Isolation and Properties of the Vaccinia Virus DNA-dependent RNA Polymerase*

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A vaccinia virus-specified DNA-dependent RNA polymerase was isolated from the cytoplasm of infected HeLa cells. The enzyme appeared in cells only after infection; it differed in its chromatographic properties from all cellular RNA polymerases; and its subunit composition (seven subunits ranging in size from 13,300 to 135,000 daltons) was completely different from that of HeLa cell RNA polymerases I and II. The latter two enzymes were also isolated and their subunit compositions were found to resemble closely those of mouse plasmacytoma MOPC 315 RNA polymerases I and II. The three largest subunits of the isolated vaccinia virus-specified RNA polymerase migrated in sodium dodecyl sulfate-polyacrylamide gels at the same rate as virion core polypeptides VP1c, VP1d, and VP2c; the enzyme may therefore be the same as the vaccinia virus core-associated RNA polymerase.

The purified enzyme was free of detectable exonuclease and poly(A) polymerase activity. It was maximally stimulated by 0.08 M KCl and 0.001 M MnCl2; Mg2+ could not substitute for Mn2+ to a significant degree. The enzyme was very resistant to α-amanitin. It was 10 times more active with denatured calf thymus or vaccinia virus DNA as template than with the corresponding native DNAs, but transcribed the synthetic double-stranded poly(d(A-T)) almost as rapidly as single-stranded natural DNA. It transcribed all sequences of native vaccinia virus DNA.

Vaccinia virus was the first virus shown to possess a nucleic acid polymerase: Kates and McAuslan reported in 1967 that vaccinia virus cores supplied with the four ribonucleoside triphosphates were capable of transcribing portions of the viral genome within them into RNA (1). Subsequent work showed that this RNA can be transcribed in cell-free protein-synthesizing systems, that is, that it is messenger RNA (2-4), is both capped and methylated at its 5' end (5), and is polyadenylated at its 3' end (6).

The RNA polymerase present in vaccinia virus cores has not so far been isolated. This is in contrast to the several other enzymes that have been isolated from vaccinia virus, such as two nucleoside phosphohydrolases (7, 8), one or two deoxyribonucleases (9, 10), a protein kinase (11), a poly(A) polymerase (12, 13), a RNA guanylyltransferase, and a RNA (guanine-7-methyltransferase (14). The vaccinia virus RNA polymerase is not unique in its refractoriness to being extracted in active form from virions: the RNA polymerases of reovirus, orthomyxoviruses, paramyxoviruses, and rhabdoviruses likewise have not yet been isolated in pure form from virions, and the only nucleic acid polymerase for which this has been accomplished so far is the DNA polymerase of RNA tumor viruses.

In seeking an alternate source of the vaccinia virus-specific RNA polymerase, we turned to extracts of infected HeLa cells. Cytoplasmic extracts of such cells have been demonstrated to contain DNA-protein complexes capable of synthesizing both early and late viral mRNA, but no soluble DNA polymerase (15). We found, however, that such extracts do indeed contain a soluble RNA polymerase activity that is not present in uninfected cells, and the isolation of this enzyme forms the substance of this paper. Like HeLa cell RNA polymerases I and II, which we also purified, the vaccinia virus RNA polymerase is a complex structure consisting of numerous subunits, none of which it shares with the cellular RNA polymerases. Further, at least three of its subunits were identified with three virion structural proteins, indicating that the RNA polymerase isolated from infected cells is identical with the vaccinia virus core-associated enzyme.

In the following paper we show that HeLa cells infected with vaccinia virus contain several poly(A) polymerases, that one is present only in infected cells, and that this enzyme is identical with the poly(A) polymerase isolated from vaccinia virus cores (12, 15). This is another example of a virion-associated nucleic acid polymerase that exists in the free form within infected cells in amounts high enough to permit purification, isolation, and characterization. The purification of RNA polymerases and poly(A) polymerases, both cell-coded and virus-coded, from cells infected with vaccinia virus is part of a wider project aimed at elucidating the mechanism by which the 3' terminus of messenger RNA molecules is polyadenylated.

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**EXPERIMENTAL PROCEDURES**

**Cells and Virus**

HeLa S3 cells and mouse strain L fibroblasts were grown in suspension culture in Eagle's Minimal Essential Medium (Joklik's modification, Grand Island Biologicals) containing 5% fetal calf serum. Vaccinia virus strain WR was grown and purified as follows. L cells, at a concentration of 1 x 10^9/ml, were infected by adding 150 virus particles per cell (in the form of highly purified virus suspensions). After incubating at 37° for 20 h, the cells were placed at 4° and allowed to settle overnight. Most of the medium was removed by suction and the remaining cells were pelleted by centrifuging at 10,000 x g for 5 min. They were washed twice with Earle's salt solution (16) and then stored frozen at -20°. For virus purification, the frozen cell pellets were thawed, resuspended in 10 mM NaHPO₄, and centrifuged at 24,000 rpm for 45 min at 4° in an SW27 rotor. The pellets were resuspended in 1 mM NaHPO₄, layered onto a 25 to 40% sucrose (w/w in 1 mM Na₂HPO₄ density gradient, and centrifuged at 4° for 40 min at 15,000 rpm in an SW27 rotor. Virus bands were collected, diluted 1:1 with 1 mM NaHPO₄, and centrifuged at 20,000 rpm for 30 min. The virus pellets were resuspended in 1 mM NaHPO₄, reband in sucrose density gradients, and virus was again collected as described above. The pellets were resuspended in 1 mM NaHPO₄, and the A_m was measured. One A_m was taken to be equivalent to 1.2 x 10^9 virus particles or 64 µg of virus protein (17). Purified virus was stored frozen at -20°.

**Preparation of Cell Extracts**

HeLa cells were grown to a density of 1 x 10^9 cells/ml, usually in batches of 6 liters. Purified vaccinia virus was then added (440 virus particles/cell) and the culture was incubated at 37° for 20 h; this sample was used for the preparation of early vaccinia mRNA. The remainder of the infected cells were incubated at 37° for 2 h; this sample was used for the preparation of late vaccinia mRNA.

**Extracts of Whole HeLa Cells**—These extracts, infected or uninfected, were prepared as follows: all operations were carried out at 0-4°. Washed cells were suspended in 0.15 M NaCl, 0.05 M Tris/HCl (pH 7.9), at a concentration of 5 x 10^9 cells/ml and homogenized in a Dounce homogenizer. EDTA, dithiothreitol, glycerol, and ammonium sulfate were then added to give final concentrations of 1 mM, 15% (w/w), and 0.3 M, respectively; the final Tris/HCl concentration was 0.50 M, pH 7.9. The homogenates were sonicated four times for 15 s, each time with a Branson sonicator model W140D at a defined as the incorporation of 1 pmol of UMP into RNA in 30 min

**Cytoplastic Extracts**—For the preparation of cytoplastic extracts, freshly harvested washed cells were resuspended in 10 mM Tris/HCl, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, and 50 µg/ml actinomycin D at a concentration of 5 x 10^9 cells/ml, permitted to swell for 5 min, and ruptured with five strokes of a Dounce homogenizer. Nuclei were pelleted by centrifuging at 1,000 x g for 10 min and washed once with RSB. The combined cytoplastic fractions were adjusted to contain 15% (w/v) glycerol, 50 mM KCl, 50 mM Tris (pH 7.9), 1 mM EDTA, and 1 mM dithiothreitol. These were then centrifuged at 140,000 x g for 2 h, and the supernatants were dialyzed against two changes of 10 volumes of 0.05 M Tris/HCl, 1 mM EDTA, 1 mM dithiothreitol, and 15% (w/v) glycerol, pH 7.9 (Buffer A), containing 0.05 mM KCl. A heavy precipitate which formed during dialysis was removed by centrifugation at 140,000 x g for 1 h and the resulting supernatant was used immediately for enzyme isolation or stored at -80°.

**Enzyme Purification**

The first purification step involved the use of DEAE-Sephadex. For batches of 6 x 10^9 cells, 4 x 35 cm columns, equilibrated with Buffer A containing 0.5 mM KCl, were employed. The 140,000 x g supernatants were applied at a flow rate of 80 ml/h. The columns were washed with 500 ml of Buffer A containing 0.1 M KCl and developed with 3,000-m1 linear gradients of 0.1 to 0.5 M KCl in Buffer A. Fractions of 20 ml were collected. Enzyme assays were carried out on 25-µl samples of alternate fractions.

**Extraction of RNA**

**Preparation of RNA Polymerase**

Cloned vaccinia virus was pelleted and resuspended in 0.02 M Tris/HCl (pH 8.0), 2 mM EDTA at a concentration of 5 mg/ml. SDS1 and proteinase K were then added to give final concentrations of 0.5% and 1 mg/ml, respectively, and the mixture was incubated at 37° for 2 h. By this time the suspension had become completely clear. The DNA was then gently extracted twice with phenol (saturated with 0.1 M NaCl), 10 mM Tris/HCl, 5 mM EDTA, pH 7.5 (0.1 M STE) at room temperature. The final aqueous phase was carefully overlaid with 2 volumes of cold ethanol and the DNA precipitate was collected by spoiling onto a glass rod. The DNA was washed with 70% ethanol, dried, and dissolved overnight in 20 mM Tris/HCl (pH 8.0) containing 1 mM EDTA.

**Preparation of Early and Late Vaccinia Virus mRNA**

HeLa cells (1 x 10^9) were infected with 400 virus particles per cell as described before (19). One-half of the infected cells were then incubated at 37° in the presence of 5 x 10^-5 M cytosine arabinoside for 2 h; this sample was used for the preparation of early vaccinia mRNA. The remainder of the infected cells were incubated at 37° for 5 h and used for the preparation of late viral mRNA. Both cultures were centrifuged, washed twice with Earle's saline, and cytoplastic extracts were prepared from each by homogenizing with a Dounce homogenizer and extracting the RNA by means of the high-salt SDS procedure of Warner et al. (20). After precipitating the RNA with ethanol, it was dissolved in 0.1 mM EDTA.

**Hybridization**

Vaccinia virus RNA was hybridized to vaccinia virus DNA on filter disks essentially as described by Gillespie and Spiegelman (21). In brief, DNA (20 µg/ml) was denatured by heating to 100° for 10 min followed by quick cooling in an alcohol/ice bath. The solution was then diluted 40-fold with 0.06 M NaCl, 0.06 M sodium citrate, pH 7.4 (4 x SSC) and slowly filtered through presoaked 8.5-mm diameter Millipore filters. The filters were washed with 50 ml of 4 x SSC and then allowed to dry at room temperature, followed by heating

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1 The abbreviation used is: SDS, sodium dodecyl sulfate.
Vaccinia Virus RNA Polymerase

for 4 h in vacuo at 80°C. The amount of DNA bound to the filters was determined by including a small amount of "C-labeled vaccinia virus DNA in the DNA solution and measuring radioactivity on the filters after binding.

Hybridization was performed at 64°C in 4 x SSC containing 0.1% SDS for 18 h. The discs were then washed with 2 x SSC, incubated with 20 μg/ml pancreatic RNase in 2 x SSC for 60 min at 22°C, and again washed. Radioactivity bound to them was then measured.

Conductivity and Protein Measurements

Salt concentrations were measured using a Radiometer conductivity meter. Protein was measured essentially as described by Lowry et al. (22). Protein in bands in SDS-polyacrylamide gels was measured by staining with Coomassie brilliant blue and comparing their A$_{590}$ with that of bands containing standard amounts of bovine serum albumin.

SDS-Polyacrylamide Gel Electrophoresis

Protein samples were prepared for electrophoresis by precipitating with 10% trichloroacetic acid, and washing once with 5% trichloroacetic acid and twice with acetone. After drying, the precipitates were dissolved in 50 μl of 62.5 mM Tris (pH 6.8), 10% (w/w) glycerol, 1% SDS, 0.7 M 2-mercaptoethanol, and 0.005% bromophenol blue, and heated at 100°C for 2 min. The gel system was that of Laemmli (23) and a slab-gel apparatus similar to that described by Studier (24) was used. Electrophoresis was at 15 mA for 4 h. The gels were stained with a solution containing 0.2% Coomassie brilliant blue, 10% trichloroacetic acid, and 25% isopropanol for 4 to 16 h at 25°C. They were then destained in 7% acetic acid at 37°C. Densitometric profiles of gels were obtained using a Quick Scan (Helena Instruments) interfaced with a Digital PDP-11 computer.

Preparation of Ion Exchange Resins

DEAE-Sephadex (A-25) was swollen in Buffer A containing 0.05 M KCl. The buffer was changed repeatedly during the swelling procedure. After packing, the columns were washed further with the same buffer until the pH of the effluent was 7.9.

Phosphocellulose (Whatman P-11) was washed with 0.5 M NaOH, followed by 0.5 M HCl. After washing with water, it was suspended in Buffer A and titrated to pH 7.9 with Tris base.

Materials

Unlabeled nucleotides and calf thymus DNA were purchased from Sigma, [3H]UTP from New England Nuclear, α-amanitin from Calbiochem, and poly[d(A-T)] from Miles Corp.

RESULTS

Demonstration of a "Soluble" RNA Polymerase in Cytoplasm of HeLa Cells Infected with Vaccinia Virus

Mammalian cells contain three readily detectable RNA polymerases, I, II, and III, identified by their elution properties from DEAE-Sephadex (25) and their sensitivity to α-amanitin (26, 27). Most of these enzymes are located in the nucleus after infection.

HeLa cells were infected with vaccinia virus at an added multiplicity of 400 virus particles per cell and at 0, 4/2, and 10 h thereafter cells were harvested, the cytoplasmic fractions prepared as described under "Experimental Procedures" and centrifuged at 100,000 x g for 60 min. RNA polymerase activity was assayed in the pellets (after addition of NP40) and in the supernatant ("soluble") fraction, with and without the addition of denatured calf thymus DNA as template. The results are shown in Table I. Cytoplasmic fractions from uninfected HeLa cells possessed very little activity, either soluble or particulate. By contrast, the level of RNA polymerase in the pellet fraction of infected cell extracts had increased sharply by 4/2 h after infection (that is, 30 to 60 min after the time when the first mature virus particles had been formed) and increased another 3-fold between then and 10 h. Added calf thymus DNA did not increase the level of this activity, indicating that it represented "packaged" enzyme present in immature or mature vaccinia virus particles. There was no detectable increase in RNA polymerase activity in the soluble fraction in the absence of added DNA; but when the assay was performed in the presence of added denatured calf thymus DNA, a significant increase in activity was demonstrated by 4/2 h after infection, and there was a further small rise by 10 h. It was this enzyme, apparently both virus-specified and free, that we wished to purify and compare with the host-specified RNA polymerases.

Purification of Vaccinia Virus-specified RNA Polymerase

Chromatography on DEAE-cellulose

The starting material for the purification of the vaccinia virus RNA polymerase was the cytoplasmic fraction of infected HeLa cells. Extracts of whole uninfected cells were used for comparison so as to eliminate the possibility of a "new" enzyme in the cytoplasmic fraction being a host enzyme that was normally located in the nucleus.

Fig. 1A shows the DEAE-Sephadex elution profile of RNA polymerase activity in whole uninfected HeLa cell extracts. Two major peaks of activity, the second with a shoulder, were obtained. The activity in the first peak, which eluted at 0.2 M KCl, was completely resistant to 0.5 μg/ml α-amanitin; it corresponded to RNA polymerase I. The activity in the second peak, which eluted at about 0.3 M KCl, was about 60% sensitive to 0.5 μg/ml α-amanitin, and represented a mixture of RNA polymerases II and III. Schwartz et al. (27) also found that these two enzymes cochromatographed on DEAE-Sephadex.

The DEAE-Sephadex elution profile of RNA polymerase activity in the cytoplasm of vaccinia virus infected HeLa cells is shown in Fig. 1B. Most of the activity eluted at a lower KCl concentration (0.16 to 0.18 M) than the cellular enzymes.

<table>
<thead>
<tr>
<th>Time after infection (h)</th>
<th>Pellet RNA polymerase activity (cpm)</th>
<th>Supernatant RNA polymerase activity (cpm)</th>
</tr>
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<tbody>
<tr>
<td>0 (uninfected)</td>
<td>33</td>
<td>60</td>
</tr>
<tr>
<td>4.5</td>
<td>1287</td>
<td>55</td>
</tr>
<tr>
<td>10</td>
<td>4151</td>
<td>56</td>
</tr>
</tbody>
</table>
Two minor peaks that eluted at about 0.2 and 0.3 M KCl, respectively, were also found; both were resistant to 0.5 μg/ml α-amanitin. They represented cellular RNA polymerases I and III, respectively; no cellular RNA polymerase II was present in the cytoplasmic fraction of infected cells.

The major RNA polymerase peak in the cytoplasm of infected HeLa cells clearly represented a new and presumably virus-specified enzyme, and further work was aimed at isolating it.

An interesting feature of the vaccinia RNA polymerase was that it occasionally eluted as a double peak (see Fig. 1B). Sometimes the shoulder resolved into a second peak, smaller than the first. However, when purified as described below, the enzymes derived from both peaks showed the same sedimentation characteristics, were identical in subunit composition as judged by SDS-polyacrylamide gel electrophoresis, possessed identical specific activities, and were indistinguishable in all other properties that were investigated (see below).

**Chromatography on Phosphocellulose**

The enzyme-containing fractions eluted from DEAE-Sepharose were combined, diluted to reduce the KCl concentration to 0.1 M, and applied to a phosphocellulose column which was eluted as described under "Experimental Procedures." Fig. 2 shows that a single peak of RNA polymerase activity resulted.

**Centrifugation in Glycerol Density Gradients**

The enzyme-containing fractions were concentrated by ammonium sulfate precipitation and sedimented into density gradients of 15 to 35% (w/w) glycerol (see "Experimental Procedures"). The enzyme sedimented as a sharp peak (Fig. 3A) at about the same rate as HeLa cell RNA polymerase I (Fig. 3B) and slightly more slowly than *Escherichia coli* RNA polymerase (Fig. 3B) ($M_r = 488,000$ (34, 35)).

**Purification of HeLa Cell RNA Polymerases I and II**

HeLa cell RNA polymerases I and II were purified by the same steps as vaccinia virus RNA polymerase. HeLa cell RNA polymerase III was present in amounts too low to permit isolation; however it was purified sufficiently to permit the conclusion that none of its component subunits had the same electrophoretic mobility as any of the subunits of the vaccinia virus RNA polymerase.

**Summary of Purification Procedures**

Table II summarizes the purification of the vaccinia virus-
specified RNA polymerase. The yield of enzyme was low, most probably because it was quite labile. This lability may explain why it has so far been impossible to isolate it from virus particles. The overall purification factor was 167. The specific activity of the purified enzyme with denatured DNA as template was 1.78 x 10⁶ units/mg of protein. The specific activity of the purified enzyme with native DNA as template was far lower; the single-stranded/double-stranded DNA activity ratio was 18. The activity ratio was some 6 times lower than that of the enzyme in the original cell extracts; this may indicate either the loss during purification of a factor facilitating transcription of native DNA, or the removal of endonucleases which, by nicking, could provide additional sites for transcription initiation. After the glycerol density gradient purification step, the viral RNA polymerase contained no detectable amounts of exonucleases for RNA or DNA, poly A polymerase, and polynucleotide phosphorylase.

Subunit Composition of Vaccinia Virus-specified RNA Polymerase and of HeLa Cell RNA Polymerases I and II

The subunit composition of all three purified enzymes was examined in 10% polyacrylamide-SDS slab gels. The results are shown in Fig. 4. The virus-specified enzyme profile revealed six major bands, the largest of which actually contained two polypeptides of very similar size (see below, Fig. 6). The reasons for postulating that the seven polypeptides were the component subunits of the viral RNA polymerases are: (a) all were present in equimolar amounts (Fig. 5 and Table III); (b) all were present in the same relative proportions during the final two stages of purification (chromatography on phosphocellulose and sedimentation in glycerol density gradients); and (c) their summed molecular masses totalled 425,000 daltons (Table III), a value compatible with that postulated on the basis of the sedimentation analysis presented in Fig. 3.

The purified vaccinia virus RNA polymerase also contained several polypeptides that were present in far lower than equimolar amounts, especially in the size range above 135,000 daltons. These polypeptides were judged to be impurities. Similar large and difficult to remove impurities have been reported in purified preparations of cellular polymerases.

The subunit compositions of HeLa cell RNA polymerases I and II and the sizes of their subunits, based upon the gels shown in Fig. 4, are given in Table IV. All polypeptides present in approximately equimolar amounts were considered to be enzyme subunits; in addition several polypeptides that showed significant departures from equimolar ratios were

![Fig. 5. Densitometer scans of the electrophoretograms of vaccinia virus RNA polymerase subunits shown in Figs. 4 and 6 (inset). The direction of electrophoresis was from left to right. The gels were stained with Coomassie brilliant blue and scanned at 550 nm using a Quick Scan (Helena Laboratories). The profiles were analyzed using a Digital PDP-11 computer.](http://www.jbc.org/)

<table>
<thead>
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<th>TABLE III</th>
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<tr>
<td>Molar ratios and molecular weights of subunits of vaccinia virus RNA polymerase</td>
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<td>Subunit</td>
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<td>---------</td>
</tr>
<tr>
<td>a</td>
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<tr>
<td>b</td>
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<td>f</td>
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<td>g</td>
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![Fig. 4. SDS-polyacrylamide gel electrophoretograms of vaccinia virus RNA polymerase, HeLa cell RNA polymerases I and II, and Escherichia coli RNA polymerase (holoenzyme). Ten to twenty micrograms of each enzyme was used. For details, see “Experimental Procedures.” The direction of electrophoresis was from top to bottom. A, vaccinia virus RNA polymerase; B and C, HeLa cell RNA polymerases I and II, respectively; D, E. coli RNA polymerase.](http://www.jbc.org/)

![TABLE IV](http://www.jbc.org/)

<table>
<thead>
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<th>TABLE IV</th>
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<tr>
<td>Sizes of putative subunits of HeLa cell RNA polymerases I and II</td>
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<tr>
<td>Subunit</td>
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</tr>
<tr>
<td>a</td>
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<td>j</td>
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<td>k</td>
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</table>
also considered to be enzyme components since they were present in relatively large amounts and could not be removed by further purification procedures. These were subunit g of RNA polymerase I and subunits e and i of RNA polymerase II which were present in significantly higher amounts, and subunits b and c of polymerase II which were present in significantly smaller amounts. On this basis, HeLa cell polymerases I and II comprised 8 and 11 subunits, respectively, that ranged in size from 22,000 to over 200,000 daltons. These subunit complements were very similar to those reported for RNA polymerases I and II from calf thymus (36) and the murine plasmacytoma MOPC 315 (37-39).

It is unlikely that all polypeptides listed in Table IV are components of all HeLa cell RNA polymerase I and II molecules, for their aggregate molecular masses are 552,000 and 1,012,000 daltons, respectively, which is in excess of that expected from the enzymes' sedimentation rates (see Fig. 3). It is more likely that both enzymes exist in more than one form which share many, but not all, of the subunits. Evidence that this is true for the RNA polymerases of calf thymus, plasmacytoma, Xenopus, etc., has already been presented (36, 37). It is also interesting that some of the subunits of HeLa cell RNA polymerases I and II may be common to both enzymes, as is readily appreciated by inspecting the gels shown in Fig. 4. Irrespective of which of these subunits are actually identical, it is clear from Fig. 4 that none of the subunits of HeLa cell RNA polymerases I and II are the same as any of the subunits of the vaccinia virus RNA polymerase.

HeLa cell RNA polymerase III was not present in HeLa cells in amounts sufficiently large to permit isolation. However, the polypeptide composition of the purest enzyme preparation that could be obtained revealed no subunits that electrophoresed at the same rate as any of the seven polypeptides of the vaccinia virus RNA polymerase.

Comparison of Vaccinia Virus RNA Polymerase Subunits and Vaccinia Virus Structural Polypeptides

Vaccinia virus cores possess RNA polymerase activity. It was therefore of interest to determine whether the subunits of the vaccinia virus RNA polymerase that had been purified from the cytoplasm of infected cells could be identified among the virion structural polypeptides. Fig. 6 shows that this was indeed the case. In order to resolve enzyme subunits a and b, only one-fifth of the amount of enzyme protein was used as in the analysis shown in Fig. 4 and the polyacrylamide concentration was lowered to 7.5%. The three largest enzyme subunits corresponded exactly to virion structural polypeptides: subunit a corresponded to VP1c, subunit b to VP1d, and subunit c to VP2c. Further, these three polypeptides were present in equimolar amounts not only in the enzyme, but also in the virus. Comparison of the smaller enzyme subunits with virion polypeptides was uncertain owing to the complex nature of the virion polypeptide profile in the region below molecular masses of less than 70,000 daltons.

The average number of vaccinia virus RNA polymerase molecules per virus particle could be calculated assuming that the molecular mass of the viral DNA is 122 x 10^6 daltons (41), that the proportion of DNA in the virus is 5% (17), that the sizes of the subunits a, b, and c are as shown in Table III, and that they comprise 2.5% of the virion protein mass. It turns out to be between 150 and 200. Others have estimated that there are about 100 molecules per virion of poly(A) polymerase (13), phosphohydrolase (7), deoxyribonuclease (9), and guanylyl- and methyltransferases (14).

**Properties of Enzyme Reaction**

**Template Dependence**

Table V shows that the vaccinia virus RNA polymerase was completely dependent for activity on added DNA. Vaccinia and calf thymus DNAs were used about equally efficiently; single-stranded DNA was 20 to 70 times as effective as double-stranded DNA. The enzyme resembled in this respect cellular RNA polymerase II (25); both are engaged in the synthesis of messenger RNA. The synthetic double-stranded polynucleotide poly[d(A-T)] was transcribed about 20 times more efficiently than natural double-stranded DNAs.

**Effect of DNA Concentration on Reaction Rate**

Fig. 7 shows the effect of varying the template concentration on the rate of transcription catalyzed by vaccinia virus RNA polymerase. For both denatured vaccinia virus DNA and denatured calf thymus DNA the $K_m$ values were between 0.5 and 1 μg/ml.

**Fig. 6. Electrophoretograms of (A) vaccinia virus RNA polymerase subunits (600 units, 3.5 μg) and (B) vaccinia virus structural polypeptides (25 μg) in 7.5% acrylamide-SDS slab gels. The direction of electrophoresis was from top to bottom. The virion structural polypeptides are labeled according to Sarov and Joklik (40).**

**Table V**

<table>
<thead>
<tr>
<th>Template dependence of vaccinia virus RNA polymerase</th>
<th>Template$^*$</th>
<th>UMP incorporated pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Calf thymus DNA</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Denatured calf thymus DNA$^*$</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Vaccinia virus DNA</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Denatured vaccinia virus DNA$^*$</td>
<td>50.8</td>
<td></td>
</tr>
<tr>
<td>Poly[d(A-T)]$^*$</td>
<td>16.7</td>
<td></td>
</tr>
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</table>

$^*$ All templates were present at 100 μg/ml.
$^*$ For conditions of denaturation, see "Experimental Procedures."
Vaccinia Virus RNA Polymerase

**Fig. 7 (left).** The effect of DNA concentration on the rate of transcription catalyzed by vaccinia virus RNA polymerase. Standard reaction mixtures were used containing denatured vaccinia virus (○—○) or denatured calf thymus DNA (■—■).

**Fig. 8 (left center).** Time course of RNA synthesis by the vaccinia virus RNA polymerase. About 20 units of enzyme were incubated in 300-μl standard reaction mixtures containing (A) denatured and (B) native calf thymus DNA, each at 100 μg/ml. Samples of 50 μl were removed at the indicated times and trichloroacetic acid-insoluble radioactivity was measured.

**Fig. 9 (right center).** Effect of KCl concentration on the activity of vaccinia virus RNA polymerase. Standard reaction mixtures were used with the indicated concentrations of KCl.

**Fig. 10 (right).** Effect of pH on the activity of vaccinia virus RNA polymerase. Standard reaction mixtures containing 0.05 M Tris/HCl buffers at the indicated pH values were used. The pH values of the buffers were determined at a concentration of 0.5 M at 25°C.

**Fig. 11 (left).** Effect of divalent cation concentration on the activity of vaccinia virus RNA polymerase. Standard reaction mixtures containing either MgCl₂ or MnCl₂ at the indicated concentrations were used.

**Fig. 12 (right).** Sucrose density gradient sedimentation analysis of the RNA transcribed by vaccinia virus RNA polymerase from denatured DNA. About 95 units of enzyme were incubated in 200-μl reaction mixtures (see "Experimental Procedures") at 37°C for 30 min with either denatured calf thymus DNA (A) or denatured vaccinia virus DNA (B), both at saturating concentrations. The mixtures were then made 0.05 M with respect to EDTA and 0.5% with respect to SDS and layered onto 11-ml density gradients of 15 to 30% (w/w) sucrose in 0.01 M Tris/HCl (pH 7.5), 0.1 M NaCl, 0.001 M EDTA, and 0.5% SDS. Centrifugation was at 29,000 rpm in an SW41 rotor at 20°C for 16 1/2 h.

**Time Course of RNA Synthesis**

As shown in Fig. 8, the vaccinia virus polymerase catalyzed the incorporation of UMP into RNA for about 40 min, both with native and with denatured DNA as template.

**Effects of Ionic Strength and pH**

Fig. 9 shows that the activity of vaccinia virus RNA polymerase was dependent upon the presence of KCl; the optimum concentration was 80 mM. Above this concentration, the reaction was inhibited.

The optimum pH for the polymerase reaction was about 8.5 when Tris/HCl was the buffer. This is shown in Fig. 10.

**Divalent Cation Requirements**

The activity of vaccinia virus RNA polymerase was dependent upon the presence of Mn²⁺, which was required in equimolar concentration with the nucleoside triphosphates that were present. The effect of varying the Mn²⁺ concentration on enzyme activity at [NTP] = 1.5 mM is shown in Fig. 11. Mg²⁺ substituted for Mn²⁺ only to a very limited extent.

**Effect of Inhibitors**

**Actinomycin D** — Actinomycin D, which binds to DNA rather than to polymerases, completely inhibited the vaccinia virus RNA polymerase, even at concentrations as low as 2 μg/ml.

**Rifampicin** — Rifampicin inhibits the multiplication of vaccinia virus (42, 43), but fails to inhibit the RNA polymerase in vaccinia virus cores (44, 45). This raises the possibility that it might not be able to penetrate into cores. However, it was found that rifampicin, even at concentrations as high as 200 μg/ml, has no effect on the activity of highly purified vaccinia virus RNA polymerase.

**α-Amanitin** — Even at concentrations as high as 250 μg/ml, α-amanitin had no effect on the activity of the vaccinia virus RNA polymerase. This concentration is sufficient to inhibit completely not only cellular RNA polymerase II, but also cellular RNA polymerase III (27). It is interesting that the cellular enzyme that transcribes messenger RNA is highly sensitive to α-amanitin, whereas the corresponding viral enzyme is highly resistant to it.

**Nature of RNA Transcripts**

**Size** — A sucrose density gradient size analysis of the RNA transcribed from denatured vaccinia virus and calf thymus
Absence of Poly(A) — The transcripts synthesized by the vaccinia virus RNA polymerase contained no poly(A) sequences as demonstrated by the fact that less than 1% of them were retained when passed through poly(U)-cellulose columns.

Nature of Sequences Represented — The proportion of vaccinia virus DNA that was transcribed by the viral RNA polymerase in vitro was determined by measuring the ability of early and late vaccinia messenger RNA to compete in hybridization experiments with transcripts synthesized by the isolated enzyme. It is known that early vaccinia virus messenger RNA (that is, messenger RNA transcribed before vaccinia DNA has begun to replicate, typically before 2½ h after infection) and late vaccinia messenger RNA are transcribed from about 40 and 100% of the viral genome respectively (31). Fig. 13 shows that the RNA polymerase occasionally eluted from DEAE-Sephadex in a trailing fraction. The enzyme was completely prevented from hybridizing to vaccinia virus-infected HeLa cells as described under "Experimental Procedures," where the conditions for hybridization are also outlined.

DNA is shown in Fig. 12. The median sedimentation coefficient of the transcripts (or of transcripts complexed with template) was about 10 S.

The vaccinia virus RNA polymerase is a complex enzyme, with a molecular mass of about 425,000 daltons, and is composed of seven polypeptide subunits. Two of them, a and b, are very large (over 125,000 daltons); one, c, is medium sized (about 77,000 daltons); one, d, is small (about 34,000 daltons); and three, e, f, and g, are very small (13,000 to 19,000 daltons). None of these subunits are components of either HeLa cell RNA polymerase I or II which we also isolated and found to comprise 8 and 11 subunits, respectively. The subunit complement of these very complex enzymes is not easy to define since some subunits apparently dissociate readily causing them to be present in lower than equimolar amounts, while some very large polypeptides that are not enzyme subunits tend to remain associated with the enzyme throughout the purification procedure. In spite of these complications, the subunit complements of the two HeLa cell RNA polymerases, as purified by us, were very similar to those of MOPC 315 RNA polymerases I and II (37) and HeLa cell RNA polymerases I and II (48). The summed molecular masses of the 8 and 11 subunits that we found to be associated with HeLa cell RNA polymerases I and II, respectively, exceeded the estimates of their sizes determined by density gradient analysis, which suggests that these two enzymes may exist in more than one molecular form, with some subunits restricted to one form. This situation has already been described for other RNA polymerases (36, 37). This problem did not arise with the vaccinia virus RNA polymerase, the summed subunit molecular weight of which was consistent with the enzyme's sedimentation behavior.

Like the host cell RNA polymerases, the vaccinia virus RNA polymerase is functionally unstable. No doubt its complexity on the one hand, and its consequent lability on the other, are among the primary reasons why it has so far proved impossible to extract it from the viral core, which is a rather stable structure, not easily dissociated by gentle means.

As extracted from infected cells, the vaccinia virus RNA polymerase occasionally eluted from DEAE-Sephadex in a heterogeneous manner. When the enzymes in the leading and trailing fractions of DEAE-Sephadex eluate peaks were isolated and compared, they were found to be identical in all respects that could be tested, including specific activity and subunit composition as determined by SDS-polyacrylamide gel electrophoresis. The most likely explanation of this phenomenon was that the vaccinia virus RNA polymerase exists in two or more forms that differ in charge. Phosphorylation is a charge modification that could give rise to this type of behavior. Experiments are currently being carried out to determine whether any of the subunits of the vaccinia virus RNA polymerase are phosphorylated.

As for the kinetic properties of the vaccinia virus RNA polymerase, the enzyme was synthesized only after infection, using 50 μCi/ml of α-[3H]UTP and 100 pg/ml native vaccinia virus DNA. The incubation period was 30 min. The transcripts were extracted with phenol, precipitated with ethanol, and dissolved in 4× SSC containing 0.1% SDS. Early and late vaccinia virus messenger RNAs were prepared from vaccinia virus-infected HeLa cells as described under "Experimental Procedures," where the conditions for hybridization are also outlined.
Vaccinia Virus RNA Polymerase

polymerase, it is a typical DNA-dependent RNA polymerase. In its purified form it uses single-stranded DNA as template much more efficiently than double-stranded DNA; however, the synthetic double-stranded polynucleotide polyd(A-T) is transcribed almost as efficiently as single-stranded natural DNAs. In the crude state the enzyme transcribes double-stranded DNA much more efficiently than in the pure state; but the nature of the factor that is responsible for activity on double-stranded DNA has not yet been clarified. One possibility is that it is a nuclease; we have found, in this respect, that, during the purification of the enzyme, nucleases are removed and purified enzyme preparations contain no detectable exonucleases. The enzyme exhibits no preference for any particular type of DNA, vaccinia virus and calf thymus DNA being used equally efficiently. All vaccinia DNA sequences are transcribed by the enzyme. This was expected, since the enzyme was isolated from infected cells in the late period of the multiplication cycle, when the entire viral genome is transcribed (31). The nature of the constraint(s) that causes only about one-half of the viral genome to be transcribed in the core, or during the early period of the multiplication cycle, is not known. Among the obvious possibilities are modification of the enzyme, constraints on DNA structure, and regulatory proteins.

The enzyme was active in vitro for about 40 min. It was completely resistant to α-amanitin; this is of considerable interest, since the host enzyme that transcribes messenger RNA is extremely sensitive to this inhibitor. It was almost completely dependent on the presence of Mn2+ for activity; Mg2+ was essentially inactive. It differs in this respect from cellular RNA polymerases I, II, and III, all of which are active in the presence of Mg2+ (27).

The purified vaccinia virus RNA polymerase is now being used in vitro studies on the factors that specify the efficiency of transcription of specific regions of the vaccinia virus genome, and, in conjunction with the purified vaccinia virus poly(A) polymerase (49), in studies of the mechanism by which vaccinia virus messenger RNAs are polyadenylated.

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