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Purification and Characterization of a DNA-dependent RNA Polymerase from Vaccinia Virions*

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A DNA-dependent RNA polymerase has been extracted from vaccinia virions and purified to near homogeneity as judged by glycerol gradient sedimentation and polyacrylamide gel electrophoresis. The native enzyme has a molecular weight of approximately 500,000 and can be dissociated into putative subunits of 140,000, 137,000, 37,000, 35,000, 31,000, 22,000, and 17,000 daltons. Activity was dependent on all four ribonucleoside triphosphates, Mn^{2+}, and a DNA template. Optimal activity occurred at pH 7.9 in the presence of 90 mM KCl or 40 mM $(NH_4)_2SO_4$. All single-stranded DNAs tested served as templates. By contrast, linear double-stranded DNAs were not effectively transcribed and very low activity was obtained with circular supercoiled DNAs which contain small single-stranded regions. The synthetic alternating copolymer poly(dA-dT), which forms a completely base-paired structure, also was not transcribed, whereas poly(dA,dT) and other random copolymers served as templates. Of the four homopolydeoxribonucleotides, only poly(dC) and poly(dT) were transcribed, suggesting that initiation specifically occurs with purine ribonucleotides. In support of this, higher K_m values were obtained for GTP and ATP (333 and 80 μM, respectively) than for UTP and CTP (22 and 12 μM, respectively) using a DNA template. The RNA polymerase was inhibited by polyanions but was resistant to rifampicin and α-amanitin.

Poxviruses, unlike other DNA viruses that infect animal cells, contain a complete array of enzymes necessary for synthesis and modification of mRNA. These enzymes are located within the core of the infectious virus particle and can be activated *in vitro* to transcribe the endogenous genome (1, 2). Upon disruption of the virus core with sodium deoxycholate, many enzymes are liberated in soluble form and some have been extensively purified and characterized. These include enzymes which are clearly involved in mRNA biosynthesis (such as poly(A) polymerase (3), RNA guanylyltransferase (4), RNA guanine-7-methyltransferase (4), and RNA (nucleoside-2'-)methyltransferase (5)) and others that may have roles as yet undefined in transcription (including two separate nucleic acid-dependent nucleoside triphosphate phosphohydrolases (6), protein kinase (7), single strand-specific deoxyribonuclease (8, 9), topoisomerase (10), RNA triphosphatase (11), and 5'-phosphate polynucleotide kinase (12)). An enzyme, conspicuous by its absence from this list, is the DNA-dependent RNA polymerase itself. Nevins and Joklik (13), however, found that a small per cent of the cytoplasmic RNA polymerase activity in vaccinia virus-infected cells was nonparticulate. They isolated the soluble enzyme, which was dependent on a single-stranded DNA template and Mn^{2+}, and showed that it differed from host RNA polymerase in chromatographic properties, polypeptide composition, and sensitivity to α-amanatin. A description of an RNA polymerase with similar properties, now purified from vaccinia virions, is described here.[1]

EXPERIMENTAL PROCEDURES

Cells and Virus—Vaccinia virus (strain WR) was grown in HeLa S3 cells and purified from the cytoplasmic fraction by sedimentation through a cushion of 36% (w/v) sucrose and two successive 15 to 30% (w/v) sucrose gradient sedimentations using slight modification of the procedure originally described by Joklik (14).

Enzyme Purification—All procedures were carried out at 0 to 4°C. Approximately 90 to 95 mg of purified vaccinia virus was incubated in 12 ml of 0.25 M NaCl, 0.1 M Tris-HCl (pH 8.5), 0.01 M dithiothreitol, and 0.2% sodium deoxycholate for 30 min at 0°C. Insoluble proteins were removed by centrifugation in an SW 41 rotor at 35,000 rpm for 30 min at 4°C. The supernatant was forced two times through a 0.5-inch, 26-gauge hypodermic needle to shear released DNA. Glycerol, Triton X-100, and EDTA were added to final concentrations of 10%, 0.1%, and 0.1 mM, respectively, and the total volume was increased so as to reduce the NaCl concentration to 0.2 M. The material then was applied at 20 ml/h to a DEAE-cellulose column (3.0 × 1.5 cm) equilibrated with Buffer A (0.15 M Tris-HCl (pH 8.4), 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% Triton X-100, and 10% glycerol) containing 0.2 M NaCl. After washing the column with additional Buffer A containing 0.2 M NaCl, the flow-through fractions containing RNA polymerase activity were combined.

The pooled material was diluted and adjusted to reduce the Tris-HCl and NaCl concentrations to 0.05 M, while maintaining the previous concentrations of dithiothreitol, EDTA, glycerol, and Triton X-100, and then applied at 20 ml/h to a DEAE-Bio-Gel column (5 × 1 cm) which was equilibrated with Buffer B (0.05 M Tris-HCl (pH 8.4), 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% Triton X-100, and 10% glycerol) containing 0.05 M NaCl. After washing the column with the latter buffer, a 60-ml linear gradient from 0.05 to 0.4 M KCl in Buffer B was applied. Fractions that contained RNA polymerase activity were pooled, diluted 2-fold with Buffer C (0.05 M Tris-HCl (pH 7.9), 1 mM dithiothreitol, 0.1 mM EDTA, 0.1% Triton X-100, 10% glycerol), and then applied at 10 ml/h to a phosphocellulose column (7 × 0.7 cm) that had been equilibrated with Buffer C containing 0.1 M KCl. After washing the column with 8 ml of the latter buffer, a 120-ml linear gradient from 0.1 to 0.6 M KCl in Buffer C was applied. Fractions with RNA polymerase activity were pooled, diluted 2-fold with Buffer C, and applied at 10 ml/h to an aminopentyl-agarose column (7 × 0.7 cm) equilibrated with Buffer C containing 0.075 M KCl. The column was washed with 15 ml of the latter buffer and a 150-ml linear gradient from 0.075 to 0.8 M KCl in Buffer C was applied. Fractions containing RNA polymerase activity were pooled and concentrated 14-fold using a Millipore immersible CX ultrafiltra-

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[1] An RNA polymerase with similar properties has also been purified from vaccinia virions by E. Spencer, S. Schuman, and J. Hurwitz (personal communication).

tion unit. Approximately 0.3 ml of concentrated enzyme was applied to each 11 ml of 15 to 35% (v/v) glycerol gradient in Buffer C containing 0.3 M KCl. Centrifugation at 39,000 rpm was for 18 h at 4°C in an SW 41 rotor. After collecting the gradient in equal fractions, enzyme was pooled and stored at −70°C. Under these conditions, the enzyme was stable for many months. For analysis of polypeptide composition, enzyme purified through the glycerol gradient step was used; enzyme purified through the phosphocellulose step was used for other studies.

RNA Polymerase Assay—Vaccinia virus RNA polymerase was routinely assayed in 0.1-ml mixtures containing: 50 mM Tris-HCl (pH 7.9); 2 mM dithiothreitol; 1 mM concentrations of ATP, GTP, and CTP; 0.04 mM [^{3}H]UTP (222 cpm/pmol); 4 mM $MnCl_2$; 90 mM KCl; and 5 μg of denatured calf thymus DNA. Incubations were carried out at 37°C for 30 min and stopped by adding 10 μg of bovine serum albumin and 3 ml of cold 5% trichloroacetic acid. After 15 min, the precipitate was collected on fiberglass filters which were dried and then counted in a toluene-based scintillation fluid. By definition, 1 unit of enzyme incorporates 1 pmol of UMP into RNA in 30 min at 37°C.

Polyacrylamide Gel Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out in slabs (10 × 14 × 0.15 cm) as described by Laemmli (15). The concentration of polyacrylamide in the resolving gel was 12.5%, while that in the stacking gel was 6%. Gels were stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol, 10% acetic acid and destained with several washes of a solution containing 10% methanol and 10% acetic acid. Densitometer tracings were made with an E-C Apparatus Corp. instrument and areas under the curves were calculated with a Numonics digitizer interfaced with a Wang 2200 computer.

Nondenaturing gel electrophoresis was carried out in 5% polyacrylamide slabs (16). For two-dimensional electrophoresis, a cylindrical nondenaturing 5% polyacrylamide gel (15 × 0.5 cm) was used for the first dimension. The gel then was soaked for 30 min in 0.625 M Tris-HCl (pH 6.8), 2.3% sodium dodecyl sulfate, 10% glycerol, and 0.7 M β-mercaptoethanol at room temperature and then heated at 80°C for 5 min. The gel cylinder then was placed on top of a sodium dodecyl sulfate (12.5%)-polyacrylamide slab gel for the second dimension.

Materials—All special reagents for preparation of polyacrylamide gels as well as Coomassie brilliant blue R-250, low molecular weight protein markers, and DEAE-Bio-Gel were obtained from Bio-Rad. DE52 and P-11-cellulose were products of Whatman. Aminopentyl-agarose was purchased from Bethesda Research Laboratories and sodium dodecyl sulfate, glycine, and aurintricarboxylic acid were from Sigma. Heparin sodium was supplied by Hynson, Wescott and Dunning, Inc., and α-amanitin and rifampicin were supplied by Boehringer Mannheim and Mann Research Laboratories, respectively. Radioisotopes were obtained from Amersham Corporation and glass fiber filters were from Enzo Biochemicals.

RESULTS

Purification of RNA Polymerase from Vaccinia Virus—Release of RNA polymerase from vaccinia virions required both a detergent, such as sodium deoxycholate, and a reducing agent and was enhanced by NaCl (Fig. 1). Highest activity was obtained with 0.2% sodium deoxycholate, 10 mM dithiothreitol, and NaCl at concentrations of 0.1 M or higher. These conditions are slightly different than those previously used to extract other vaccinia virus core associated enzymes (3–8, 10–12).

After disruption of virus by this method, insoluble structural proteins were removed by high speed centrifugation. Passage of the supernatant through a DEAE-cellulose column served to remove endogenous DNA. The RNA polymerase then was bound to a DEAE-Bio-Gel column while poly(A) polymerase (3), RNA guanylyltransferase·guanine-7-methyltransferase complex (4), RNA (nucleoside-2′-)methyltransferase (5), and DNA-dependent nucleoside triphosphate phosphohydrolases (6) all flowed through. Further purification of the RNA polymerase was achieved by chromatography on columns of phosphocellulose and aminopentyl-agarose (Fig. 2). In these experiments, [^{35}S]methionine-labeled virus was used to monitor purification since insufficient amounts of protein were present for conventional analysis. An 800-fold increase in specific

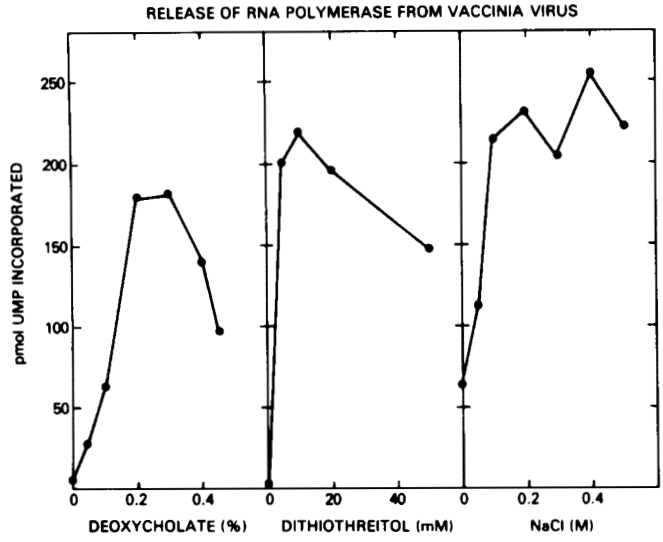

FIG. 1. **Release of RNA polymerase from vaccinia virus.** The effect of sodium deoxycholate concentration on release of RNA polymerase was determined by incubating 0.64 mg of purified virus in 0.1 ml of 0.05 M Tris-HCl (pH 8.5), 0.25 M NaCl, 0.05 M dithiothreitol, and various concentrations of sodium deoxycholate for 30 min at 0°C. At the end of this time, particulate material was sedimented in a microcentrifuge for 5 min at 12,800 × *g*. Samples (5 μl) of the supernatant were assayed for RNA polymerase activity using a single-stranded calf thymus DNA template as described under "Experimental Procedures." The effects of varying concentrations of dithiothreitol on release of RNA polymerase were evaluated using mixtures containing 0.05 M Tris-HCl (pH 8.5), 0.2% sodium deoxycholate, and 0.25 M NaCl. Similarly, the effect of NaCl was determined in mixtures containing 0.05 M Tris-HCl (pH 8.5), 0.2% sodium deoxycholate, and 0.05 M dithiothreitol. Enzyme assays were carried out in duplicate.

enzyme activity was obtained (Table I); however, the true purification was difficult to estimate because of a 4-fold increase in enzyme units that occurred during early stages of the isolation procedure presumably due to removal of inhibitors, such as deoxycholate.

The purified polymerase appeared to be homogeneous by co-sedimentation of radioactively labeled protein and enzyme activity on glycerol gradients (Fig. 3, *upper panel*). By taking advantage of distinctive properties of *Escherichia coli* and vaccinia virus RNA polymerases specifically to assay one in the presence of the other, we could demonstrate that the two enzymes co-sedimented (Fig. 3, *lower panel*). Similar results were obtained when the enzymes were centrifuged in separate gradient tubes.

Further evidence for the purity of the vaccinia virus RNA polymerase was obtained by electrophoresis on nondenaturing 5% polyacrylamide slab gels. A single radioactively labeled protein band (Fig. 4, *Lane 3*) corresponding to a single Coomassie brilliant blue staining band (Fig. 4, *Lane 2*) was detected. Under these conditions, the vaccinia virus RNA polymerase has a higher electrophoretic mobility than the *E. coli* enzyme (Fig. 4, *Lane 1*).

Molecular Weight and Subunit Composition—The sedimentation rate of the viral enzyme in glycerol gradients suggested that it has a molecular weight similar to that of *E. coli* RNA polymerase, which is about 490,000 (17). To determine the polypeptide composition of the vaccinia virus RNA polymerase, fractions obtained during successive steps in purification and after glycerol gradient centrifugation were treated with sodium dodecyl sulfate and mercaptoethanol and electrophoresed on 12.5% polyacrylamide slab gels. After electrophoresis, the gels were stained and autoradiographed. Examination of Fig. 5 reveals that the pooled enzyme fractions from the aminopentyl-agarose column (*Lane 5*), as well as from peak

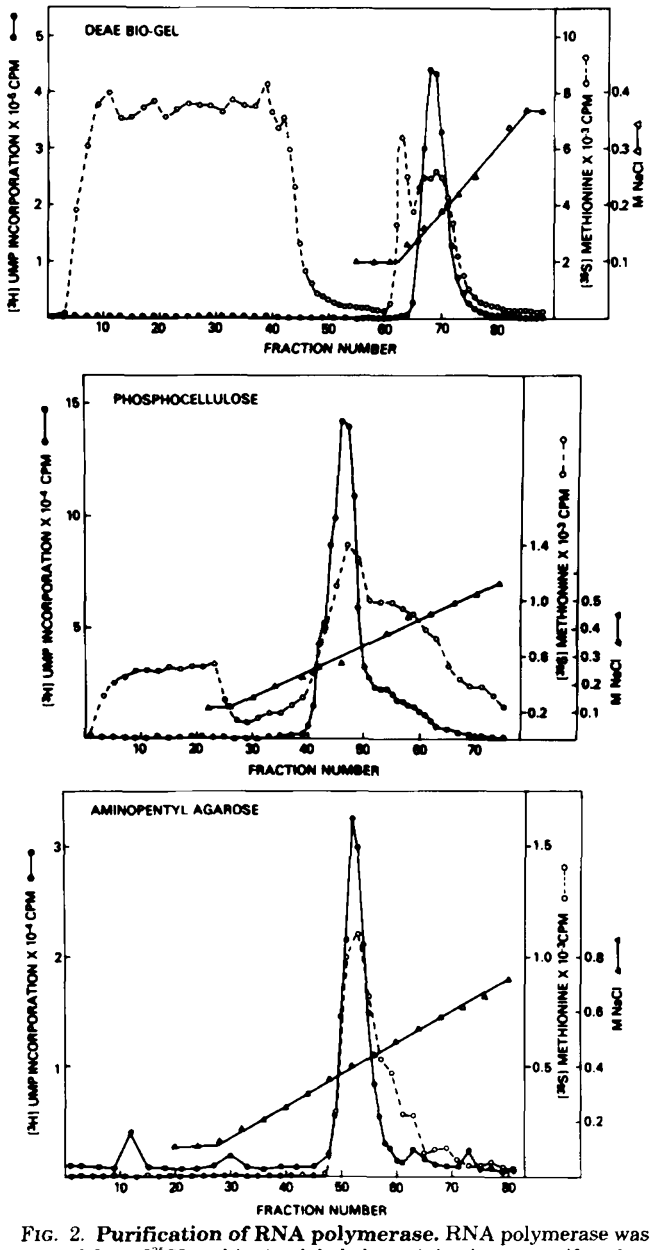

FIG. 2. **Purification of RNA polymerase.** RNA polymerase was extracted from [^{35}S]methionine-labeled vaccinia virus, centrifuged at high speed to remove insoluble structural proteins, passed through a DEAE-cellulose column to remove DNA, and purified by chromatography on successive columns of DEAE-Bio-Gel, phosphocellulose, and aminopentyl agarose. Enzyme assays were performed using single-stranded calf thymus DNA as template.

TABLE I

Purification of vaccinia virus RNA polymerase

Fraction	Volume	Protein[a]	Enzyme[b]	Specific activity	Yield	Purification factor
	ml	*mg*	*units*	*units/mg*	*%*	*-fold*
Disrupted virus	12.0	95.0	243,676	2,565		
High-speed supernatant	11.5	56.0	495,100	8,841	203	3
DEAE-cellulose	21.5	46.0	548,734	12,060	225	5
DEAE-Bio-Gel	15.5	3.4	923,577	273,248	379	107
Phosphocellulose	15.6	0.55	562,514	1,017,204	231	397
Aminopentyl-agarose	10.6	0.08	167,413	1,993,012	69	777

[a] Determined using [^{35}S]methionine-labeled virus with a specific activity of 10^6 cpm/mg calculated using the relationship of 64 μg of protein/$A_{260\ nm}$ of virus.

[b] Expressed as picomoles of UMP incorporated in 30 min at 37°C.

glycerol gradient fractions (*Lanes 6* to *8*), contained polypeptides of three size classes: high molecular weight polypeptides of 140,000 and 137,000; medium molecular weight polypeptides of 37,000, 35,000, and 31,000; low molecular weight polypeptides of 22,000, 21,000, and 17,000.

A two-dimensional procedure was used to demonstrate that polypeptides of all three size classes remain associated during electrophoresis on a nondenaturing polyacrylamide gel. After electrophoresis of the native RNA polymerase, the gel tube was heated in a solution containing sodium dodecyl sulfate and mercaptoethanol and placed on top of a polyacrylamide gel slab for electrophoresis in the second dimension. As shown in Fig. 6, a small amount of streaking occurred during the first dimension. However, the M_r = 140,000, 137,000, 35,000, 31,000, 22,000, and 17,000 polypeptides clearly fall on the same vertical axis, indicating that they migrated as a unit on the first dimension nondenaturing gel. With shorter autoradiographic

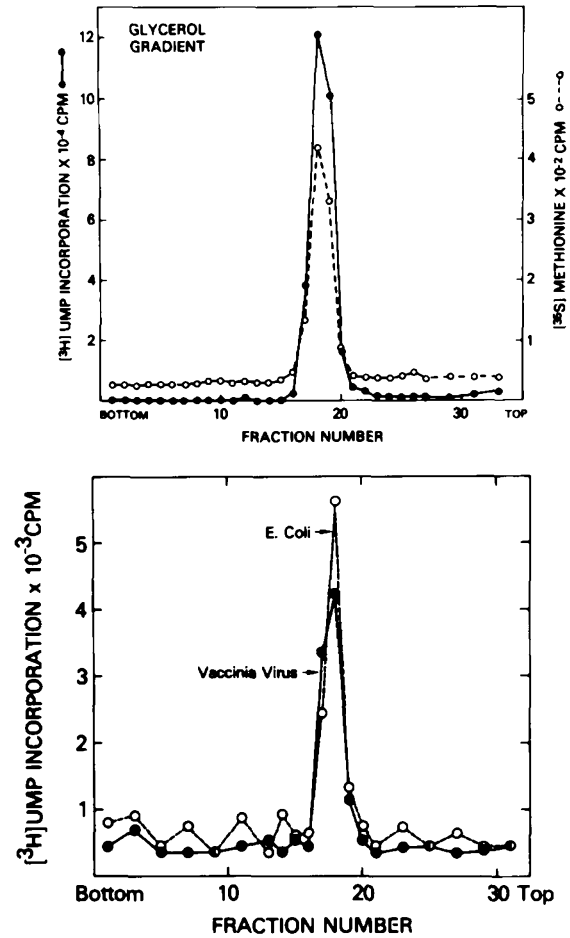

FIG. 3. **Glycerol gradient centrifugation of purified RNA polymerase.** *Upper panel,* RNA polymerase purified from [^{35}S]methionine-labeled vaccinia virus was centrifuged in 15 to 35% glycerol gradients as described under "Experimental Procedures" and assayed using single-stranded calf thymus DNA and Mn^{2+}; *lower panel,* 10 units of vaccinia virus RNA polymerase were mixed with *E. coli* RNA polymerase and centrifuged in a 15 to 35% glycerol gradient. To measure vaccinia virus RNA polymerase specifically, samples were incubated in reaction mixtures lacking ribonucleoside triphosphates but containing 150 μg of rifampicin/ml for 10 min at 4°C. At the end of this period, ribonucleoside triphosphates were added and the assay was performed as usual. To measure *E. coli* RNA polymerase specifically, double-stranded DNA and Mg^{2+} were used in the assay without rifampicin. The specificities of the assays were confirmed in control experiments, and the separate RNA polymerases were also analyzed on parallel gradients.

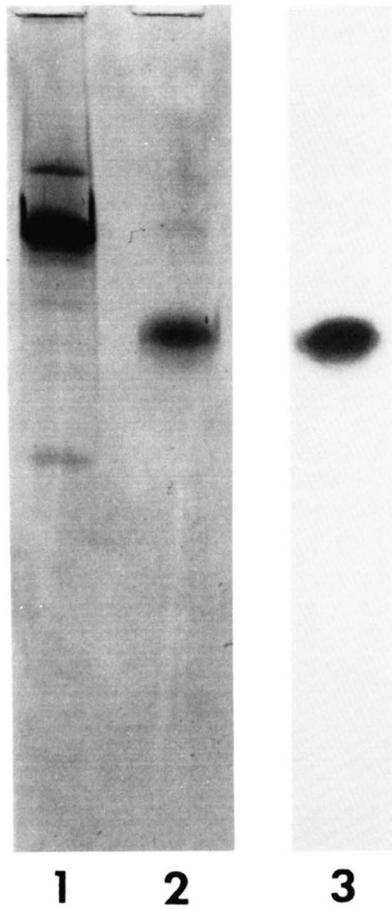

FIG. 4. **Polyacrylamide gel electrophoresis of undenatured RNA polymerase.** Column chromatography and glycerol gradient-purified RNA polymerase from [^{35}S]methionine-labeled vaccinia virus was run in parallel with *E. coli* RNA polymerase on a 5% polyacrylamide slab gel. After electrophoresis, the gel was stained and autoradiographed. *Track 1*, stained *E. coli* RNA polymerase; *Track 2*, stained vaccinia virus RNA polymerase; *Track 3*, autoradiograph of vaccinia virus RNA polymerase.

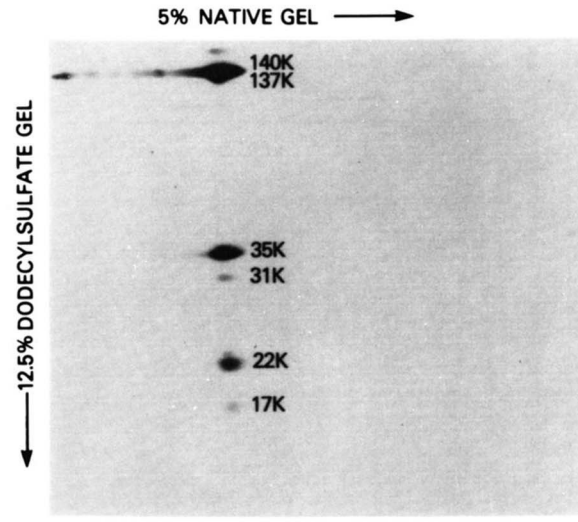

FIG. 6. **Two-dimensional gel electrophoresis of [^{35}S]methionine-labeled vaccinia virus RNA polymerase.** Approximately 10 μg of glycerol gradient-purified RNA polymerase was electrophoresed in a nondenaturing cylindrical 5% polyacrylamide gel. The gel then was incubated in sodium dodecyl sulfate and mercaptoethanol and applied to the top of a 12.5% polyacrylamide slab gel containing sodium dodecyl sulfate as described under "Experimental Procedures." After electrophoresis in the second dimension, the gel was stained and an autoradiograph was prepared. The autoradiograph is shown.

exposure and on photographs of the stained gel, the M_r = 140,000 and 137,000 polypeptides were seen as two distinct bands; however, neither the M_r = 37,000 nor the minor M_r = 21,000 polypeptides were resolved.

Densitometer tracings were made of stained one-dimensional denaturing gels to estimate the molar ratios of the putative subunits (Fig. 7; Table II). The M_r = 140,000, 137,000, 37,000, and 31,000 polypeptides appeared to be present in equivalent molar ratios; however, the M_r = 35,000 and 22,000 polypeptides appeared to be 2 and 4 M, respectively. Whether the high molar ratios indicate multiple copies of the same

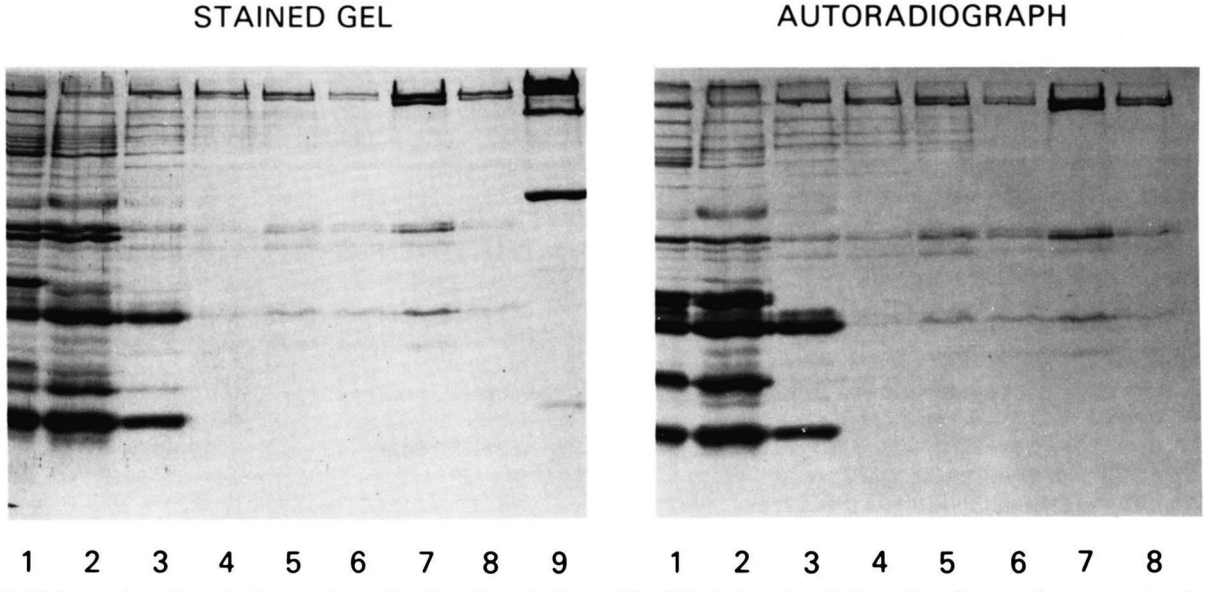

FIG. 5. **Polyacrylamide gel electrophoresis of sodium dodecyl sulfate- and mercaptoethanol-dissociated polypeptide subunits.** Samples, obtained at the following stages in the purification of vaccinia virus RNA polymerase, were analyzed on a 12.5% polyacrylamide gel: *1*, high speed supernatant; *2*, DEAE-cellulose; *3*, DEAE-Bio-Gel; *4*, phosphocellulose; *5*, aminopentyl-agarose; *6* to *8*, consecutive peak fractions from glycerol gradient; and *9*, purified *E. coli* RNA polymerase. Nearly equivalent amounts, approximately 6000 units, of RNA polymerase were applied in *Samples 1* to *5*.

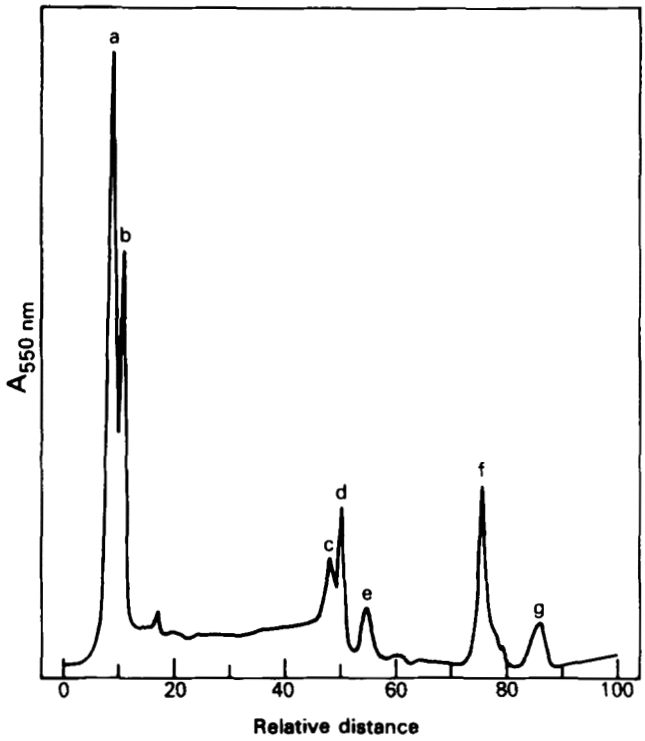

FIG. 7. **Densitometer tracing of RNA polymerase subunits.** Glycerol gradient-purified RNA polymerase was dissociated with sodium dodecyl sulfate and mercaptoethanol, and polyacrylamide gel electrophoresis was carried out as in Fig. 5. After staining with Coomassie brilliant blue, the gel was destained and scanned with an E-C Apparatus Corp. densitometer.

TABLE II

Molecular weights and molar ratios of polypeptides associated with vaccinia virus RNA polymerase

The molecular weights of the two largest polypeptides were determined on 5% polyacrylamide gels using subunits of *E. coli* RNA polymerase (165,000, 155,000, 95,000, and 39,000 as standards); 12.5% polyacrylamide gels with bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), and lysozyme (14,300), were used to determine the molecular weights of the remaining polypeptides. Molar ratios were calculated from densitometer scans of 12.5% gels such as the one shown in Fig. 6, by dividing the area under each peak by the indicated molecular weight. In Experiment 1, the 140,000 and 137,000 subunits were not sufficiently resolved to accurately determine the areas under each. In both experiments, the results were normalized by setting the molar ratio of the sum of the large polypeptides as 2.

Polypeptide	Molecular weight	Experiment 1	Experiment 2
a	140,000	2.0	1.1
b	137,000		0.9
c	37,000	0.9	1.1
d	35,000	2.0	1.5
e	31,000	1.2	0.9
f	22,000	3.9	3.6
g	17,000	2.2	0.9

polypeptide or different polypeptides with similar molecular weights was not distinguished. Variable results, 0.9 to 2.2 M, were obtained for the M_r = 17,000 polypeptide; the amount of the M_r = 21,000 polypeptide, which appears as a slight shoulder on the densitometer tracing, could not be estimated but appears to be less than 1 M. From the molecular weights and molar ratios of the individual polypeptides, a total molecular weight of 517,000 was obtained, in reasonable agreement with the value of approximately 500,000 determined for the native enzyme by glycerol gradient centrifugation.

Requirements for RNA Polymerase Activity—A DNA template, all four ribonucleoside triphosphates, and a divalent cation were essential for RNA polymerase activity (Table III). Omission of dithiothreitol from the reaction mixture had only a small effect; however, this reducing agent was present in the enzyme preparation.

Divalent Cation Requirement—Mn^{2+}, at concentrations approximately equimolar with ribonucleoside triphosphates, was required for RNA polymerase activity (Fig. 8). By contrast, very little activity was obtained when Mg^{2+} was substituted for Mn^{2+} (Fig. 8).

Monovalent Cation Requirement—Activity was enhanced by addition of monovalent cations. Maximal activity was obtained either with 90 mM KCl or 40 mM $(NH_4)_2SO_4$ (Fig. 8).

pH Optimum—Maximal activity, with Tris-HCl buffers, occurred at approximately pH 7.9 (Fig. 8).

Effect of Ribonucleoside Triphosphate Concentration—Under standard conditions, RNA polymerase activity was linear for approximately 30 min and continued for more than 100 min. Initial reaction velocities were measured as a function of ribonucleoside triphosphate concentration. Data were plotted in a double reciprocal fashion and K_m values were calculated by linear regression (Fig. 9). K_m values of 333, 80, 22,

TABLE III

Requirements for RNA polymerase activity

Reaction mixture	UMP incorporated
	pmol
Complete[a]	60.7
−DNA	0.0
−ATP	0.0
−GTP	5.7
−CTP	0.0
$-Mn^{2+}$	0.0
−Dithiothreitol	45.0

[a] Complete refers to the standard reaction mixture described under "Experimental Procedures."

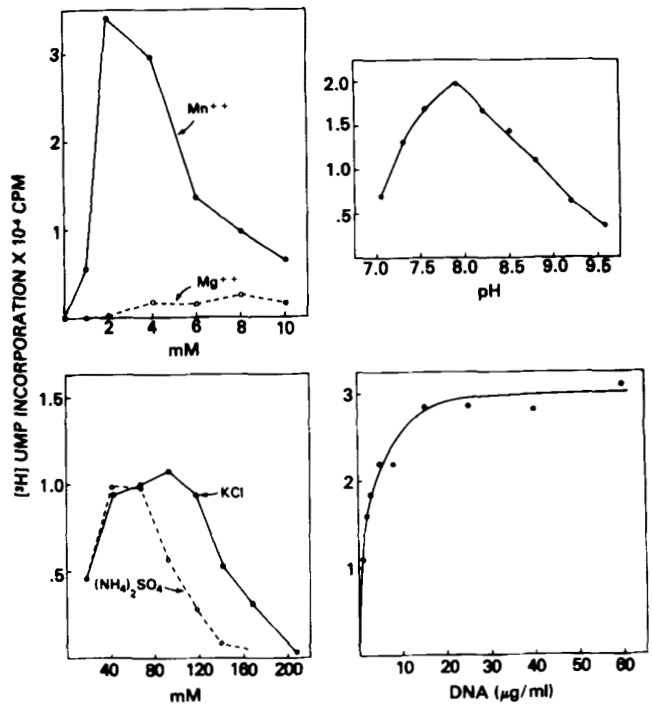

FIG. 8. **Optimal conditions for RNA polymerase activity.** Standard reaction conditions were used with varying divalent and monovalent cation concentrations, pH, and denatured calf thymus DNA concentrations. All reactions were carried out in duplicate.

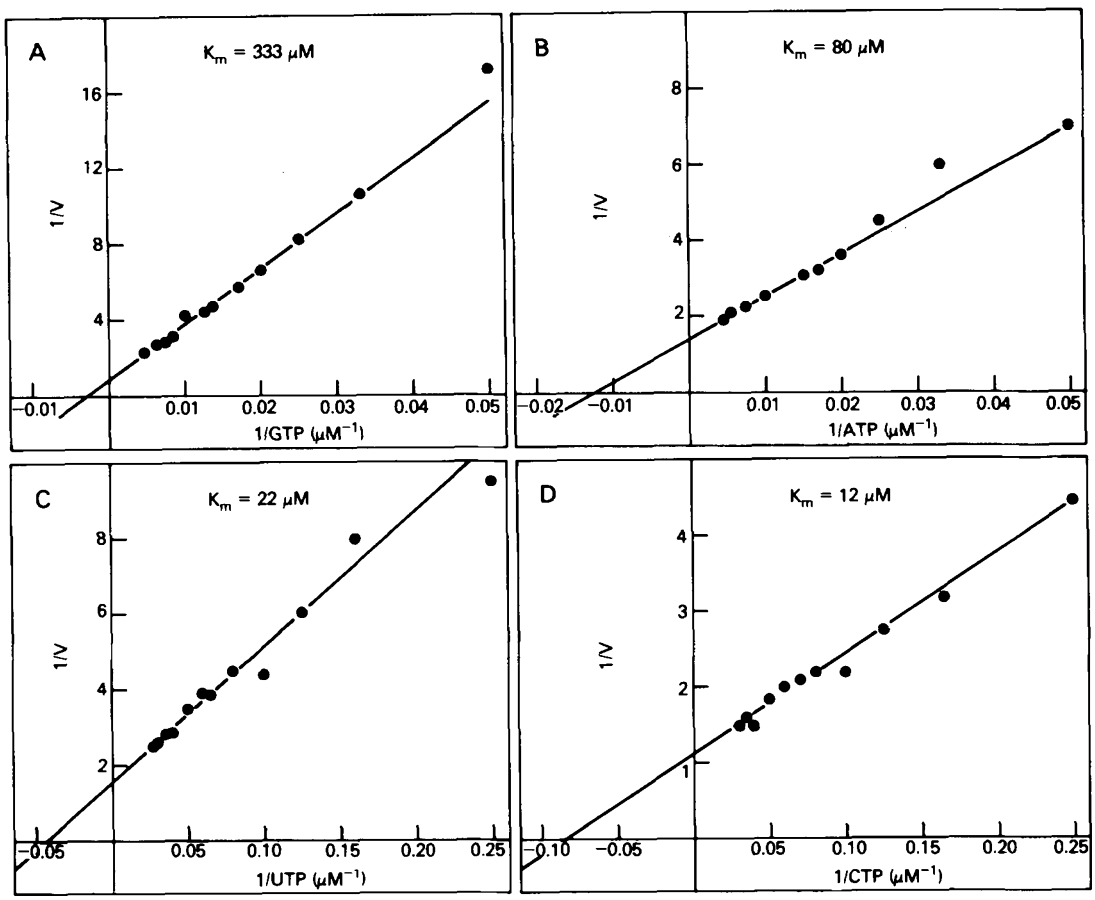

FIG. 9. **Effect of ribonucleoside triphosphate concentration on RNA polymerase activity.** K_m values for GTP (*A*), ATP (*B*), and CTP (*C*) were determined using 0.1 mM [^{3}H]UTP (89 cpm/pmol), while 0.1 mM [^{3}H]CTP (89 cpm/pmol) was used to determine the K_m for UTP (*D*). The concentrations of the other two ribonucleoside triphosphates that were held constant were 1 mM each. $MnCl_2$ was present at a concentration equimolar to that of the combined ribonucleoside triphosphates. RNA polymerase activity was measured after 10-min incubations at 37°C. All reactions were done in triplicate.

and 12 μM were obtained for GTP, ATP, UTP, and CTP, respectively.

Template Specificity—Denatured calf thymus DNA was used in our standard assay. Maximal RNA polymerase activity was obtained with about 15 μg/ml of this template (Fig. 8). Other DNAs and synthetic polydeoxyribonucleotides were also tested. Three different types of DNA were examined: linear double-stranded DNA (calf thymus, vaccinia virus, T4 phage, adenovirus, and λ phage), double-stranded circular superhelical DNA (pBR322 plasmid and simian virus 40 virus), and single-stranded circular DNA (f1 phage). Results, summarized in Table IV, indicate that, among the DNAs in their native forms, only single-stranded f1 DNA was transcribed effectively. Linear double-stranded DNAs, including λ which has short terminal single-stranded segments (18) and superhelical DNAs which have short internal single-stranded regions (19, 20), were poor templates. However, upon denaturation, all DNAs tested promoted RNA synthesis. Indeed, under present conditions, there was relatively little difference in the relative template activities of the different DNAs.

The size of RNA products synthesized on single-stranded calf thymus DNA was approximately 8 S. Transcription was demonstrated by using vaccinia virus DNA as a template and hybridizing the ^{32}P-labeled RNA to vaccinia DNA restriction fragments immobilized on a nitrocellulose membrane (not shown).

A variety of synthetic polymers were also tested (Table V). Of the four homopolydeoxyribonucleotides, only poly(dC) and poly(dT) served as templates, and the former was about 6 times better than the latter. The alternating copolymer, poly(dA-dT), was ineffective, presumably because of its extensive base-pairing. All of the random copolymers, however, including poly(dA,dT), poly(dA,dC), poly(dC,dT), and poly(dC,dG), served as templates. Of these, poly(dC,dT) was by far the best.

TABLE IV

DNA template dependence of vaccinia virus RNA polymerase

DNA template	UMP incorporated	
	Native	Denatured[a]
	pmol	*pmol*
None	0	
Calf thymus	13.0	140
Vaccinia virus	2.4	112
T4	0.6	48
Adenovirus	1.4	144
λ	0.3	121
Simian virus 40	0.5	113
f1	290.0	
pBR322	5.0	68

[a] Denatured forms of DNAs were obtained by heating in 10 mM Tris-HCl (pH 7.9), 1 mM EDTA for 10 min at 100°C and rapidly cooling the reaction mixture.

Inhibitors of RNA Polymerase—It was of interest to test the effects of known inhibitors of prokaryotic and eukaryotic RNA polymerases on vaccinia virus RNA polymerase activity. Neither rifampicin, a specific inhibitor of prokaryotic RNA polymerase (21), nor α-amanitin, a specific inhibitor of eukar-

TABLE V
Polydeoxyribonucleotides as templates

Template[a]	Nucleotide incorporated	Amount incorporated
		pmol
Poly(dT)	AMP	108
Poly(dC)	GMP	604
Poly(dG)	CMP	0
Poly(dA)	UMP	1
Poly(dA,dC)	GMP	82
	UMP	107
Poly(dC,dT)	AMP	460
	GMP	897
Poly(dC,dG)	GMP	98
	CMP	48
Poly(dA,dT)	AMP	39
	UMP	39
Poly(dA-dT)	AMP	0
	UMP	0

[a] All templates were present at 2.2 μg/0.1 ml. Poly(dA-dT) is an alternating copolymer, whereas poly(dA,dT) and the other copolymers are random.

TABLE VI
Inhibition of vaccinia virus RNA polymerase

Compound[a]	Concentration	RNA polymerase activity[b]
	μg/ml	%
Heparin	10	4
Aurintricarboxylic acid	0.05	8
Poly(L-glutamic acid)	200	31
Actinomycin D	5	26
α-Amanitin	250	100
Rifampicin	150	121
Isatin-β-thiosemicarbazone	15	66

[a] Approximately 6.5 units of RNA polymerase was incubated with each compound in 36 μl of 140 mM Tris-HCl (pH 7.9) for 10 min at 0°C. At the end of this time, the remainder of the polymerase assay mixture was added and the incubation was continued at 37°C for 30 min. In the case of actinomycin D, the enzyme was added last.

[b] Activity is expressed as percentage of that without inhibitor but with appropriate low concentration of ethanol in the case of actinomycin D, dimethyl sulfoxide in the case of rifampicin, and acetone in the presence of isatin-β-thiosemicarbazone.

yotic RNA polymerases (22), interfered with vaccinia virus RNA polymerase activity (Table VI). By contrast, polyanions such as heparin, aurintricarboxylic acid, and poly(L-glutamic acid), which are more general inhibitors of RNA polymerases, were potent inhibitors of the viral enzyme.

The failure of rifampicin to inhibit vaccinia virus RNA polymerase was of particular interest since this compound prevents vaccinia virus growth (23, 24). Inhibition occurs at a specific stage in assembly, however, and does not prevent synthesis of early or late mRNA (25, 26).

Another specific inhibitor of vaccinia virus growth, isatin-β-thiosemicarbazone (27), had only a small effect on RNA polymerase activity (Table VI). *In vivo*, this drug inhibits late protein synthesis and decreases the stability of late mRNA but has little or no effect on the synthesis of early or late mRNA species (28, 29).

DISCUSSION

A DNA-dependent RNA polymerase that is resistant to rifampicin and α-amanitin has been isolated from vaccinia virus particles. Previous difficulties in extracting this enzyme appear to have been due to multiple factors, including its instability and apparent change in divalent cation and template requirements after solubilization. Thus, the core-associated complex transcribes the endogenous double-stranded DNA genome optimally in the presence of Mg^{2+} but the soluble enzyme requires a single-stranded template and Mn^{2+}. These altered requirements were apparent in crude soluble virus extracts and were not a result of extensive enzyme purification. Thus, if a protein analogous to the σ factor of *E. coli* RNA polymerase was dissociated from the viral enzyme, it must have occurred during virus disruption. Although alteration of the RNA polymerase might have resulted from use of sodium deoxycholate, similar results were obtained with other detergents.[2] Moreover, Nevins and Joklik (13) reported similar properties for RNA polymerase isolated without detergents from vaccinia virus-infected cells. An inability to transcribe double-stranded DNA efficiently is also a frequent characteristic of purified eukaryotic RNA polymerases. Nevertheless, the latter enzymes exhibit considerable activity with circular supercoiled DNA because of its short single-stranded regions (22). By contrast, the vaccinia virus RNA polymerase has very low activity with supercoiled viral and plasmid DNAs, suggesting that it might not be able to separate strands for elongation. Assuming that the enzyme we have isolated is the native transcriptase and that the natural template is double-stranded DNA, the possibility that specific DNA unwinding proteins might be involved in initiation and elongation steps of transcription is suggested. In this regard, either of the two DNA-dependent nucleoside triphosphate phosphohydrolases previously isolated in our laboratory from vaccinia virus cores (6, 30) are good candidates since unwinding proteins from *E. coli* (31) and bacteriophage T4 (32) catalyze a similar DNA-dependent hydrolysis of ATP. That virus cores have a special ATP requirement for transcription is revealed both by a need for high ATP concentrations and specific inhibition of transcription by ATP analogs with nonhydrolyzable γ-phosphates (33, 34). To what degree this ATP requirement reflects the activity of the DNA-dependent nucleoside triphosphate phosphohydrolases (6, 30), the protein kinase (7), or still other unidentified enzymes present in vaccinia virus cores is not known.

Although purified RNA polymerase was able to transcribe a variety of single-stranded DNAs, some selectivity was shown using synthetic polydeoxyribonucleotides. Of the four homopolydeoxyribonucleotides, only poly(dC) and poly(dT) served as templates. Additionally, all random copolymers that contained either dC or dT were transcribed. Failure to use poly(dA) or poly(dG) as templates suggests that initiation does not occur with a pyrimidine ribonucleoside triphosphate. Specific use of purine ribonucleoside triphosphates for initiation is also consistent with the higher K_m values found for GTP and ATP compared to CTP and UTP using denatured calf thymus DNA as template and with our preliminary analysis of the 5′ ends of such transcripts labeled with the four α-^{32}P-labeled ribonucleoside triphosphates.[2] Moreover, mRNA synthesized *in vitro* by vaccinia virus cores and *in vivo* at both early and late times contains only adenosine and guanosine capped 5′ ends (35, 36).

The purified vaccinia virus RNA polymerase appeared homogeneous, by two criteria: co-sedimentation of enzyme activity with [^{35}S]methionine-labeled protein on glycerol gradients, and detection of a single Coomassie brilliant blue-stained band corresponding to a single [^{35}S]methionine-labeled protein on nondenaturing polyacrylamide gel electrophoresis. That the multiple polypeptides resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis are intimately associated with the RNA polymerase was demonstrated by analysis of glycerol gradient fractions and by two-dimensional gel electrophoresis. To determine accurately the molecular weights

[2] B. M. Baroudy, unpublished observation.

of these polypeptides, both 5 and 12.5% polyacrylamide gels were used. With *E. coli* RNA polymerase subunits as standards, the high molecular weight polypeptides associated with the vaccinia virus RNA polymerase were estimated to be 140,000 and 137,000. The lower molecular weight polypeptides were estimated to be 37,000, 35,000, 31,000, 22,000, 21,000, and 17,000. Molar ratios were determined from the amounts of dye adsorbed to each of the polypeptides. As previously discussed (37), this method is subject to considerable error. Nevertheless, the results indicated that the 140,000-, 137,000-, 37,000-, and 31,000-dalton polypeptides were present in molar ratios of 1, whereas the 35,000- and 22,000-dalton polypeptides were present in molar ratios of 2 and 4, respectively. The precision of the measurements was not adequate to determine whether the 17,000-dalton polypeptide was 1 or 2 M; in addition, the 21,000-dalton polypeptide appeared to be submolar. A molecular weight of 517,000 for the RNA polymerase was calculated from the polypeptide composition, in excellent agreement with the value of 500,000 for the native enzyme estimated by glycerol gradient centrifugation. Whether each of the polypeptides is a true subunit required for catalytic activity, however, can only be ascertained from dissociation-reassociation experiments or through the isolation and characterization of appropriate mutants.

The large size and general structure of the vaccinia virus RNA polymerase is similar to that of *E. coli* RNA polymerase (17) and eukaryotic RNA polymerases (22). Like the other RNA polymerases, the viral enzyme contains two subunits of high molecular weight with molar ratios of 1, and several smaller subunits, some of which appear to have molar ratios greater than 1. However, the large viral subunits appear to be somewhat smaller than those of eukaryotic enzymes (22). Since host protein synthesis is rapidly and effectively inhibited (38), the labeling with [^{35}S]methionine during virus growth of all RNA polymerase subunits suggests that they are virus-coded. Nevertheless, direct proof for viral coding is lacking.

Nevins and Joklik (13) reported that the subunit composition of the viral RNA polymerase isolated from infected HeLa cells is completely different from that of HeLa cell RNA polymerases I and II. The infected cell polymerase contains molar subunits of 135,000, 130,000, 77,000, 34,000, 19,500, 16,500, and 13,500 daltons (13). Upon comparison of the RNA polymerases isolated from vaccinia virions and infected cells, the major difference appears to be the absence of a 77,000-dalton polypeptide in the former. In view of the difficulties in assigning accurate molecular weights to polypeptides of M_r > 100,000, we attribute no significance to the small differences in molecular weights of the largest subunits in virion and infected cell RNA polymerases. Although Nevins and Joklik (13) reported only 1 molar polypeptide of 34,000 similar to our polypeptide of 35,000, inspection of photographs and densitometer tracings of their gels reveal additional slightly larger and smaller submolar polypeptides which could correspond to our 37,000- and 32,000-dalton polypeptides. The 22,000- and 17,000-dalton polypeptides in virion RNA polymerase may correspond to the 19,500- and 16,500-dalton polypeptides of infected cell RNA polymerase. In addition, we have noted a diffuse staining band of about 12,000 daltons upon analysis of the virion RNA polymerase which could correspond to the 13,500-dalton polypeptide of Nevins and Joklik (13). Thus, except for the presence of the 77,000-dalton polypeptide exclusively in the infected cell RNA polymerase, the subunit compositions of the two enzymes appear quite similar. Although the possibilities that the 77,000-dalton polypeptide was dissociated from the virion polymerase by the deoxycholate procedure or that it nonspecifically co-eluted with the infected cell polymerase must be considered, a more interesting alternative is that this polypeptide is specifically involved in transcription of "late" genes. We suggest this idea because the nonparticulate RNA polymerase was isolated from cells at 12 h after vaccinia virus infection (13).

In conclusion, a multisubunit enzyme from vaccinia virions which retains the essential catalytic activity for transcription of DNA has been purified. Whether this RNA polymerase can be persuaded to utilize double-stranded DNA templates, to substitute Mg^{2+} for Mn^{2+}, and most importantly to initiate synthesis at correct promoter sites remains to be determined.

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