might be cleaved from the enzyme by proteolysis. Proteolysis is already known to cleave the 5′ → 3′ exonuclease from DNA polymerase I (38). Since the 5′ → 3′ exonuclease which we detected sediments slightly faster than the bulk of the polymerase activity (6.5 S compared to 6.3 S) it is possible that the 6.5 S proteins are polymerase molecules which retain the 5′ → 3′ exonuclease activity. However, evidence for this speculation is lacking.

Like several other exonucleases, the 3′ → 5′ exonuclease of vaccinia polymerase hydrolyzes short DNA chains more rapidly than long DNA chains. This size dependence is more pronounced on single-stranded DNA than on duplex DNA. One possible explanation for this phenomenon, first suggested by Huang and Lehman (31), is that polymerase molecules bind nonproductively to internal nucleotides, and therefore activity increases as the ratio of terminal to internal nucleotides increases. We have tested this possibility directly, challenging the exonuclease with circular DNA, either single-stranded ϕX174 DNA or duplex SV40 DNA. Low concentrations of single-stranded ϕX174 DNA inhibit the exonuclease dramatically, indicating that nonproductive binding may explain the size dependence for single-stranded DNA. However, added single-stranded ϕX174 DNA does not inhibit the activity of the polymerase on a ϕX174 template primed with a strand of a restriction fragment (18). Covalently closed circular duplex SV40 DNA fails to inhibit the exonuclease, even at relatively high ratios of SV40 DNA to linear substrate DNA. Therefore, we do not have a good explanation for the size dependence of exonuclease activity on double-stranded DNA.

The availability of purified DNA polymerase is a first step in our approach toward defining the enzymatic mechanisms involved in vaccinia DNA replication. In the accompanying paper we describe a study of the effect of template secondary structure on the activity of the vaccinia DNA polymerase. We are currently studying the effect of other vaccinia replication proteins on the activity of the polymerase on defined DNA substrates.

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