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Studies on Vaccinia Virus-Directed Deoxyribonucleic Acid Polymerase

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A vaccinia-directed deoxyribonucleic acid (DNA) polymerase has been partially purified from the cytoplasmic fractions of virus-infected HeLa cells. The utilization of natural and synthetic templates by this enzyme resembles that of the host cell DNA-dependent DNA polymerases. The vaccinia DNA polymerase cannot copy ribopolymers or ribonucleic acid but is very effective with an "activated" DNA as template. An exonuclease preferring single-stranded DNA as substrate is found in the most highly purified preparations of the enzyme. The molecular weight of the vaccinia DNA polymerase seems to be about 110,000. The viral DNA polymerase is also found to be associated with purified, infected cell nuclei, and this association may be due, at least in part, to nonspecific adsorption of the vaccinia DNA polymerase by nuclei.

Infection of HeLa cells by vaccinia virus leads to the appearance of a new deoxyribonucleic acid (DNA)-dependent DNA polymerase (hereafter referred to as D-DNA polymerase) in cytoplasmic fractions of the infected cells (3, 8, 12). This new D-DNA polymerase differed from the one host HeLa cell D-DNA polymerase then known in its chromatographic behavior on diethylaminoethyl (DEAE)-cellulose, response to primer DNA, inactivation by heat and *p*-chloromercuribenzoate, association with nuclease activity, and inhibition by vaccinia antiserum (3). The immunological data strongly implied that the viral genome directly coded for at least a portion of the vaccinia D-DNA polymerase molecule.

Recently, it has been shown that nuclear extracts of uninfected HeLa cells contain two clearly distinct D-DNA polymerase activities, designated nuclear D-DNA polymerase I and II. A major portion of D-DNA polymerase activity is also found in the cytoplasm of these cells and is very similar, and probably identical, to nuclear D-DNA polymerase II (15, 17). Another minor, but distinct, D-DNA polymerase is found within mitochondria; this polymerase is easily separable from the major cellular D-DNA polymerases by the removal of mitochondria from cytoplasmic fractions or chromatography on DEAE-cellulose (9, 13). In addition, a polyriboadenylic acid-dependent DNA polymerase (R-DNA polymerase) has been found in several eucaryotic cells and has been purified 800-fold from cytoplasmic fractions of HeLa cells (5). This R-DNA polymerase is distinguished from cellular D-DNA polymerases by its distinctive ability to copy the polyriboadenylic acid strand of the oligomer-homopolymer [dT_{12}·poly(rA)].

Increased knowledge of the various DNA polymerase activities present in HeLa cells prompted us to reinvestigate the properties of the vaccinia-directed DNA polymerase found in vaccinia-infected HeLa cells. In this paper, we have investigated the intracellular distribution of the vaccinia-directed DNA polymerase and compared its properties to the DNA polymerases found in the normal host cell.

MATERIALS AND METHODS

Cells, virus, and infection of cells. HeLa S-3 cells were grown in suspension cultures at 37 C in F-13 medium (Gibco, Grand Island, N.Y.) supplemented with 5% heat-inactivated fetal calf serum (Gibco) and 1% lactalbumin hydrolysate (Gibco). Cells were harvested at 5×10^5 per ml, washed with 0.001 M KPO_4 (*p*H 7.3) containing 0.32 M sucrose and 0.002 M $MgCl_2$, and stored in liquid N_2. For virus infection, cells grown to 5×10^5 per ml were collected by centrifugation and resuspended at 5×10^6 per ml in supplemented F-13 media with $MgCl_2$ added to a final concentration of 0.02 M. Vaccinia virus, strain WR, was added at 2.5 to 3.5 plaque-forming units per cell, and the virus was allowed to adsorb for 1 hr at 37 C. The cells were diluted to 5×10^5 per ml with supplemented F-13 medium, incubated at 37 C for 6 hr, harvested, washed with 0.001 M KPO_4 (*p*H 7.3)

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containing 0.32 M sucrose and 0.002 M $MgCl_2$, and stored in liquid N_2.

Column chromatography. Whatman DEAE-cellulose (DE-52; H. Reeve Angel Co., Clifton, N.J.; exchange capacity, 1.0 meq/g dry) and Whatman P-11 phosphocellulose (exchange capacity, 7.4 meq/g dry) were equilibrated with 1.0 M KPO_4 (*p*H 7.5 and 8.9, respectively, for DE-52 and P-11). Immediately before chromatography of cellular extracts, the DE-52 and P-11 were equilibrated with 0.02 M KPO_4 containing 5×10^{-4} M dithiothreitol (DTT: Calbiochem, Los Angeles, Calif.) at *p*H 7.5 and 8.9, respectively.

Preparation of cellular extracts. Cytoplasmic fractions were prepared by a modification of the procedure described by Berkowitz et al. (2). Frozen cells were suspended in 20 volumes of 0.01 M NaCl and 0.001 M KPO_4 (*p*H 7.3) per gram of cells and broken by 10 strokes with a Dounce homogenizer to obtain nuclei and cytoplasm. Nuclei were separated from the cytoplasm by centrifugation at $1,000 \times g$ for 10 min. Mitochondria were removed from the cytoplasmic fractions by centrifugation at $12,000 \times g$ for 20 min and, when desired, ribosomes were also removed by centrifugation at $100,000 \times g$ for 4 hr. Although removal of ribosomes results in the loss of a large amount of DNA polymerase which adsorbs nonspecifically to the ribosomes, it facilitates separation of the vaccinia DNA polymerase from host DNA polymerase in subsequent DEAE-cellulose chromatography. Nuclei were resuspended in 10 volumes of 0.32 M sucrose, 0.001 M $MgCl_2$, 0.001 M KPO_4 (*p*H 7.0) and 0.3% Triton N-101 (Ruger Chemical Co., Inc., Irvington-on-Hudson, N.Y.), given 10 strokes with the Dounce homogenizer, and centrifuged at $1,000 \times$ g for 10 min. The supernatant fluid of nuclei (Triton wash) and the cytoplasmic extract were used as the sources of enzyme for further purification.

When desired, more highly purified nuclei were prepared by washing 2 to 4 times in 10 volumes of 0.32 M sucrose, 0.001 M $MgCl_2$, 0.3% Triton N-101, and 0.001 M KPO_4 (*p*H 7.0) before being pelleted through 1.0 M sucrose containing 0.001 M $MgCl_2$, 0.3% Triton N-101, and 0.001 M KPO_4 (*p*H 7.0); centrifugation was at $1,000 \times g$ for 15 min.

DNA, nucleotides, and polynucleotides. DNA, nucleotides, and polynucleotides and the sources from which they were obtained were: ^{3}H-labeled ribonucleoside and deoxyribonucleoside triphosphates (Schwartz-Mann, Orangeburg, N.Y.); unlabeled ribonucleoside and deoxyribonucleoside triphosphates and salmon sperm DNA (Calbiochem); poly(rA), poly(dT), poly(rU), poly(rC), poly(dT)·poly(rA), poly(rA)·poly(rU), and poly[d (A–T)] (Miles Laboratories, Elkhart, Ind.); oligodeoxynucleotides $(dT)_{12-18}$ and $(dA)_{12-18}$ (Collaborative Research, Waltham, Mass.), oligonucleotide $(rU)_{29}$ was generously provided by S. Pestka, Roche Institute of Molecular Biology. The annealing of oligonucleotides to homopolymers was done as previously reported at a proportion of three parts of oligonucleotide to seven parts of homopolymer by weight [4:1 for $(dT)_{12-18}$. polio RNA] (6). The preparation of HeLa DNA (3) and activated salmon sperm DNA (pancreatic deoxyribonuclease treated) were previously reported (15).

D-DNA and R-DNA polymerase assays. The standard reaction mixtures used to assay the HeLa cell D-DNA and R-DNA polymerase activities and the vaccinia DNA polymerase activity are listed below.

(i) The DNA-dependent DNA polymerase (D-DNA) assay required: bovine serum albumin (BSA, Armour Pharmaceutical Co., Kankakee, Ill., fraction V), 90 μg; tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 8.5), 10 μmoles; $MgCl_2$, 1.5 μmoles; DTT, 0.1 μmole; ^{3}H-deoxyadenosine triphosphate (5×10^4 counts/min per nmole), 20 nmoles; deoxycytosine triphosphate, 20 nmoles; deoxyguanosine triphosphate, 20 nmoles; deoxythymidine triphosphate, 20 nmoles; activated salmon sperm DNA, 0.5 to 75 μg as required; and 5 to 30μliters of enzyme solution in a final volume of 200 μliters.

(ii) The R-DNA polymerase assay required: BSA, 90 μg; Tris-hydrochloride buffer (*p*H 7.5), 10 μmoles; $MnCl_2$, 0.1 μmole; DTT, 0.5 μmole; $(dT)_{12}$·poly(rA), 7.5 μg; ^{3}H-thymidine triphosphate (5×10^4 counts/min per nmole), 20 nmoles; KCl, 25 μmoles; and 5 to 50 μliters of enzyme solution in a final volume of 200 μliters.

(iii) The vaccinia DNA polymerase assay required: BSA, 90 μg; KPO_4 buffer (*p*H 7.5), 10 μmoles; DTT, 0.1 μmole; $MgCl_2$, 1.0 μmole; ^{3}H-deoxyadenosine triphosphate (5×10^4 counts/min per nmole), 20 nmoles; deoxycytosine triphosphate, 20 nmoles; deoxythymidine triphosphate, 20 nmoles; activated salmon sperm DNA, 0.5 to 75 μg as required; and 5 to 30 μliters of enzyme solution in a final volume of 200 μliters.

The HeLa cell and the vaccinia D-DNA polymerase assay mixtures were incubated at 37 C for 30 min, and the HeLa cell R-DNA polymerase assays were incubated at 30 C for 30 min. Acid precipitation of the reaction products and assay for radioactivity were done as previously described (15).

Deoxyribonuclease assay. The standard reaction mixture contained: BSA, 90 μg; KPO_4 (*p*H 7.5), 10 μmoles; $MgCl_2$, 1 μmole; DTT, 0.1 μmole; ^{3}H-HeLa cell DNA (native or heat denatured), 2.4 μg (1.1×10^5 counts/min); and 5 to 15 μliters of enzyme solution in a final volume of 200 μliters. After incubation at 37 C for 60 min, the mixture was ice-chilled and 200 μliters of heat-denatured salmon sperm DNA (2.5 mg/ml) and 100 μliters of 6% perchloric acid were added. After standing for 5 min at 0 C, the mixture was centrifuged at $10,000 \times g$ for 5 min, and 400 μliters of supernatant fluid was placed in a scintillation vial together with 0.6 ml of water and 10 ml of fluor containing toluene, 667 ml; Triton X-100 (Beckman Instruments, Inc., Fullerton, Calif.), 337 ml; 2,5-diphenyloxazole, 5.5 g (Beckman); 1,4-bis-2-(5-phenyloxazolyl) benzene, 100 mg (Beckman). Samples were counted in a Beckman LS-250 liquid scintillation counter.

Protein determination. Protein concentration was determined by the modification of the Lowry procedure described by Pricer and Weissbach (14).

Electrofocusing of vaccinia DNA polymerase. Par-

tially purified vaccinia DNA polymerase (210-fold purified enzyme after P-11 chromatography) was electrofocused on an LKB Ampholine column (LKB Instruments, Inc., Rockville, Md.). The enzyme was introduced into a thermostated column (1.5 C) together with a linear sucrose gradient (47 to 0%, w/v) containing 1% carrier ampholytes (LKB; *p*H range, 3 to 10). After 44 hr at an applied voltage of 300 v, 3-ml fractions were collected, and the *p*H (at 2 C) and refractive index (at 25 C) were determined for each fraction. DNA polymerase activity was determined before and after dialysis of the fractions against 0.1 M KPO_4 (*p*H 7.5) containing 5×10^{-4} M DTT.

Preparation of vaccinia antiserum. Monolayer cultures of rabbit kidney cells (7- to 10-day-old rabbits), grown at 37 C in supplemented F-11 medium (Gibco), were infected with vaccinia virus at 10 plaque-forming units per cell. Cells were harvested 7 hr after exposure to the virus, washed with phosphate-buffered normal saline, and stored in liquid N_2. Samples of these cells, containing 0.25 mg of virus-infected cell protein, were emulsified with 0.5 ml of Freund complete adjuvant (Difco, Detroit Mich.) and injected subcutaneously into a 2-kg rabbit at biweekly intervals. Serum was collected 2 weeks after the third inoculation of antigen. A partially purified gamma globulin fraction was prepared by adding $(NH_4)_2SO_4$ to one-third saturation at 0 C and centrifuging at 10,000 × *g* for 10 min (repeated twice). The final precipitate was resuspended in 0.15 M NaCl and 0.001 M Tris-hydrochloride (*p*H 8.5), stored in liquid N_2, and used for all titrations of vaccinia and HeLa cell D-DNA polymerase activities.

RESULTS

Intracellular distribution of DNA polymerase activity. Subcellular fractions of HeLa and vaccinia-infected HeLa cells were prepared as described (see Materials and Methods). For the purpose of comparison, the distribution of DNA polymerase activity among these fractions was determined by using assay conditions which were optimal for the HeLa-cell DNA polymerases. The data presented in Table 1 indicate that virus infection of HeLa cells does not result in a significant change in the distribution of DNA polymerase activity between cytoplasm and nuclei. The total DNA polymerase activity in vaccinia-infected cells is apparently the same as that in normal cells. It is pertinent to note that the activity of purified vaccinia DNA polymerase is reduced by 50% when assayed under conditions optimal for HeLa cell DNA polymerases.

TABLE 1. *Distribution of total DNA polymerase activity in normal and vaccinia-infected HeLa cells*[a]

Cell fraction	Total polymerase activity (%)	
	Normal HeLa	Vaccinia-infected HeLa
Purified nuclei	8–10	9–13
Cytoplasm (including ribosomes)	68–75	67–74
Triton wash of nuclei	14–15	7–14
Mitochondria	2–5	4–5

[a] Assay conditions were optimal for HeLa cell D-DNA polymerases (see Materials and Methods). Assays were carried out at 37 C for 30 min and contained 3 to 5 μliters of cell fractions to be in the proportional range of protein concentration.

A rough estimate of the amount of vaccinia polymerase present in subcellular fractions prepared from virus infected cells can be obtained by using vaccinia antiserum. As shown in Table 2, vaccinia antiserum (gamma globulin preparation; see Materials and Methods) does not inhibit partially purified D-DNA polymerases obtained from uninfected cells. Thus, partially purified D-DNA polymerase II, obtained from either the nuclei or cytoplasm of uninfected HeLa cells, as well as D-DNA polymerase I are not affected by vaccinia antisera. Nor is the DNA polymerase activity found in the cellular fractions prepared from uninfected cells inhibited by vaccinia antisera. By contrast, this antiserum inhibits purified vaccinia DNA polymerase (obtained as described later in this paper) by about 80%. Preimmune serum did not inhibit either vaccinia or HeLa cell DNA polymerase activity. In infected cells, both cytoplasm and nuclei, the latter purified by washing 2 to 4 times in Triton-containing buffers and by further sedimentation through sucrose, contain the vaccinia DNA polymerase, as indicated by the ability of the vaccinia antiserum to inhibit the DNA polymerase activity in these cell fractions (Table 2). Because vaccinia virus replicates in the cytoplasm of cells, it was surprising to find evidence of the vaccinia-directed DNA polymerase associated with "purified" nuclei from infected cells. Control experiments, in which purified nuclei from uninfected cells were mixed with infected cell cytoplasm, showed that normal nuclei could adsorb large amounts of vaccinia DNA polymerase. Furthermore, this nuclear-adsorbed viral polymerase could be only partially removed by washing with Triton-containing buffers and by sedimentation through sucrose. It appears that the vaccinia DNA polymerase associated with our purified infected-cell nuclei is, in part, and perhaps wholly, adsorbed onto these nuclei in a nonspecific fashion.

Partial purification of vaccinia DNA polymerase: DEAE-cellulose chromatography. Berns

TABLE 2. *Inhibition of DNA polymerase activity by vaccinia antiserum*[a]

Enzyme source	Total cellular polymerase activity (%)	Deoxynucleotide incorporated (pmoles)		Percent of inhibition (−) or stimulation (+)
		Minus γ-globulin	Plus γ-globulin	
Normal HeLa:				
Purified nuclei	10	103	116	+13
Cytoplasm including ribosomes	73	271	272	0
Triton wash of nuclei	15	234	248	+6
Vaccinia-infected HeLa:				
Purified nuclei	13	1,828	512	−72
Cytoplasm including ribosomes	74	1,241	425	−66
Triton wash of nuclei	9	149	55	−63
Purified vaccinia polymerase		107	24	−78
Purified HeLa nuclear I polymerase		54	67	+24
Purified HeLa nuclear II polymerase		890	907	+2
Purified HeLa cytoplasmic polymerase		260	279	+7

[a] Assays were carried out by using conditions optimal for vaccinia polymerase (see Materials and Methods). A 2- to 5-μliter sample of each enzyme solution was incubated at 37 C for 30 min in assay mixtures containing a 15-μliter sample of vaccinia antiserum (γ-globulin preparation containing 17.3 mg of protein per ml) but lacking primer DNA and deoxynucleoside triphosphates. Control reaction mixtures, without γ-globulin added, were similarly prepared and incubated. Then assay mixtures were ice-chilled and 75 μg of activated salmon sperm DNA primer and a mixture of four deoxynucleoside triphosphates (75 to 125 ^{3}H counts/min per pmole of deoxynucleoside triphosphate) were added per assay. Assay mixtures were reincubated at 37 C for 30 min before being processed for counting. Partially purified HeLa DNA polymerases obtained from P-11 column fractions (as previously described) (17) and a partially purified vaccinia polymerase from Sephadex G-200 gel filtration (as described in Results) were used.

et al. (3) have used DEAE-cellulose chromatography to separate the vaccinia DNA polymerase. We have used this technique to examine both cytoplasmic and Triton wash extracts. Cytoplasmic fractions, prepared as previously described, were chromatographed on DE-52 columns. Representative elution profiles of cytoplasmic extracts from normal and infected cells are shown in Fig. 1a and b, respectively. More than 97% of the applied polymerase activity was retained on the columns. The peak of D-DNA polymerase activity eluted by 0.08 M KPO_4 in the infected-cell extract (Fig. 1b) has always been observed upon chromatography of cytoplasmic extracts derived from vaccinia-infected HeLa cells and has never been seen in uninfected cells. Both uninfected and infected cells contain a peak of D-DNA polymerase activity eluted at about 0.16 M KPO_4, and this host enzyme has been previously described (15, 17). In addition, column fractions were assayed for the presence of polyriboadenylic acid-dependent DNA polymerase (5) which normally represents about 1% of the total DNA polymerase activity in the cell. Although not shown, the peak of R-DNA polymerase activity was found to elute between 0.1 and 0.12 M KPO_4 (5) and partially overlapped the peak of the vaccinia D-DNA polymerase activity eluted at 0.08 M KPO_4. Infected cells showed no increase in R-DNA polymerase activity, which again comprised about 1% of the total DNA polymerase activity. Chromatography of the Triton wash fractions prepared from normal and virus-infected cells resulted in elution profiles of DNA polymerases which were essentially the same as those shown by the respective cytoplasmic extracts.

Additional purification of vaccinia DNA polymerase obtained from DEAE-cellulose chromatography of cytoplasmic and Triton wash frac-

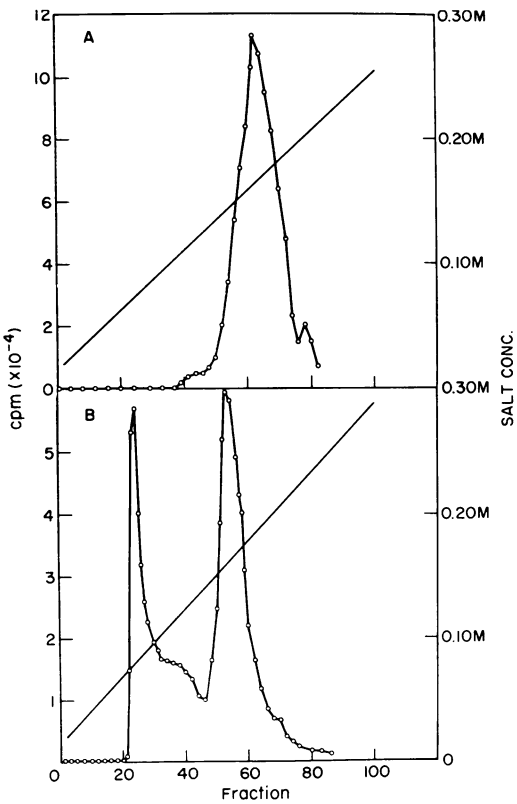

FIG. 1. *DEAE-cellulose chromatography of D-DNA polymerase activity of (A) normal HeLa cytoplasmic extract and (B) vaccinia-infected HeLa cytoplasmic extract (with ribosomes removed). Columns were loaded at a proportion of 4 to 5 mg of protein per gram of DEAE-cellulose and washed with one column volume of 0.02 M KPO_4 (pH 7.5) containing 5 × 10^{-4} M dithiothreitol (DTT), and then were eluted with 10 column volumes of a linear gradient from 0.02 to 0.30 M KPO_4 (pH 7.5) containing 5 × 10^{-4} DTT. Fractions equivalent to 20% of the column volume were collected, and 30-μliter samples of each fraction were assayed for polymerase activity by using assay conditions optimal for HeLa cell D-DNA polymerases (see Materials and Methods).*

tions: phosphocellulose chromatography. Fractions from DE-52 columns containing the highest levels of vaccinia DNA polymerase activity were pooled, and the *p*H was adjusted to 8.9 with 1.0 M KOH. This pooled material was loaded onto a phosphocellulose column at a proportion of 4 to 5 mg of protein per g of P-11 phosphocellulose. The column was washed with one column volume of 0.02 M KPO_4 (*p*H 8.9) containing 5 × 10^{-4} M DTT, and then was eluted with eight column volumes of a linear gradient from 0.02 to 0.40 M KPO_4 (*p*H 8.9) containing 5 × 10^{-4} M DTT. An excellent separation of vaccinia polymerase (eluted at 0.14 M KPO_4) from the contaminating polyriboadenylic acid-dependent DNA polymerase (eluted at 0.22 M KPO_4) was obtained (Fig. 2). Recovery of vaccinia polymerase activity was greater than 85%. No additional peak of D-DNA polymerase activity was detected.

Sephadex (G-200) gel filtration. A 1-ml sample of vaccinia polymerase (obtained from a P-11 column containing 66 μg of protein) and a 1-ml sample of pig heart lactic dehydrogenase (Boehringer; molecular weight of 109,000 and containing 25 μg of protein) were consecutively run on a G-200 column (40 by 1 cm) previously equilibrated with 0.05 M KPO_4 (*p*H 8.5) and 5 × 10^{-4} M DTT. Both enzymes were eluted with the same buffer, and the exclusion volume of the column was determined by using blue dextran 2000 (Sephadex). A comparison of the elution

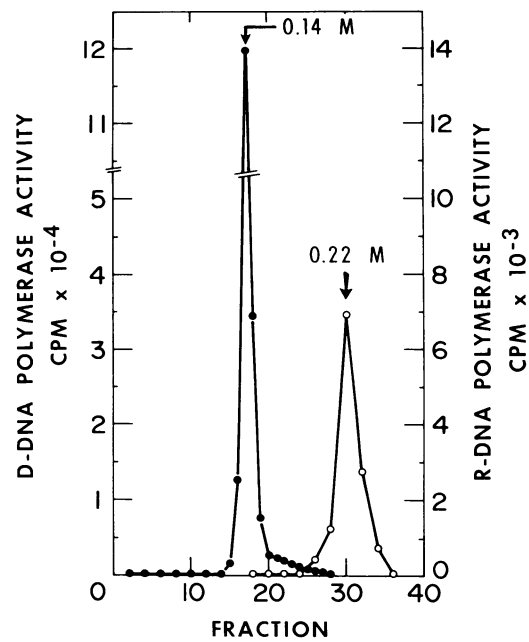

FIG. 2. *Phosphocellulose chromatography of the pooled vaccinia DNA polymerase fractions obtained after DEAE-cellulose chromatography of the Triton wash and cytoplasmic fractions of viral-infected cells. Thirty μliters of each fraction (5.5 ml) were assayed for D-DNA polymerase activity (●) and for R-DNA polymerase activity (○); see Materials and Methods. The specific activity of the ^{3}H-thymidine triphosphate in the R-DNA polymerase assays was 50 counts/min/pmole; the specific activity of the ^{3}H-deoxyadenosine triphosphate in the D-DNA polymerase assays was 12.5 counts/min/pmole. The D-DNA and R-DNA polymerase assays were carried out for 30 and 60 min, respectively, at the appropriate temperatures.*

profiles obtained indicated a molecular weight of 110,000 ± 10,000 for vaccinia polymerase (Fig. 3).

Characterization of vaccinia polymerase: requirements for optimal activity. The *p*H optimum for polymerase activity was 7.5 to 8.5 with KPO_4 or imidazole-hydrochloride at 50 mM. However, the level of activity was fourfold higher when KPO_4 (50 mM) instead of imidazole-hydrochloride (50 mM), was used. The addition of KCl (at 138 mM) to imidazole-hydrochloride-buffered assays raised the level of polymerase activity to that observed when KPO_4 at 50 mM alone was used as buffer; therefore, KPO_4 (50 mM) was routinely used.

A mixture of four deoxynucleoside triphosphates was required for maximal activity; omission of a single deoxynucleoside triphosphate resulted in a decline in activity of 83%. The enzyme incorporated a single deoxynucleoside triphosphate (^{3}H-deoxyadenosine triphosphate) into an acid-insoluble product at a rate of 3% or less than that observed with a mixture of four deoxynucleoside triphosphates. No incorporation of ribonucleoside triphosphates was detected.

Magnesium was optimal at 5 mM. We have not investigated the effect of substituting other metal ions for magnesium (3). Polymerase activity was 30% higher when assayed at 37 C rather than at 30 C. Bovine serum albumin, at 90 μg per assay, was added to stabilize enzyme activity. Mercaptoethanol, or DTT, has not been shown to be required but was added to assays routinely. Vaccinia DNA polymerase showed no activity in the absence of a DNA template.

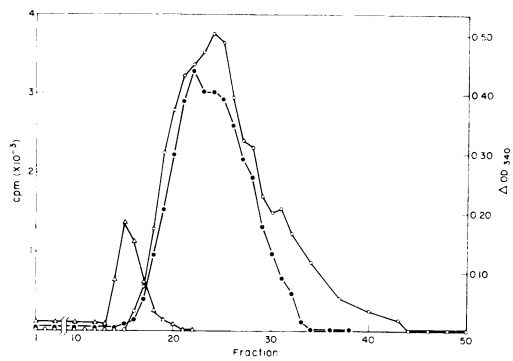

FIG. 3. *Gel filtration of vaccinia DNA polymerase and pig heart lactic dehydrogenase on a Sephadex G-200 column (1 by 40 cm). A 30-μliter sample of each fraction was assayed for polymerase activity (●) by using assay conditions optimal for vaccinia polymerase (see Materials and Methods). Lactic dehydrogenase activity (○) was assayed by incubation, at 25 C, of 5-μliter samples of each fraction with 0.7 ml of KPO_4 (0.1 M, pH 6.7) containing 28 μg of DPNH and 70 μg of sodium pyruvate and by measuring the change in absorbancy at 340 nm for five minutes. The exclusion volume is indicated by the blue dextran marker (△); absorbancy was at 630 nm. A 1.14-fold increase in purification of vaccinia polymerase was obtained. Fractions of 0.92 ml were collected.*

Utilization of DNA and RNA templates by vaccinia DNA polymerase. Berns et al. (3) used heat-denatured DNA as primer-template to assay DNA polymerase activity. In view of the known efficiency of other primers for DNA-dependent DNA polymerases we have investigated the template requirements of vaccinia polymerase for optimal activity (Table 3).

Vaccinia DNA polymerase obtained by P-11 chromatography and additional Sephadex gel filtration shows maximal activity with deoxyribonuclease-treated "activated" DNA (Table 3).

TABLE 3. *Utilization of templates by vaccinia polymerase*

Template	Deoxynucleotide incorporated (pmoles)[a]
Activated, native salmon sperm DNA	112.6
Activated, native HeLa DNA	109.1
Native HeLa DNA	1.9
Denatured HeLa DNA	0.7
$(dG)_{12}$·poly(dC)	83.6
poly(dG)·poly(dC)	62.1
$(dA)_{12}$·poly(dT)	10.9
poly[d(A-T)]	49.5
$(dT)_{12}$·poly(rA)[b]	<0.3
poly(dT)·poly(rA)	0.0
oligo$(rU)_{28}$·poly(rA)[b]	0.0
$(dG)_{12}$·poly(rC)[b]	0.0
poly(dG)·poly(rC)	0.4
poly(rA)·poly(rU)	0.4
$(dT)_{12}$·polio RNA	0.0
$(dT)_{12}$	0.5
No primer	0.0

[a] Assays were performed for 30 min at 37 C by using conditions optimal for vaccinia polymerase (see Materials and Methods). Each assay contained 10 μg of template and deoxynucleotide triphosphate substrates appropriate for that assay, i.e., with activated native DNA (^{3}H-deoxyadenosine triphosphate plus the other three unlabeled deoxynucleotides), $(dA)_{12}$·poly(dT), and ^{3}H-deoxythymidine triphosphate. Each nucleoside triphosphate was added at 10 nmoles per assay, and the labeled compounds had specific activities of 5×10^5 counts/min per nmole. The vaccinia polymerase used was obtained after Sephadex G-200 chromatography.

[b] These templates were also tested by using assay conditions optimal for R-DNA polymerase (see Materials and Methods).

It utilizes such an "activated" DNA 50 to 100 times better than untreated single- or double-stranded DNA and thus resembles the HeLa DNA-dependent DNA polymerases (6, 15). Synthetic polydeoxynucleotide complexes such as poly(dG)·poly(dC), poly[d(A-T)], and others are also effective templates. However, oligomer-homopolymer complexes which contain a polyribomer strand are not utilized. This is illustrated in Table 3 if one compares the utilization of $(dG)_{12}$·poly(rC) or poly(dG)·poly(rC) with $(dG)_{12}$·poly(dC) or poly(dG)·poly(dC). As expected, a natural RNA such as polio RNA is not an effective template even in the presence of a primer such as $(dT)_{12}$. The inability of the small primer molecule $(dT)_{12}$ to stimulate polydeoxynucleotide synthesis alone indicates that the vaccinia DNA polymerase has no significant deoxynucleotidyl terminal transferase activity (4). In all respects, vaccinia DNA polymerase acts like the DNA-dependent DNA polymerases of HeLa cells (15) and is markedly different from either the R-DNA polymerase found in HeLa cells (5) or the RNA-dependent DNA polymerases of the RNA tumor viruses (1, 7).

As Berns et al. reported (3), the vaccinia DNA polymerase is saturated at relatively low levels of template. Figure 4 shows the response of the HeLa and vaccinia DNA polymerases to varying amounts of an activated DNA template. Both HeLa nuclear I DNA polymerase and the vaccinia DNA polymerase respond well to low concentrations of template, whereas DNA polymerase II (from either nucleus or cytoplasm) prefers high template concentrations. Vaccinia DNA polymerase differs from the nuclear I polymerase because, under the conditions shown in Fig. 4, the former is saturated by 5 μg of template (per 200-μliter assay) and nuclear I polymerase is not saturated even by 25 μg of template in the assay.

Experiments performed by Paolo La Colla, in this laboratory, have indicated that vaccinia DNA obtained from purified vaccinia virus is no more effective a template for vaccinia DNA polymerase than is native HeLa or salmon sperm DNA.

Association of deoxyribonuclease activity with partially purified vaccinia polymerase. Berns et al. (3) reported the presence of a deoxyribonuclease activity in their partially purified preparation of vaccinia polymerase. Similarly, we have found deoxyribonuclease activity in our preparations of vaccinia polymerase obtained after P-11 chromatography and Sephadex gel filtration. The activity of this nuclease was 2 times higher when assayed with denatured DNA rather than native DNA. Polymerase obtained from a P-11 column was electrofocused on an Ampholine column in an attempt to separate nuclease from vaccinia polymerase activity (Fig. 5). Column fractions 16, 17, and 18 were assayed for polymerase activity (with activated salmon sperm DNA as primer) and deoxyribonuclease activity (with ^{3}H-native and denatured HeLa-cell DNA as substrate). The DNA polymerase peak obtained by electrofocusing contained a corresponding peak of nuclease activity (Table 4), supporting the hypothesis that this nuclease activity may be part of the vaccinia DNA polymerase molecule.

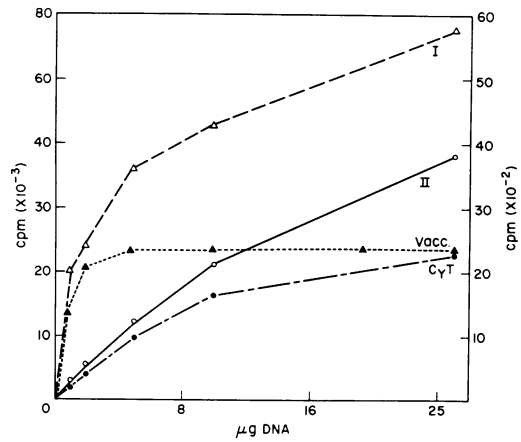

FIG. 4. *Response of HeLa and vaccinia DNA polymerases to primer concentration. Each assay contained the indicated amount of activated salmon sperm DNA as primer. Polymerase assays of HeLa enzymes and of vaccinia DNA polymerase were carried out as described in Materials and Methods. HeLa enzymes were obtained from P-11 column fractions (17), and vaccinia DNA polymerase was obtained from a Sephadex G-200 column fraction. Protein concentrations of the enzyme samples added per assay were: HeLa D-DNA polymerase nuclear I, 8.8 μg; D-DNA polymerase nuclear II, 3.8 μg; D-DNA polymerase II (cytoplasmic), 3.0 μg; vaccinia DNA polymerase, 0.05 μg. The (10^{-2}) counts/min ordinate is for the nuclear I enzyme. HeLa polymerase assays contained 20 nmoles of each unlabeled deoxynucleoside triphosphate and 20 nmoles of ^{3}H-deoxyadenosine triphosphate (dATP) (5×10^4 counts/min per nmole); vaccinia polymerase assays contained 10 nmoles of each unlabeled deoxynucleoside triphosphate and 10 nmoles of ^{3}H-dATP (5×10^5 counts/min per nmole).*

Time course and end point of DNA synthesis by vaccinia polymerase. The incorporation of deoxynucleoside triphosphates into an acid-insoluble product was monitored over a 24-hr period. Additional enzyme was added at 4-hr-intervals, up to hr 12, to compensate for loss of enzyme activity. When activated salmon sperm DNA was used as template, the incorporation of deoxynucleotide monophosphates ceased when

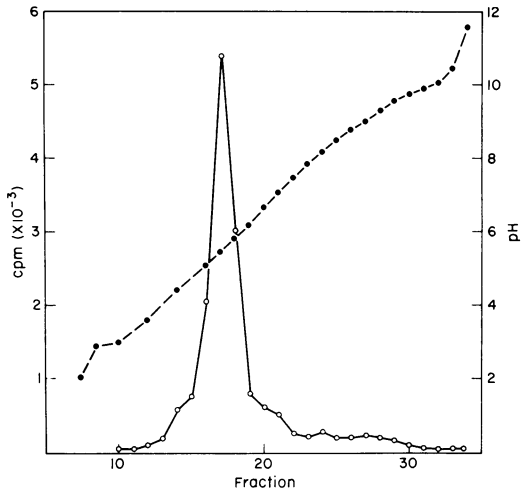

FIG. 5. *Ampholine column electrofocusing of vaccinia polymerase obtained after P-11 chromatography. A 15-μliter sample of each fraction was assayed for polymerase activity by using conditions optimal for vaccinia polymerase (see Materials and Methods). Recovery of polymerase activity was 36%.*

the amount of product formed was equivalent to 9 to 12% of the amount of template initially present. In like manner, when native and denatured HeLa DNA were used as templates, incorporation of deoxynucleoside triphosphate ceased at levels equivalent to 0.2 and 1.1%, respectively, of the amount of template initially present; preincubation of these templates for 60 min at 37 C with vaccinia DNA polymerase raised these levels to 0.4 and 1.7%, respectively. It appeared that the vaccinia polymerase, like the host-cell DNA polymerases, can only repair "gaps" in these primer-templates and cannot carry out net synthesis. Preincubation of the primer-templates with vaccinia DNA polymerase and its associated nuclease activity apparently widened preexisting gaps.

We have obtained similar results with the HeLa-cell D-DNA polymerases which also cannot carry out net synthesis with activated DNA as a template. The maximal incorporation of deoxynucleotide phosphate is, again, about 12 to 13% of the amount of template present.

DISCUSSION

The vaccinia-specified DNA polymerase, described by Berns et al. (3), has been purified (ca. 240-fold after sequential chromatography on DEAE-cellulose and phosphocellulose and by Sephadex gel filtration) from the cytoplasmic and Triton wash (detergent wash of nuclei) fractions of virus-infected HeLa cells. The viral polymerase differed from the host cell DNA polymerase in its chromatographic behavior on DEAE-cellulose, its response to DNA template concentrations, the conditions required for optimal activity, and inhibition by vaccinia antiserum. In addition, the molecular weight of vaccinia DNA polymerase (about 110,000, as estimated by Sephadex gel filtration) was found to be different from the molecular weights of D-DNA polymerase I (30,000) and D-DNA polymerase II (over 200,000), the normal host cell DNA polymerases (17). However, when a variety of RNA and DNA primer-templates were tested for utilization by the vaccinia and the HeLa cell DNA polymerases, no distinction between the vaccinia DNA polymerase and the host-cell DNA polymerases was noted. Like the other DNA polymerases, vaccinia polymerase was unable to carry out net synthesis of DNA and appeared to be limited to the repair of "gaps" in an activated DNA primer.

A deoxyribonuclease activity, preferring single-stranded DNA as substrate, was associated with our purest preparations of vaccinia polymerase. The nuclease appears to be an exonuclease which releases 5'-mononucleotides: ^{3}H-thymidine 5'-phosphate was the major acid-soluble product formed (94%) and identified by paper chroma-

TABLE 4. *DNA polymerase and deoxyribonuclease activity of ampholine column fractions*

Fraction no.[a]	Activity assayed	Deoxynucleotide (pmoles)		Nuclease activity-polymerase activity (ratio)
		Rendered acid soluble	Incorporated acid insoluble	
16	Nuclease[b]	21		0.13
	Nuclease[c]	42		0.26
	Polymerase[d]		161	
17	Nuclease[b]	32		0.10
	Nuclease[c]	52		0.16
	Polymerase[d]		325	
18	Nuclease[b]	20		0.11
	Nuclease[c]	29		0.16
	Polymerase[d]		187	

[a] See Fig. 5. Vaccinia DNA polymerase and deoxyribonuclease assays were carried out for 1 hr as described in Materials and Methods. Assays contained 20-μliter samples of the column fractions.

[b] Substrate was 2.4 μg of ^{3}H-native HeLa DNA (1.1×10^5 counts/min).

[c] Substrate was 2.4 μg of ^{3}H heat-denatured HeLa DNA (1.1×10^5 counts/min).

[d] Activated salmon sperm DNA (28 μg per assay) was used as primer DNA.

tography (16) when ^{3}H-thymidine-labeled HeLa DNA was used as substrate for nuclease reactions. At present, we do not have sufficient information to determine whether this nuclease corresponds to one of the several nuclease activities appearing during viral infection (10, 11) or whether the nuclease is a host enzyme. This nuclease activity was not inhibited when tested with vaccinia antiserum, but this may result from a deficiency of the antiserum because viral-polymerase activity was inhibited only about 80%. The failure to obtain 100% inhibition of viral-polymerase activity with the antiserum preparation remains unexplained; Berns et al. (3) have reported similar results.

The immunological data reported herein, together with that reported by Berns et al. (3) and Magee and Miller (12), who have shown that crude HeLa DNA polymerase antiserum does not inhibit the "new" DNA polymerase in infected cell extracts, strongly support the contention that the viral genome directly codes for at least a portion, and perhaps all, of the vaccinia DNA polymerase molecule. An alternative hypothesis is that the virus induces the formation of a new DNA polymerase in rabbit cells which is immunologically related to a viral-induced polymerase in HeLa cells. Additional data are required to settle these points of present uncertainty.

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