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Sedimentation of an RNA Polymerase Complex from Vaccinia Virus That Specifically Initiates and Terminates Transcription

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A high-molecular-weight protein complex that is capable of accurate transcription initiation and termination of vaccinia virus early genes without additional factors was demonstrated. The complex was solubilized by disruption of purified virions, freed of DNA by passage through a DEAE-cellulose column, and isolated by glycerol gradient sedimentation. All detectable RNA polymerase activity was associated with the transcription complex, whereas the majority of enzymes released from virus cores including mRNA (nucleoside-2′-*O*)methyltransferase, poly(A) polymerase, topoisomerase, nucleoside triphosphate phosphohydrolase II, protein kinase, and single-strand DNase sedimented more slowly. Activities corresponding to two enzymes, mRNA guanylyltransferase (capping enzyme) and nucleoside triphosphate phosphohydrolase I (DNA-dependent ATPase), partially sedimented with the complex. Silver-stained polyacrylamide gels, immunoblots, and autoradiographs confirmed the presence of subunits of vaccinia virus RNA polymerase, mRNA guanylyltransferase, and nucleoside triphosphate phosphohydrolase I, as well as additional unidentified polypeptides, in fractions with transcriptase activity. A possible role for the DNA-dependent ATPase was suggested by studies with ATP analogs with γ-S or nonhydrolyzable β-γ-phosphodiester bonds. These analogs were used by vaccinia virus RNA polymerase to nonspecifically transcribe single-stranded DNA templates but did not support accurate transcription of early genes by the complex. Transcription also was sensitive to high concentrations of novobiocin; however, this effect could be attributed to inhibition of RNA polymerase or ATPase activities rather than topoisomerase.

Among the eucaryotic DNA viruses, only members of the poxvirus family encode their own DNA-dependent RNA polymerase. The vaccinia virus RNA polymerase resembles the corresponding cellular enzyme with regard to the number and size of subunits (3, 20, 32). The largest subunits of vaccinia virus and eucaryotic RNA polymerases II and III share extensive sequence homology (5), and similarities between other subunits are anticipated. The RNA polymerase and additional enzymes used for capping, methylation, and polyadenylation of mRNA are present in the infectious particle and permit the rapid expression of early genes in the cytoplasm of host cells. The subviral location of these enzymes also segregates them from most cellular proteins thereby greatly facilitating analysis of the components of the transcription machinery. Soluble extracts of purified vaccinia virus (11, 25), like extracts of infected cells (8, 24), are able to transcribe DNA templates containing early genes in vitro. Furthermore, this system is unique in its ability to accurately initiate and terminate mRNAs (25a). The promoters of early genes appear to be located within a short, approximately 30-base-pair region preceding the RNA start site (6; J. Weir and B. Moss, manuscript in preparation). The termination mechanism involves the recognition of a specific signal located upstream of the actual termination site (25a).

In the present study we show that after disruption of virus particles and removal of the endogenous template, the RNA polymerase is associated with a rapidly sedimenting protein complex that may include putative initiation and termination factors as well as capping enzyme and a DNA-dependent ATPase. Nonhydrolyzable ATP analogs inhibit both the latter activity and specific transcription, suggesting a role for the ATPase in mRNA synthesis.

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MATERIALS AND METHODS

DNA. A template for transcription assays was prepared from plasmid pSC16 which contains the entire vaccinia growth factor (VGF) gene (35) and a second gene of unknown function on a *Bgl*II fragment inserted into the vector pUC9 (36). The insert was cleaved from the vector and purified by agarose gel electrophoresis. Bacteriophage M13mp18 single-stranded DNA was purified by polyethylene glycol precipitation of the phage and phenol-chloroform extraction of the DNA (17).

Preparation of virus extracts and glycerol gradient sedimentation. Vaccinia virus strain WR was grown on HeLa S3 suspension cultures and purified by two rounds of centrifugation on sucrose gradients (13). Soluble virus extracts were prepared by deoxycholate disruption of the virus and passage through DEAE-cellulose as described previously (25). Extract from approximately 10^{12} virions was layered on an 11-ml 15 to 35% (vol/vol) glycerol gradient containing 0.2 M KCl, 50 mM Tris (pH 8.0), 1 mM dithiothreitol, and 0.1 mM EDTA and centrifuged for 15 h at 260,000 × *g* in a SW41 rotor. Fractions were collected by pumping from tube bottoms and were stored frozen at −70°C.

Enzyme assays. Vaccinia transcription reactions contained 100 ng of DNA template, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.9), 4 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM each ATP, CTP, and UTP, 0.1 mM GTP, 5 μCi of [α-^{32}P]GTP, and 5 μl of enzyme in a total volume of 50 μl. After 1 h at 30°C, reactions were terminated, and RNA products were electrophoresed on 4% polyacrylamide–8 M urea gels as described previously (25).

Vaccinia RNA polymerase activity was measured as described by Baroudy and Moss (3), using single-stranded M13 DNA as the template and [α-^{32}P]GTP as the radiolabeled nucleotide. After incubation of the reaction mixture for 30

min at 37°C, mixtures were spotted onto DE-81 filters (Whatman Inc., Clifton, N.J.) which were then washed with 0.5 M Na_2HPO_4 to remove unincorporated nucleotides (16). Radioactivity was determined by liquid scintillation counting.

mRNA guanylyltransferase was measured by transfer of GMP from [α-^{32}P]GTP to 5'-diphosphate poly(A) (16). Incorporation of GMP was determined on DE-81 filters as described above. Covalent labeling of guanylyltransferase with [α-^{32}P]GTP was performed as described by Shuman and Hurwitz (29). After termination of the reaction with 10 mM EDTA, protein was precipitated with trichloroacetic acid and electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel.

mRNA 2'-*O*-methyltransferase was assayed by transfer of methyl groups from *S*-[*methyl*-^{3}H]adenosylmethionine to brome mosaic virus RNA (2). As before, radioactive material bound to DE-81 filters was measured by scintillation counting.

DNA-dependent ATPase activity was determined by a modification of a previously described method (23). Reactions were conducted in 10-μl volumes containing 0.2 to 1.0 μg of heat-denatured calf thymus DNA or single-stranded M13 DNA, 100 mM HEPES (pH 7.0), 1 mM $MgCl_2$, 1 mM ATP, 0.5 mM dithiothreitol, 0.05% Nonidet P-40, and 10 μCi of [γ-^{32}P]ATP. In some cases [α-^{32}P]ATP or [α-^{32}P]dATP was used. After 5 min a 37°C, 1 μl was applied to a polyethyleneimine plate which then was developed by ascending chromatography. The solvent used was 0.75 M potassium phosphate (pH 3.5) when release of P_i was measured or 0.8 M acetic acid–0.9 M LiCl when formation of nucleoside diphosphate was determined. For assays specific for nucleoside triphosphate phosphohydrolase II (NPH II), GTP was substituted for ATP at the same concentrations (22).

DNA topoisomerase (4) was measured with 0.4 μg of supercoiled pUC19 DNA in 150 mM NaCl–10 mM Tris hydrochloride (pH 7.5)–0.1 mM EDTA. After incubation at 37°C for 30 min, DNA products were electrophoresed on 1.2% agarose gels in 90 mM Tris borate–2.5 mM EDTA and stained with ethidium bromide.

Protein kinase activity was analyzed for phosphorylation of endogenous protein under conditions resembling those used for transcription assays. Reaction mixtures contained 100 ng of DNA, 100 mM HEPES (pH 7.9), 4 mM $MgCl_2$, 1 mM dithiothreitol, and 0.2 mM [γ-^{32}P]ATP. Reactions were allowed to proceed for 30 min at 37°C. Proteins were separated by SDS-polyacrylamide gel electrophoresis (see below), and the gel was fixed in 40% methanol–10% acetic acid, dried, and exposed to X-ray film for autoradiography.

Poly(A) polymerase activity was measured by incorporation of [α-^{32}P]ATP onto a poly(C) primer (19). Incorporation of radioactivity was determined by DE-81 filter binding.

Escherichia coli RNA polymerase activity was measured in reaction mixtures containing 4 μg of plasmid pUC19, 0.1 M KCl, 50 mM Tris hydrochloride, (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM each ATP, CTP, and UTP, 0.1 mM GTP, and 5 μCi of [α-^{32}P]GTP. After 30 min at 37°C, mixtures were assayed by DE-81 filter binding.

SDS-polyacrylamide gel electrophoresis of proteins. A 200-μl sample of each glycerol gradient fraction was precipitated by bringing solutions to 200-μg/ml sodium deoxycholate and 10% (wt/vol) trichloroacetic acid. Pellets were washed twice with cold acetone before suspension in electrophoresis sample buffer. SDS-polyacrylamide gel electrophoresis was in 7.5 to 15% acrylamide gradient gels in a discontinuous buffer system (15). Gels were stained with a commercial silver stain kit (Bio-Rad Laboratories, Richmond, Calif.).

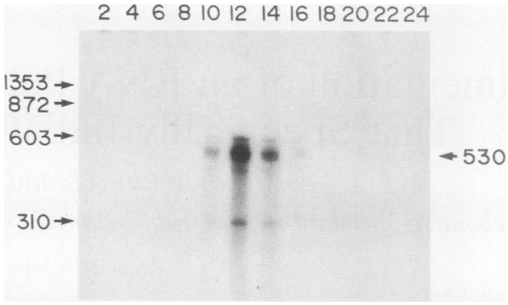

FIG. 1. Transcription analysis of glycerol gradient-fractionated vaccinia virion extract. The indicated glycerol gradient fractions (5 μl) were assayed for transcription activity on the VGF linear DNA template. The mobility of the correctly terminated VGF transcript (530 nucleotides) is indicated at right. The mobilities of selected ϕX174 *Hae*III markers (in nucleotides) are shown at left.

Antibodies and immunoblotting procedure. Antibodies were raised in rabbits immunized with purified vaccinia virus RNA polymerase (3) or nucleoside triphosphate phosphohydrolase I (NPH I) (21). For immunoblotting, proteins were electrophoretically transfered from polyacrylamide gels to nitrocellulose filters (7). Filters were probed with rabbit antibody raised against the specified enzyme, followed by incubation with ^{125}I-labeled protein A.

Materials. [α-^{32}P]GTP (400 to 800 Ci/mmol) was purchased from Amersham Corp. (Arlington Heights, Ill.) or New England Nuclear Corp. (Boston, Mass.). [γ-^{32}P]ATP, [α-^{32}P]ATP, [α-^{32}P]dATP, and ^{125}I-labeled protein A were from Amersham, and *S*-[*methyl*-^{3}H]adenosylmethionine was from New England Nuclear Corp. Unlabeled nucleotides, poly(C), and poly(A) were from Pharmacia Inc. (Piscataway, N.J.). Adenosine (β-γ-imido)triphosphate (AMPPNP), adenosine γ-thiotriphosphate (ATP-γ-S), and novobiocin were supplied by Boehringer Mannheim Biochemicals (Indianapolis, Ind.).

RESULTS

Sedimentation of transcription complex. Soluble extracts of purified vaccinia virions which have been passed through a DEAE-cellulose column to remove endogenous DNA accurately initiate and terminate transcription on templates containing vaccinia early genes (25a). In an attempt to fractionate putative initiation and termination factors from RNA polymerase, the soluble proteins were sedimented on glycerol gradients. Each fraction was assayed for transcription activity with a linear DNA template containing an early gene that codes for the VGF. Previous studies demonstrated that the unfractionated extract transcribed this template to yield the correctly initiated and terminated VGF RNA of about 530 nucleotides, a nonterminated runoff transcript of 1,000 nucleotides, and a smear of poly(A)$^+$ RNA (25a). Fractions 10 to 14 from the center of the gradient produced a major transcript of 530 nucleotides expected for the correctly terminated mRNA (Fig. 1). The width of the 530-nucleotide band reflects heterogeneity at the 3' end of the VGF mRNA, a situation which is known to occur in vivo (38). The 1,000-nucleotide runoff product was not observed in this experiment but appeared as a minor band in some others. The smear of poly(A)$^+$ RNA was absent, suggesting that the

complex was separated from the poly(A) polymerase. The minor transcript of about 300 nucleotides in length was also observed previously (25a) and appears to be the terminated RNA from an oppositely oriented gene of unknown function present in the same DNA fragment as the VGF gene.

The 530-nucleotide RNA made by the transcription complex was identical in size to the correctly initiated transcript previously made with the same template and the unfractionated extract. The unlikely possibility that the RNA polymerase complex initiated transcription at an aberrant site 530 nucleotides from the end of the DNA fragment was eliminated in two ways. When DNA fragments truncated within the VGF gene were used as templates, the major transcript was shortened, and the correct site of initiation was deduced from the size of the runoff RNA (data not shown). In addition, the 530-nucleotide transcript was the major product when the 4,200-base-pair supercoiled plasmid was used as the template (S. Broyles, unpublished data).

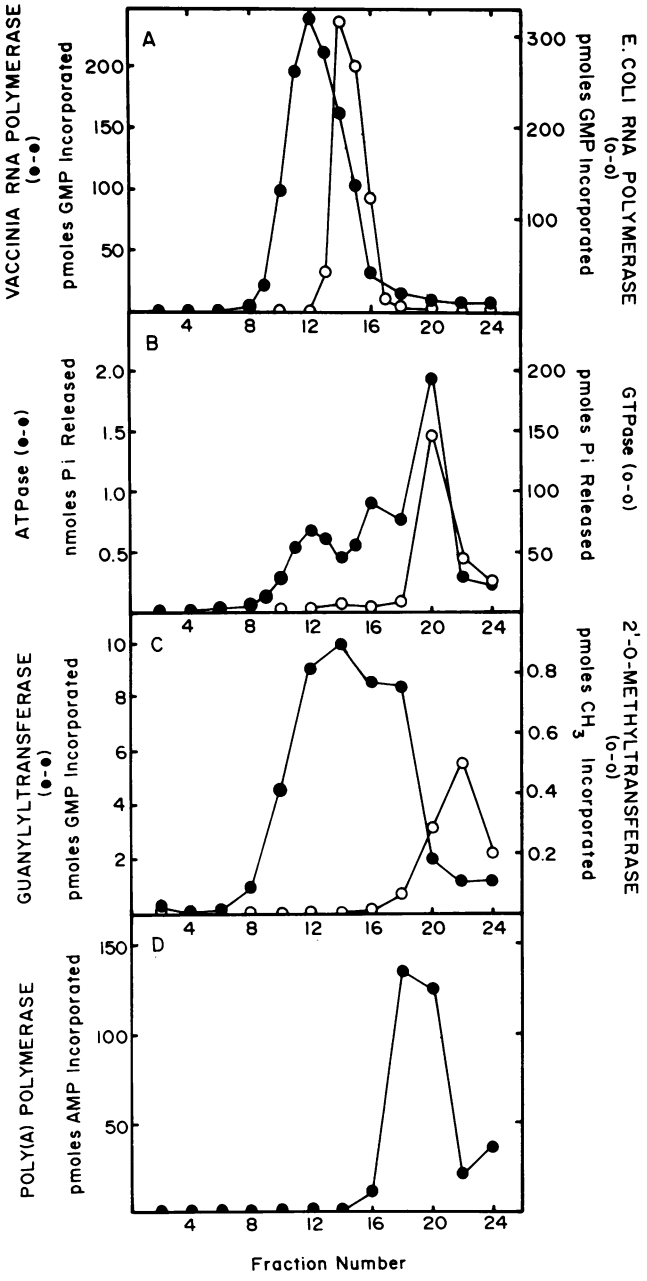

FIG. 2. Vaccinia virion enzyme activities fractionated by glycerol gradient sedimentation. Equal volumes of each fraction were assayed for the specified activities.

Transcription assays indicated that more activity was recovered in the peak glycerol fractions than was applied to the gradient, suggesting the removal of some inhibitor. Activation of transcription did not occur when the peak gradient fraction was mixed with other fractions, although termination sometimes appeared to be enhanced when material just above the peak was added. When fractions near the top of the gradient were mixed with the peak fraction, the transcripts which were produced migrated more slowly because of polyadenylation.

During the course of these studies, glycerol gradients were prepared with KCl concentrations ranging from 0.05 to 1.0 M. A concentration of 0.2 M appeared optimal since the peak of transcription activity was broader at lower salt concentrations and less activity was recovered with higher concentrations. Addition of Mg^{2+} to the glycerol did not affect the sedimentation of the transcription activity. Resedimentation of the RNA polymerase complex was attempted several times, but little activity was recovered. The RNA polymerase complex was stored at −70°C; addition of divalent cations including Mg^{2+}, Zn^{2+}, or Ca^{2+} did not increase stability.

For operational purposes, we called the rapidly sedimenting structure a transcription complex because of the possibility that protein factors in addition to RNA polymerase might be required to accurately transcribe vaccinia virus genes. In this regard, previous reports (3, 32) indicated that highly purified vaccinia virus RNA polymerase is active only with single-stranded DNA templates.

Enzymatic activities associated with transcription complex. Fractions from the glycerol gradient were assayed for a variety of enzymes known to be released from disrupted vaccinia virus cores. A nonspecific assay for RNA polymerase activity (3), depending on a single-stranded DNA template and Mn^{2+}, was used to avoid any requirement for potential transcription factors. The vaccinia RNA polymerase sedimented as a single uniform peak coinciding with the transcription activity and was not detected elsewhere in the gradient (Fig. 2A). The sedimentation coefficient of the vaccinia RNA polymerase complex was greater than that of *E. coli* RNA polymerase under these conditions.

There are at least two nucleic acid-dependent NPH activities in vaccinia virions. NPH I specifically hydrolyzes ATP to ADP and P_i, whereas NPH II hydrolyzes all four ribonucleoside triphosphates (22). Purified forms of the two enzymes are similar in size and sediment with apparent molecular weights of 61,000 to 68,000. In contrast, the ATPase activity from the virus extract showed a complex distribution across the glycerol gradient (Fig. 2B). One peak, however, coincided precisely with the RNA polymerase. The ATPase present in this fraction was totally dependent on DNA, showed a marked preference for single-stranded relative to native DNA, and was not stimulated by RNA homopolymers (data not shown). In addition, the ATPase hydrolyzed dATP (data not shown) as well as ATP, but not GTP (Fig. 2B). These characteristics are all consistent with the described properties of NPH I (22).

The slowly sedimenting peak of GTPase was DNA dependent and therefore represents NPH II (22). The ATPase activity located in these fractions, however, could include some uncomplexed NPH I as well as NPH II.

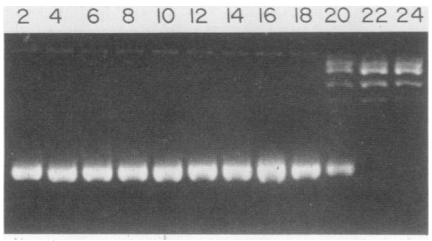

FIG. 3. DNA topoisomerase activity of glycerol gradient-fractionated vaccinia virion extract. The indicated glycerol gradient fractions were tested for topoisomerase activity with supercoiled plasmid as the substrate. The band of highest mobility is supercoiled substrate DNA, and the more slowly migrating relaxed topoisomer products can be seen in fractions 20 to 24.

Purified mRNA guanylyltransferase, commonly referred to as capping enzyme, is composed of two subunits and sediments with an apparent molecular weight of 127,000 (34). Nevertheless, the enzyme from virus extract displayed a complex sedimentation profile (Fig. 2C). Much of the capping activity was present in the middle of the gradient overlapping the profile of RNA polymerase activity, although some also was found in more slowly sedimenting fractions.

Two other enzymes which are involved in RNA modification, the 2′-*O*-methyltransferase (Fig. 2C) and the poly(A) polymerase (Fig. 2D), remained near the top of the gradient in positions consistent with their known sedimentation constants (2, 19).

Because of a previously suspected role in transcription (see below), the activity of the vaccinia DNA topoisomerase was measured across the glycerol gradient. The majority of topoisomerase activity, however, was located in fractions 20 to 24 at the top of the gradient, and none appeared to be associated with the transcription complex (Fig. 3). The single-strand-specific nuclease of vaccinia virus (26) was also located in the fractions at the top of the gradient (M. Merchlinsky, unpublished data). Protein kinase activity, measured by incorporation of phosphate from [γ-^{32}P]ATP into polypeptides without additional substrate, was only located at the top of the glycerol gradient (data not shown). The latter result suggests that the vaccinia protein kinase (14) was not the source of any of the ATPase activity associated with the RNA polymerase and further indicates that this kinase is not required for in vitro transcription.

Analysis of polypeptides in the glycerol gradient. Since the major structural proteins of vaccinia virus are not solubilized by the deoxycholate extraction procedure and are removed by centrifugation, there is a considerable enrichment for enzymes involved in RNA synthesis and modification (3). The polypeptide composition of the glycerol gradient fractions was analyzed by SDS-polyacrylamide gel electrophoresis. The sedimentation position of the vaccinia RNA polymerase was readily determined by the prominent high-molecular-weight doublet bands in fractions 10 through 14 (Fig. 4). These polypeptides measure 145 and 135 kilodaltons (kDa), respectively, and are constituents of purified vaccinia RNA polymerase (3, 20, 32). Additional polypeptides of 73, 37, 23, 21.5, and 20 kDa also appear to cosediment with the RNA polymerase as judged by the maximal intensity of each band in fraction 12. Polypeptides similar in molecular weight to the four smaller ones noted above have been observed to be constituents of highly purified enzyme isolated from virus particles (3). The 73-kDa polypeptide was previously observed in preparations of purified RNA polymerase from infected cells (20) but not from viral particles (3). It is likely that other small polypeptides known to be part of the RNA polymerase are also present in the complex but are undiscernible owing to the many low-molecular-weight proteins distributed throughout the glycerol gradient.

Recently it was reported that a 170-kDa form of the large subunit of the host RNA polymerase II is present in the rabbitpox virion in association with the viral RNA polymerase (18). Inspection of the data in Fig. 4, however, revealed no polypeptide of this size in fractions 10 to 14 containing the vaccinia RNA polymerase, whereas a more slowly sedimenting polypeptide of about 170 kDa was seen in fractions 16 to 22.

Identification of enzyme subunits. The enzymatic assays described above suggested an association of an ATPase and the mRNA capping enzyme with the vaccinia RNA polymerase. RNA polymerase subunits were identified by immunoblot analysis of glycerol gradient fractions (Fig. 5A). Immunoreactive polypeptides of about 145, 37, 35, and 22 kDa cosedimented with the RNA polymerase activity centered around fraction 12 (the two large subunits were not resolved by this blot). When an identical protein blot was probed with antibodies specific for NPH I, the 63-kDa polypeptide corresponding to the ATPase was located in fractions 10 to 24 (Fig. 5B). This distribution is consistent with the ATPase enzymatic activity profile seen in Fig. 2. The sedimentation position of the mRNA guanylyltransferase or capping enzyme was confirmed by covalent labeling of the enzyme with [α-^{32}P]GTP. During the course of catalysis, the 95-kDa capping enzyme subunit forms a covalent intermediate with GTP (29), providing a sensitive and highly specific assay for the presence of the enzyme. The labeled 95-kDa polypeptide (Fig. 5C) was present in the same glycerol gradient fractions as capping enzyme activity and overlapped the position of the RNA polymerase.

Effect of ATP analogs on transcription. ATP analogs with a nonhydrolyzable β,γ bond do not support specific transcription of early genes in permeabilized vaccinia virus cores (10, 30) but are used by purified vaccinia virus RNA polymerase for nonspecific transcription of single-stranded DNA templates (30). Similarly, we found that neither AMPPNP nor

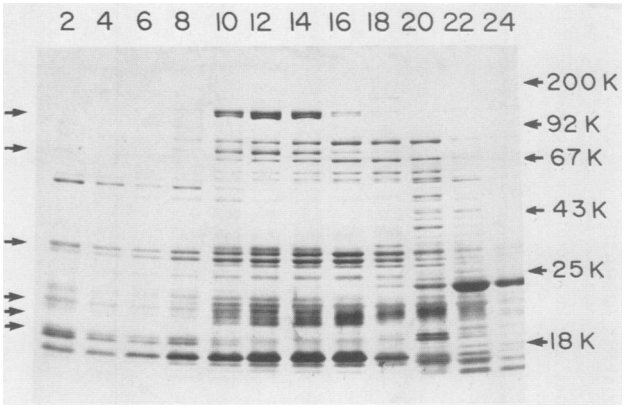

FIG. 4. SDS-polyacrylamide gel analysis of the components of glycerol gradient-fractionated virion extract. A portion of the indicated glycerol gradient fraction was electrophoresed on an SDS-polyacrylamide gel which was then silver stained. The mobilities of molecular weight standards are indicated at right (K, 10^3). The mobilities of polypeptides which appear to cosediment with RNA polymerase activity (centered around fraction 12) are shown to the left.

ATP-γ-S were able to replace ATP for specific template-dependent in vitro transcription by the RNA polymerase complex (Fig. 6). The effect of each analog could be partially reversed, however, if an equal concentration of ATP was included in the reaction. By contrast, ATP-γ-S had little effect on the ability of the transcription complex to synthesize RNA on a nonspecific single-stranded DNA template, and AMPPNP reduced the rate of RNA synthesis by about one half (Fig. 7A). The same general pattern was observed when these analogs were used as substrates for *E. coli* RNA polymerase which is known not to require an hydrolyzable β,γ bond in ATP (Fig. 7B). These experiments demonstrate an important distinction between RNA polymerase activity and accurate transcription. The nonspecific vaccinia virus RNA polymerase activity functions independently of hydrolysis of the β,γ bond in ATP, whereas the specific transcription of the VGF gene apparently has an absolute requirement for the hydrolysis.

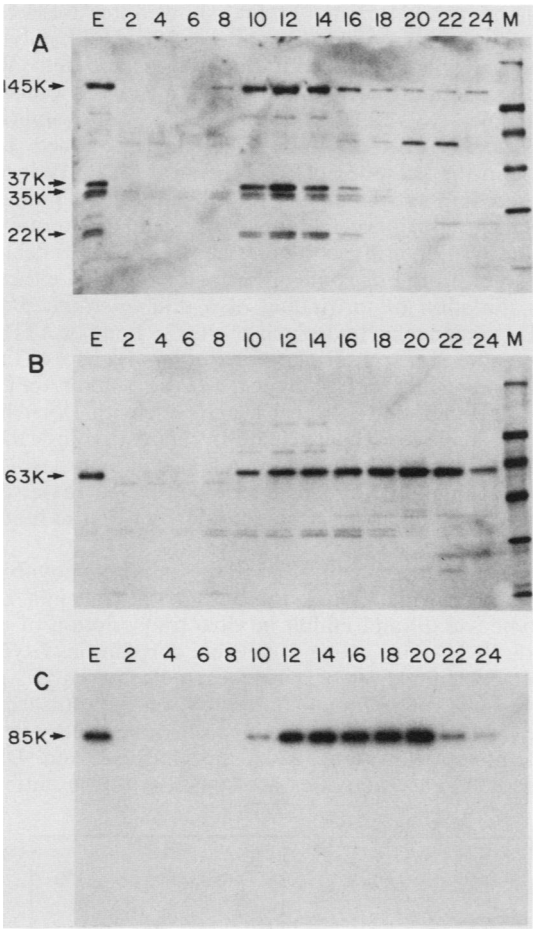

FIG. 5. Subunit analysis of glycerol gradient-fractionated virion extract. Identical volumes from the indicated glycerol gradient fractions were electrophoresed on SDS-polyacrylamide gels as described in the legend to Fig. 6. Polypeptides were transfered to nitrocellulose and probed with antibody directed against purified vaccinia RNA polymerase (A) or NPH I (B). In panel C, protein was reacted with [α-^{32}P]GTP to locate the large subunit of the mRNA capping enzyme. Lane E is unfractionated extract, and lane M is molecular weight standards: myosin heavy chain, 200,000; phosphorylase *b*, 92,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; lysozyme, 14,000. The apparent molecular weights of identified polypeptides are indicated at left (K, 10^3).

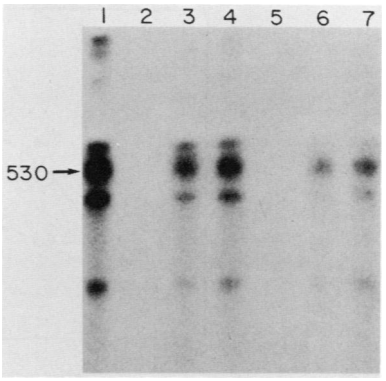

FIG. 6. Effect of ATP analogs on transcription by glycerol gradient-purified vaccinia RNA polymerase. Transcription reaction mixtures contained 1 mM ATP (lane 1), 1 mM ATP-γ-S (lane 2), 1 mM ATP and 1 mM ATP-γ-S (lane 4), 1 mM AMPPNP (lane 5), and 1 mM ATP and 1 mM AMPPNP (lane 6). Lanes 4 and 7 are identical to lanes 3 and 6, respectively, except the analog was added 10 min after preincubation with ATP. The mobility of the 530-nucleotide VGF transcript is shown at left.

Effect of novobiocin on in vitro transcription. Novobiocin blocks the action of the vaccinia DNA topoisomerase (9) and also inhibits in vitro RNA synthesis in permeabilized viral cores (9), extracts from solubilized viral cores (11), or infected cells (8). Based on these observations it has been suggested that the DNA topoisomerase plays a role in vaccinia early transcription. Nevertheless, the RNA polymerase complex was separated from topoisomerase by glycerol gradient sedimentation (Fig. 2) but retained specific transcriptional activity. Surprisingly, however, transcription by the glycerol gradient complex remained sensitive to novobiocin (Fig. 8). The addition of 1 mM novobiocin to RNA polymerase fractions completely inhibited the transcription reaction. Since this concentration of antibiotic was comparable to that previously demonstrated to block vaccinia transcription, we considered that other enzymes associated with the transcription complex might be the novobiocin-sensitive targets.

When the nonspecific RNA synthetic activity of RNA polymerase on a single-stranded template was measured, an effect of novobiocin was observed (Fig. 9A). Concentrations of antibiotic up to 0.5 mM had little effect on RNA synthesis. However, 1 mM novobiocin reduced activity to less than one-fifth the normal rate. The DNA-dependent ATPase was also sensitive to novobiocin. The rate of ATP hydrolysis was reduced to about 37 and 12% by 0.5 and 1.0 mM novobiocin, respectively (Fig. 9B). These results show that the vaccinia RNA polymerase and the DNA-dependent ATPase are sensitive to the rather high concentrations of novobiocin necessary to block transcription and that effects on either of these enzymes may be the basis for sensitivity of vaccinia transcription to novobiocin.

DISCUSSION

Glycerol gradient sedimentation analysis of soluble extracts of vaccinia virions was used to demonstrate that the viral RNA polymerase exists as part of a high-molecular-weight protein complex that can accurately transcribe viral early genes. Moreover, the components necessary for initiation, elongation, and termination of RNA synthesis were associated with the complex. At least for the VGF gene, as well as two other early genes tested (unpublished observa-

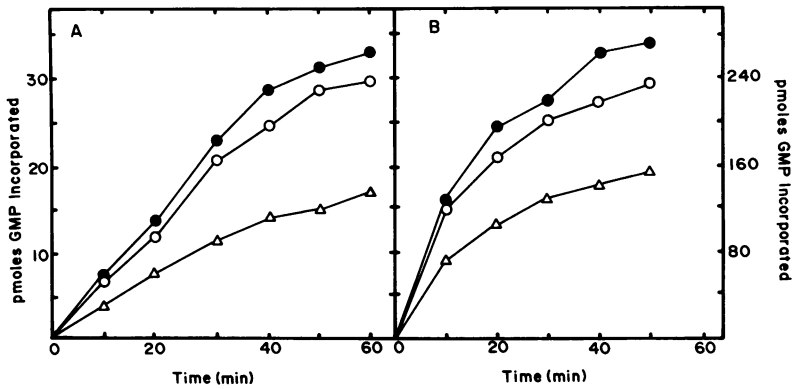

FIG. 7. Effect of nonhydrolyzable ATP analogs on nonspecific RNA polymerase activity of glycerol gradient-purified enzyme. (A) Vaccinia RNA polymerase; (B) *E. coli* RNA polymerase. Reaction mixtures contained 1 mM ATP (●), 1 mM ATP-γ-S (○), or 1 mM AMPPNP (△).

tions), no additional factors seem to be required. Evidence that the essential promoter region of several early genes extends only about 30 bp upstream of the RNA start site (6; Weir and Moss, in preparation) is consistent with the lack of a requirement for binding of an additional transcription factor.

Enzymatic analysis of glycerol gradient-fractionated viral extracts shows that most enzymes contained within vaccinia cores are not tightly bound to the transcription complex. In addition to RNA polymerase, only two known enzymatic activities, the capping enzyme and the DNA-dependent ATPase NPH I, appeared to cosediment with the complex. Even in this case, only a fraction of the total of each enzyme was associated with RNA polymerase after centrifugation in glycerol. This finding suggests either a rather weak association of the capping enzyme and ATPase with RNA polymerase or excess amounts of these enzymes. Similarly, most of the capping enzyme and DNA-dependent ATPase did not bind to DEAE-Bio-Gel, but a fraction of both activities coeluted with the RNA polymerase complex, and the latter correctly initiated and terminated transcription on the VGF template (unpublished data). An association of capping enzyme with vaccinia RNA polymerase during early stages of purification was also noted by Shuman and co-workers (31).

The importance of the association between the ATPase and the RNA polymerase was implied by the effect of ATP analogs with γ-S or nonhydrolyzable β,γ bonds on transcription. Shuman and Hurwitz (29) first showed that NPH I was unable to hydrolyze ATP-γ-S, while purified RNA polymerase was capable of incorporating the analog into RNA chains. We found that glycerol gradient-purified transcription complex was relatively unaffected in its ability to synthesize RNA on a nonspecific single-stranded DNA template in the presence of ATP-γ-S or AMPPNP. In contrast, the complex was unable to utilize these ATP analogs for specific transcription of double-stranded DNA templates. Thus, the ATP hydrolysis requirement probably reflects an inhibition of NPH I or some other factor required either for correct initiation or unwinding of a double-stranded template. Dependence on hydrolysis of the β,γ bond of ATP also has been noted for transcription by RNA polymerase II (28). In some respects, NPH I appears to be similar to DNA helicases. Helicases that form a complex with DNA polymerase are believed to participate in replication fork movement and have DNA-dependent ATPase activity, which is maximally stimulated by single-stranded DNA (reviewed in reference 1). By analogy, a possible DNA helicase function of NPH I may be required for transcription.

Relatively high concentrations of the antibiotic novobiocin have been shown to block the activity of vaccinia DNA topoisomerase (9) and inhibit in vitro transcription of early genes (8, 9, 11), suggesting a possible correlation. Glycerol gradient centrifugation of virion extracts, however, gave effective separation of topoisomerase from the transcription complex, yet transcription remained sensitive to novobiocin. Our demonstration that RNA polymerase and DNA-dependent ATPase activities are sensitive to the antibiotic

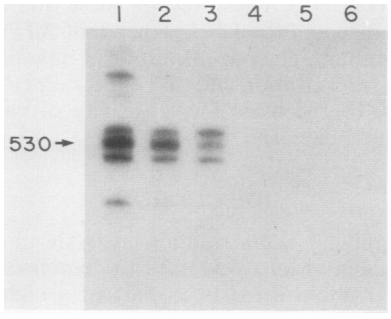

FIG. 8. Effect of novobiocin on transcription of VGF gene by glycerol gradient-purified vaccinia RNA polymerase. Reaction mixtures contained 0 (lane 1), 0.25 (lane 2), 0.5 (lane 3), 0.75 (lane 4), 1.0 (lane 5), or 2.0 (lane 6) mM novobiocin. The mobility of the 530-nucleotide VGF transcript is indicated at left.

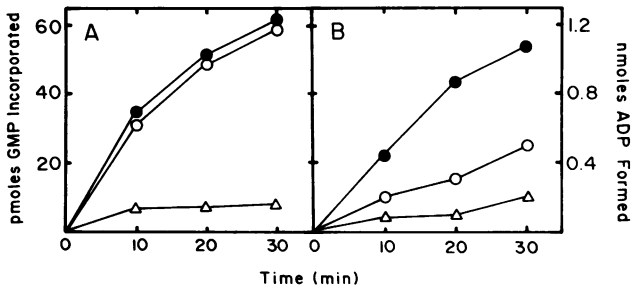

FIG. 9. Effect of novobiocin on vaccinia RNA polymerase and NPH I activities. Samples from fraction 12 of the glycerol gradient shown in Fig. 2 were tested for nonspecific RNA polymerase activity (A) or ATPase activity (B) in the presence of 0 (●), 0.5 (○), or 1.0 (△) mM novobiocin.

provides an alternative explanation for the in vitro effects of novobiocin. Intuitively, it is difficult to imagine why changing DNA topology would be important for in vitro transcription of a small linear DNA fragment. The in vivo situation might be quite different, however. It is quite possible that the DNA is constrained within the virus core and that the topoisomerase functions to relax the large molecule and provides a more favorable template for transcription. Nevertheless, our demonstration that the topoisomerase is not the only target for novobiocin shows that caution is required in interpretation of antibiotic inhibition studies. In this context, novobiocin has been shown to affect other enzymes including RNA polymerase III (12), DNA polymerase α (27), and avian retrovirus reverse transcriptase (27, 33) which bear no apparent relationship to DNA topoisomerases.

The isolation of a rapidly sedimenting vaccinia virus RNA polymerase complex which accurately initiates and terminates RNA synthesis when added to a DNA template suggests that protein-protein interactions are important in forming the active transcriptase. *E. coli* RNA polymerase also exists as a well-defined complex with σ initiation factor, but rho termination factor binds independently to RNA. A form of eucaryotic RNA polymerase III that is complexed with transcription factors IIIB and IIIC has recently been described (37). On the basis of the similarity in subunit structure of the vaccinia virus and eucaryotic RNA polymerases and evidence for extensive sequence homology of the large subunits, we believe that information obtained from the viral system may have general significance.

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