JOURNAL OF VIROLOGY, Jan. 1985, p. 205–213
0022-538X/85/010205-09$02.00/0

Vol. 53, No. 1

A Soluble Transcription System Derived from Purified Vaccinia Virions

FRED GOLINI†* AND JOSEPH R. KATES

Department of Basic and Clinical Research, Scripps Clinic and Research Foundation, La Jolla, California 92037

Received 24 July 1984/Accepted 21 September 1984

A soluble extract from purified vaccinia virus particles has been developed which displays site-specific initiation of transcription on exogenous DNA templates that carry cloned vaccinia virus early gene sequences. Bacterial plasmid vectors with segments of a strongly expressed early region of the vaccinia virus genome were active templates, whether in supercoiled or linear, truncated forms. Correct initiation, corresponding to that found in vivo, was observed for all early genes tested. The involvement of other factors besides the viral RNA polymerase was demonstrated by the loss of specific initiation upon partial purification of the enzyme. Initiation activity was restored by reconstitution of the system with factors lacking polymerase activity. The soluble system retained properties of transcription characteristic of intact viral cores, including (i) similar relative rates of initiation of various genes, (ii) multiple requirement for ATP, (iii) methylation and polyadenylation of transcripts, and (iv) inhibition by a topoisomerase antagonist.

The first example of transcriptive enzymes associated with virions was found in poxviruses (12, 25). mRNA synthesis mediated by permeabilized vaccinia virions has many features in common with mRNA biogenesis in eucaryotic cells. Vaccinia virus cores synthesize mRNAs that are capped, methylated (23, 36), and polyadenylated (11), and the enzymes mediating these processes have been purified from the virus particles (20, 24, 31). Gene splicing, however, has not been demonstrated in vaccinia virus for any of the RNA transcripts characterized to date. Studies with ATP analogs have indicated that the primary transcription process, mediated by vaccinia virus cores, involves multiple enzymatic components. Specifically, β,γ-imido ATP (AMP-PNP) and γ-thiophosphate ATP (ATPγS), which are suitable substrates for purified vaccinia virus RNA polymerase in vitro, do not support transcription in cores. A number of enzymes whose functions have not been defined, but which may play a role in the transcription process, have also been isolated from virions. These include two nucleic acid-dependent nucleoside triphosphatases and a type I topoisomerase (2, 7, 27).

Individual segments of the vaccinia viral genome have been cloned, and detailed transcriptional and translational maps have been derived (3, 6, 10, 14, 15, 33, 38, 39). In several studies to sequence the portion of the vaccinia virus genome containing the early genes, attention has been focused on the regions preceding the structural gene sequences. From these analyses, presumptive promoter sequences required for the initiation of early gene transcription have been roughly defined upstream from the transcriptional start point (13, 34).

Despite these recent, more detailed data, progress in understanding the biochemistry of transcription in vaccinia virus has been hindered by the unavailability of a soluble extract that consists entirely of viral components and is able to accurately transcribe added vaccinia virus DNA. Recently, by application of a method developed to study transcription in uninfected cells (18, 37), a soluble system was prepared from vaccinia virus-infected cells and shown to be capable of correctly initiating transcription from cloned fragments of early vaccinia virus genes (29). Whereas an extract prepared from whole, infected cells is applicable to defining transcriptional regulatory sequences on vaccinia virus DNA, the preponderance of contaminating cellular proteins and enzymes in a whole cell extract reduces its usefulness in identifying the specific viral components involved in various aspects of the transcription process. These cellular contaminants may also alter the action of the viral transcriptional components.

In this report we describe a soluble transcription system that consists only of viral components and that is entirely dependent upon the addition of exogenous DNA templates. The system displays highly efficient, site-specific initiation and transcription of vaccinia virus early genes. Furthermore, it displays a number of other properties that are characteristic of the transcription process mediated by whole vaccinia virus cores, either in vivo or in vitro.

MATERIALS AND METHODS

DNA templates. The cloning of a 7-kilobase *Eco*RI fragment of vaccinia virus DNA (pV7) and the *Hpa*II subfragments (pHpaA, pHpaC, and pHpaF) in pBR322 have been described elsewhere (10). Plasmids were purified from Triton X-100 lysates of *Escherichia coli* by buoyant density banding in propidium diiodide-CsCl gradients (4). M13 mp7 single-stranded DNA was extracted from virus obtained from culture supernatant by polyethylene glycol precipitation (22). Restriction enzymes were supplied by Bethesda Research Laboratories, New England Biolabs, and Boehringer Mannheim and were used as recommended by Maniatis et al (16). After digestion, DNA was deproteinized by successive extractions with phenol-chloroform (1:1) and diethyl ether and recovered after ethanol precipitation.

Soluble virus extract. Vaccinia virus strain WR was propagated in HeLa S3 suspension cells and purified by density banding in potassium tartrate gradients (10). Extracts were prepared from frozen stocks of purified virus as follows: 2×10^{12} virus particles were suspended in 0.5 ml of buffer A (100 mM Tris-hydrochloride [pH 8.0], 10 mM dithiothreitol, 250 mM KCl, and 0.1 mM EDTA) in a 1.5-ml microfuge tube. For all the subsequent steps solutions were kept between 0

* Corresponding author.
† Present address: Syntro Corp., San Diego, CA 92121.

and 4° C. Sodium deoxycholate was added from a 10% (wt/vol) stock to a final concentration of 0.2% (wt/vol). After gentle mixing, the suspension was held on ice for 60 min. Insoluble material was removed by centrifugation for 15 min at 10,000 × *g*. The viscosity of the supernatant was reduced by rapid expression through a 23-gauge syringe. This sheared supernatant was applied to a 0.5-ml column of DEAE-cellulose equilibrated with buffer A, and the column was washed with additional buffer A by gravity flow. Fractions (0.2 ml) containing protein not adsorbed to the column were pooled, dialyzed against buffer B (20 mM Tris-hydrochloride [pH 8.0], 2 mM dithiothreitol, 80 mM KCl, 0.1 mM EDTA, 15% [vol/vol] glycerol), and stored at −70° C. Protein concentrations of approximately 5 mg/ml were obtained by this procedure.

In vitro transcription. Standard 50-μl reactions contained 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 8.0), 4 mM $MnCl_2$, 40 mM KCl, 1 mM each ATP, UTP, and CTP, 0.1 mM GTP, 5 μCi of [α-^{32}P]GTP (760 Ci/mmol; New England Nuclear Corp.), 10 μM *S*-adenosylmethionine, 4 U of placental RNase inhibitor (Bethesda Research Laboratories), 0.5 to 1 μg of DNA template, and approximately 25 μg of protein of soluble virus extract. Where RNA was to be labeled with [^{3}H]UTP, incubations contained 5 μCi of [^{3}H]UTP (42.3 Ci/mmol), 0.1 mM UTP, and 1 mM each ATP, GTP, and CTP. For analysis of RNA methylation, reactions included all four nucleoside triphosphates (1 mM each) and 10 μM [^{3}H]*S*-adenosylmethionine (Amersham Corp.) at 75 Ci/mmol. Nucleotide analogs were purchased from Boehringer Mannheim. RNA synthesis by intact vaccinia virus cores was performed as previously described (10). Total incorporation of radioactivity was measured by trichloroacetic acid precipitation of a sample of the reaction. For gel analysis, incubations were terminated by the addition of 50 μl of a solution containing 8 M urea, 1% sodium dodecyl sulfate, 10 mM EDTA, and 250 μg of yeast tRNA per ml. Mixtures were extracted twice with an equal volume of phenol-chloroform. After ammonium acetate was added to a final concentration of 2 M, RNA was precipitated with 2 volumes of ethanol. RNA was denatured by treatment with glyoxal (21) and resolved on 1.5% agarose gels in 10 mM sodium phosphate (pH 7.0). Alternatively, ethanol-precipitated RNA was dissolved in 20 μl of 75% formamide, heated at 70° C for 5 min, and analyzed by electrophoresis through 4% acrylamide gels in 8 M urea–0.1 M Tris borate (pH 8.3)–2 mM EDTA. ^{3}H-labeled RNAs were visualized by fluorography after treatment with Enlightning (New England Nuclear); gels containing [^{32}P]RNAs were subjected to direct autoradiography.

Nuclease S1 mapping of transcripts. [^{32}P]RNA synthesized by viral cores (2 μg) or soluble extracts (20 ng) was hybridized to immobilized DNA filters containing the separated strands of recombinant phage clone B26, which includes the 7-kilobase-pair (kbp) region of the vaccinia virus genome (10). RNA annealing to the coding strand was recovered and combined with a molar excess of unlabeled DNA restriction fragment derived from 2 μg of pV7 DNA in a 20-μl volume of 80% formamide–0.8 M NaCl–80 mM sodium piperazine-*N*,*N'*-bis(2-ethanesulfonic acid) (pH 6.4)–10 mM EDTA. After heating for 10 min at 70°C, mixtures were incubated at 39°C for 7 h. Hybridizations were terminated by addition of 200 μl of digestion buffer (0.25 M NaCl, 30 mM sodium acetate [pH 4.5], 1 mM $ZnCl_2$, 20 μg of denatured calf thymus DNA per ml) containing 2,000 U of nuclease S1 (Boehringer Mannheim) per ml. Digestion with nuclease S1 was carried out at 39°C for 1 h. Nuclease-protected RNAs were precipitated with ethanol and analyzed by gel electrophoresis after glyoxal denaturation.

Fractionation of soluble extract. A portion (300 μl) of DEAE-eluted, soluble extract from 10^{12} virions (before dialysis) was layered on a 5-ml gradient of 15 to 35% (vol/vol) glycerol in buffer B and centrifuged at 37,000 rpm for 18 h at 4° C in a Beckman SW50.1 rotor. Fractions of 300 μl were collected from the bottom of the tube and assayed for transcriptional capacity as described above with M13 mp7 single-stranded DNA as a template. Fractions 5 through 7, containing the peak of RNA polymerase activity, were combined. Slower-sedimenting fractions 12 through 18, totally devoid of RNA polymerase, were pooled separately. Alternatively, RNA polymerase was partially purified chromatographically as follows: undialyzed extract from 10^{12} virions was diluted with 4 volumes of buffer C (50 mM Tris-hydrochloride [pH 8.0], 2 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol) to reduce the KCl concentration to 50 mM and applied to a 1-ml column of DEAE-cellulose equilibrated with 50 mM KCl in buffer C. The column was developed successively with 5-ml steps of buffer C containing 50, 100, 150, and 250 mM KCl. RNA polymerase activity eluted in the 150 mM KCl wash. The active fractions were pooled and applied to a 1.0-ml phosphocellulose column equilibrated with buffer C containing 150 mM KCl. This column was eluted stepwise with 5-ml portions of buffer C containing 150, 250, and 300 mM KCl. The peak of RNA polymerase activity was recovered in the 250 mM step.

RESULTS

General considerations. Our strategy for obtaining a soluble transcription system from vaccinia virions included two significant considerations. The first was our decision to use an unfractionated virion extract rather than purified proteins as the starting material for our extract preparation. In so doing, we could minimize the possibility of omitting an essential component of the transcription machinery. In preliminary experiments extracts were made from core particles; however, a large variation in activity was seen from one preparation to the next. Further experiments indicated that this variation was due to inhibitory effects of the detergent Triton X-100, which had been used in preparing cores from whole virus. Although the inclusion of $MgCl_2$ in the extraction buffer provided partial protection against Triton X-100 inhibition, subsequent extracts were generally made from whole virus in the absence of Triton X-100.

A second aspect of our strategy concerned the DNA template to be used in analyzing the properties of the in vitro system. To optimize the detection of a specific RNA initiation event, our assays required vaccinia virus templates whose transcriptional maps had been accurately determined and shown to contain nucleotide sequences that strongly promoted RNA synthesis. The templates we chose for these studies were the *Hpa*II subfragments of a 7-kbp *Eco*RI segment of vaccinia virus DNA (pHpaA through pHpaE), which had been previously cloned in pBR322 (pV7) and characterized (10). This 7-kbp region of the vaccinia virus genome includes six early genes that are transcribed in infected cells before the uncoating of the core particle. The segment also contains a single late gene that is expressed after the onset of viral DNA synthesis. The exceptionally abundant levels of RNA transcribed from the 7-kbp region both in early stages of infection in cells and in permeabilized cores suggested the presence of strong promoters of RNA synthesis. Therefore, in several experiments the chimeric plasmid pHpaF, containing a 422-nucleotide vaccinia virus

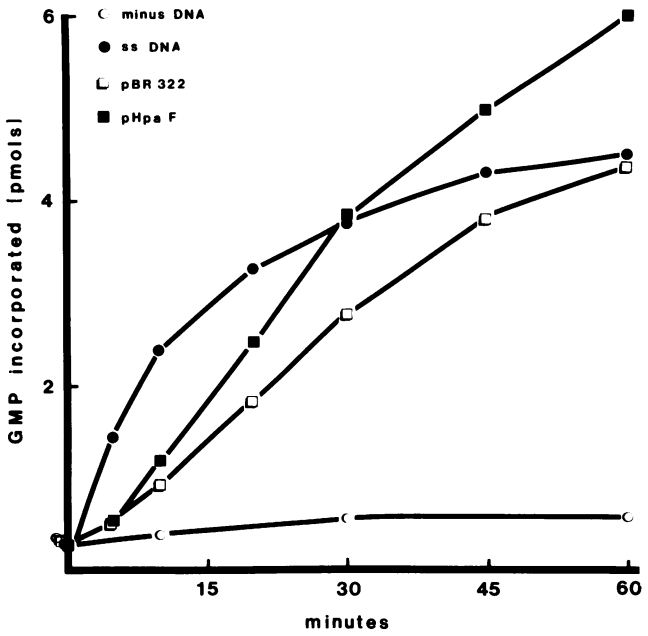

FIG. 1. RNA synthesis by a soluble extract of vaccinia virus. Standard 50-μl transcription reactions were preincubated for 4 min at 30°C before the addition of 1 μg of the indicated DNA template. RNA synthesis was measured by trichloracetic acid precipitation of 5-μl samples removed at various times. The ordinate is expressed as incorporation of GMP per 5-μl samples.

insert, was used as a template. This fragment includes the 5′ end of a major early transcription unit encoding a 38-kilodalton polypeptide and 120 nucleotides of upstream sequence. Two observations suggested that the DNA sequences required for the correct initiation of RNA transcription were located near the mapping sites of the RNA 5′ termini: the evident lack of RNA splicing in poxviruses and the correspondence of sites for RNA capping and initiation (35).

Preparation and initial characterization of the system. Treating purified vaccinia virus with 0.2% (wt/vol) sodium deoxycholate solubilized up to 50% of the protein mass, including the enzymes responsible for mRNA biogenesis. Insoluble material was removed by brief centrifugation, and the supernatant fraction was passed through a DEAE-cellulose column in 0.25 M KCl to remove deoxycholate and viral DNA. Extracts prepared in this manner were capable of supporting RNA synthesis when supplied with an exogenous template (Fig. 1). The divalent cation requirements of the extract and of permeabilized virions show distinct differences. Whereas Mg^{2+} is the divalent cation required for transcription by permeabilized virions, Mn^{2+} in 1 to 2 mM excess over the nucleoside triphosphate concentration was necessary for RNA synthesis in soluble extracts. Optimal results were obtained when reactions also included 40 mM K^+ or Na^+, 25 μg of extract, and 0.5 to 1 μg of DNA per 50-μl reaction. Under these conditions over 50 pmol of GMP was incorporated into RNA in 60 min.

The soluble extracts were able to transcribe both single-stranded and native DNAs with approximately equal efficiencies. This capacity contrasts markedly with the strong preference of purified RNA polymerase for single-stranded DNA templates (1, 26, 32). An absolute requirement for vaccinia virus sequences was not found, although a recombinant plasmid (pHpaF) containing a vaccinia virus early promoter was a more effective template than was the vector (pBR322) alone. We also noted that RNA accumulation continued for up to 60 min when transcription reactions were incubated at 30°C. With native DNA templates, a linear rate of incorporation was observed for 30 min after an initial lag of approximately 2 to 3 min. Interestingly, this lag was not seen with a single-stranded template and consequently may reflect the time required for initiation to commence with duplex DNA.

Site-specific initiation. To determine the site specificity of initiation in the soluble extract, the transcription products of circular and linear templates were analyzed by gel electrophoresis (Fig. 2). A large number of bands heterogeneous in size and up to 4 to 5 kilobases in length were synthesized in reactions containing circular pHpaF DNA (Fig. 2, lane 4). When this DNA was linearized at either the *Sal*I, *Bam*HI, or *Hin*dIII site within the vector, major bands of 930, 655, and 309 nucleotides were obtained. These were the size classes expected for runoff RNAs correctly initiated and transcribed from the 38-kilodalton (38K) gene (Fig. 2, lanes 5, 6, and 7). We also observed a background of many less intense bands that corresponded to RNAs produced in reactions programmed with vector DNA alone (Fig. 2, lane 3). The results from transcription reactions containing different DNA templates indicated that the extract recognized four other early gene promoters located within the 7-kbp region of interest. Specifically, runoff transcripts of the expected size were synthesized from templates carrying the 63, 13, 15, and 25K genes (Fig. 2, lanes 8 and 9). No evidence of correct initiation of transcription of the 40K late gene was found (data not shown).

By means of S1 nuclease mapping, transcription of closed circular pV7 DNA, a complex template containing several genes, was studied and compared with similarly treated RNA synthesized by intact viral cores in parallel reactions. [^{32}P]RNA was hybridized under conditions of DNA excess to unlabeled restriction fragments from the 7-kbp region. The hybridization reactions were then digested with S1 nuclease, and the protected RNA bands were resolved by electrophoresis. In initial experiments, a background of nuclease-resistant material was present in samples not containing any DNA probe. This background reflected self-annealing of symmetrically transcribed RNAs. We eliminated this background by preparatively hybridizing the RNA, before S1 treatment, to DNA filters containing the separated strands of a recombinant phage clone. The RNA obtained from this procedure was derived only from the sense strand (leftward reading in the vaccinia virus genome). As measured by DNA filter binding, the soluble extract transcribed 20 times as much RNA from the sense strand as from the noncoding strand. In addition, the precise correspondence between the soluble system and viral cores as to the sizes of the major S1-resistant bands (Fig. 3) indicated that both the extract and the cores use the same initiation sites. Bands 302 and 690 nucleotides in length hybridized to probes F and C (Fig. 3) and reflected transcription of the 38 and 63K genes (Fig. 3, lanes 2, 3, 7, 8). Transcription of the 13 and 15K genes, which resulted in bands 270 and 940 nucleotides long detected with the A1 probe (Fig. 3, lanes 4 and 9), and RNA initiation at the 25K gene produced a band of 630 nucleotides detected with the A2 probe (lanes 5 and 10). In both the core and the soluble extract, the 38K gene was more actively transcribed than were the 63, 13, and 15K genes. It should be noted, however, that the cores transcribed the 25K gene at a high level, but the soluble extract did not. This observation most likely reflects an incomplete promoter for the 25K gene

on the pV7 template, as the RNA initiation site on this template resides only 50 bases downstream from the terminal *Eco*RI site.

Kinetic analysis. A kinetic analysis of transcription in soluble extracts is shown in Fig. 4. To generate runoff RNAs covering a range of sizes, a single reaction was programmed with a mixed DNA template. The template mixture was prepared by linearizing pHpaF DNA at six different restriction sites in separate digests. Equal amounts of the digests were then combined for use. Samples of the reaction were withdrawn at various times, and those RNA species whose synthesis was complete were identified by gel electrophoresis. The smallest runoff transcript, 309 nucleotides in length, was first detected after 2 or 3 min of incubation. The 655-, 930-, and 1,704-nucleotide bands did not appear until 6, 8, and 20 min, respectively, and significant levels of the 2,347- and 3,892-nucleotide bands were synthesized only in the sample incubated for 60 min. From these data we calculated a rate of RNA chain elongation in the extract of 100 nucleotides per min (1.7/s). It is also apparent from Fig. 4 that RNA chain initiation is confined to the first 5 to 10 min of the reaction, since the maximum level of accumulation of each runoff species was attained shortly after its synthesis was first detected.

Requirement for multiple components. We used several different approaches to demonstrate that transcription in the soluble extract involves both RNA polymerase and other components distinct from the polymerase. In one scheme extracts were fractionated by glycerol gradient centrifugation. The peak of RNA polymerase activity was located by assaying the ability of each fraction to synthesize RNA with a single-stranded template. Slower-sedimenting fractions were pooled separately. Neither the RNA polymerase nor the pooled postpolymerase fractions alone was sufficient to correctly initiate transcription at the 38K promoter on pHpaF (Fig. 5). The correct runoff product was synthesized only in reactions containing both types of gradient fractions. In a second fractionation scheme, RNA polymerase was purified by ion-exchange chromatography on DEAE-cellulose and phosphocellulose columns. This partially purified enzyme could correctly transcribe pHpaF at significant levels only when supplemented with the pooled, postpolymerase fractions of the glycerol gradient.

Drug inhibition studies. The sensitivity of transcription activity in soluble extracts to several drugs that inhibit other transcription systems is shown in Fig. 6. The level of synthesis of the correct 309 nucleotide runoff product of pHpaF DNA was reduced in the presence of actinomycin D (Fig. 6, lanes 2 and 3), but was unaffected by the addition of alpha-amanitin or rifampin (lanes 4 and 5). The response of the soluble extract to these drugs parallels that observed for intact cores. An intriguing result was obtained with novobiocin, an inhibitor of the virion topoisomerase (7). At concentrations below 750 μM, novobiocin stimulated synthesis of

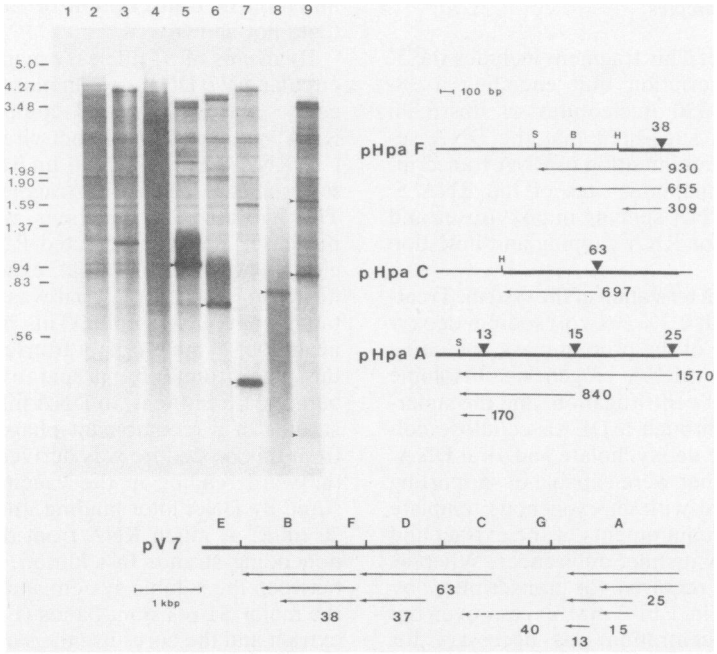

FIG. 2. Electrophoretic analysis of transcription directed by native DNA templates. RNAs synthesized by soluble extracts were analyzed by electrophoresis in a 1.5% agarose gel after denaturation with glyoxal. Standard 50-μl reactions each contained 1 μg of the following DNA templates: lane 1, no DNA; lane 2, uncleaved pBR322; lane 3, pBR322 cleaved with *Hin*dIII; lane 4, uncleaved pHpaF; lane 5, pHpaF cleaved with *Sal*I; lane 6, pHpaF cleaved with *Bam*HI; lane 7, pHpaF cleaved with *Hin*dIII; lane 8, pHpaC cleaved with *Hin*dIII; lane 9, pHpaA cleaved with *Sal*I. For reference the position and size in kilobases of glyoxal treated restriction fragments of phage lambda DNA cleaved with *Hin*dIII and *Eco*RI are given to the left of the autoradiograph. Segments of recombinant plasmid templates are depicted on the right. Thicker lines indicate sequences derived from vaccinia virus, and inverted triangles mark the location of the 5′ termini of RNAs synthesized in vivo. The number above each triangle refers to the size in kilodaltons of the polypeptide encoded by the RNA. Restriction sites for *Sal*I, *Bam*HI, and *Hin*dIII are shown by the letters S, B, and H, respectively. The arrows below each plasmid map represent the position of the runoff transcripts indicated in the autoradiograph. The predicted length of runoff transcripts (in nucleotides) is given to the right. Also shown is a transcription map of the 7-kbp *Eco*RI region of the vaccinia virus genome cloned in pV7, indicating the location of RNAs synthesized in vivo and the size of the polypeptide gene products. A dashed arrow represents the 3′-terminal heterogeneity of late mRNA specifying the 40K protein. Letters A through E refer to the *Hpa*II subfragments of the 7-kbp region.

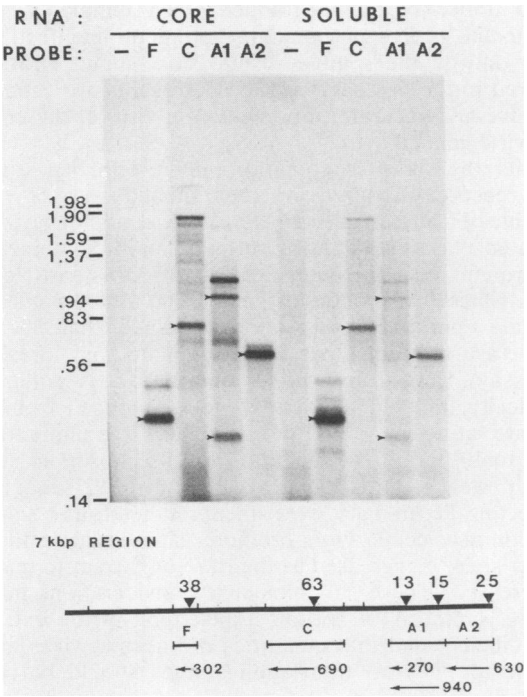

FIG. 3. Comparison of transcription initiation sites utilized in vaccinia virus cores and soluble extract by nuclease S1 analysis. ^{32}P-labeled RNA synthesized in intact viral cores or in soluble extract in response to closed circular pV7 DNA was hybridized to immobilized DNA filters containing the separated strands of the 7-kbp region. RNA transcribed from the coding strand was recovered and annealed in DNA excess to unlabeled restriction fragments derived from pV7. After nuclease S1 digestion, protected RNA fragments were resolved by gel electrophoresis in 1.5% agarose after glyoxal denaturation. Below the autoradiograph is a map of the 7-kbp region showing the *Hpa*II restriction sites and the in vivo transcription start points for the genes encoding the 38, 63, 13, 15, and 25K polypeptides. DNA probes included the *Hpa*II F and C fragments and two fragments obtained from the *Hpa*II-A region by cleavage with *Hpa*I. The arrow below each probe represent the position and size of nuclease-resistant RNA bands indicated in the autoradiograph.

the 309-nucleotide transcript in the soluble system (Fig. 6, lanes 6, 7, and 8), but at higher concentrations of the drug, the synthesis of this polynucleotide was suppressed (lane 9). In contrast, novobiocin had no effect on RNA synthesis in transcription assays programmed with a single-stranded template (data not shown).

Because transcription by vaccinia virus cores shows multiple requirements for ATP (8, 30), we decided to examine the effects of nucleotide analogs on transcription in the soluble extract. In the presence of a single-stranded DNA template, purified RNA polymerase readily incorporates the ATP analogs AMPPNP and ATPγS into RNA. In contrast, core-mediated transcription is severely reduced by substitution of either of these analogs for ATP. This evident requirement for an unmodified β-γ phosphodiester bond is specific for adenosine nucleotides, since the corresponding GTP analogs fully support transcription. RNA synthesis in soluble extracts closely resembles that of the virion system with respect to the effects of ATP analogs (Fig. 7). Synthesis of the correct runoff product in the extract was completely and specifically abolished when ATP was replaced with either AMPPNP or ATPγS, although the corresponding GTP analogs supported transcription. Furthermore, inhibition of transcription by AMPPNP in both virion cores and the soluble extract could be reversed by addition of either ATP or dATP. Unlike the result reported with permeabilized virions, however, inhibition of soluble extracts by ATPγS was not reversed by ATP (or dATP).

Since mRNA produced by intact cores is both capped and polyadenylated by virion enzymes, it was of interest to determine whether the RNAs synthesized in soluble extracts were similarly modified. Experiments with [^{3}H]*S*-adenosylmethionine as a methyl donor for RNA capping indicated that the runoff transcript, which initiates at the promoter of the 38K gene, was modified at its 5′ terminus (Fig. 8). Excision of the 309-nucleotide bands labeled with [^{3}H]*S*-adenosylmethionine or [^{3}H]UTP and quantitation of the radioactivity in a liquid scintillation counter indicated 0.6 mol of methylated nucleotide was incorporated per mol of RNA chain. Labeling by [^{3}H]*S*-adenosylmethionine was specific for the capping reaction, as only methylation and not synthesis of the runoff transcript was inhibited by *S*-adenosylhomocysteine, an antagonist of RNA capping (19).

In electrophoretic analyses of runoff transcripts from pHpaF templates truncated with *Sal*I or *Bam*HI (but not *Hin*dIII), we observed that the primary band was followed by larger, heterogeneous species (Fig. 2, lanes 5, 6, and 7). These trailing components could have resulted from polyadenylation of a fraction of the runoff transcripts. To assay for polyadenylation in the soluble extract, reactions were programmed with pHpaF DNA that had been linearized by digestion with *Sal*I. The RNA transcribed from this template was subjected to oligodeoxythymidylic acid-cellulose chromatography (Fig. 9), and gel electrophoresis was used to

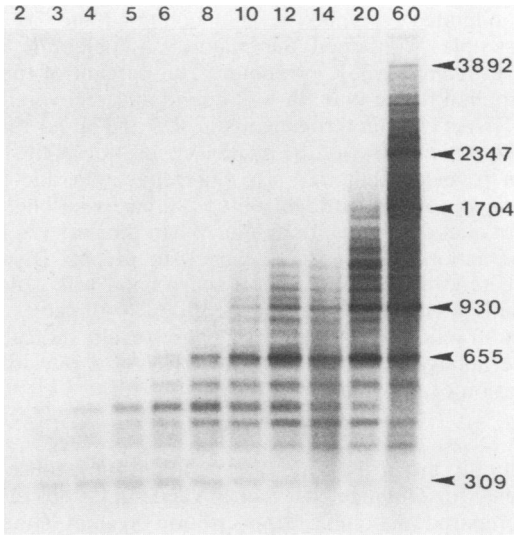

FIG. 4. Kinetic analysis of transcription in soluble extract. A mixed template was prepared by pooling equal amounts of full-length pHpaF DNA linearized by cleavage at the *Hin*dIII, *Bam*HI, *Sal*I, *Ava*I, *Pvu*II, or *Pst*I site within the pBR322 sequence. A standard transcription mixture scaled up to 100-μl volume, omitting DNA, was preincubated at 30°C for 4 min before 2 μg of the mixed template was added. At the time (in minutes) indicated above each lane a 5-μl sample was withdrawn and processed for gel analysis. RNAs were resolved by electrophoresis in a 1% agarose gel after glyoxal denaturation. The positions of runoff transcripts resulting from initiation at the vaccinia virus 38K promoter and the predicted sizes in nucleotides and indicated to the right of the autoradiograph.

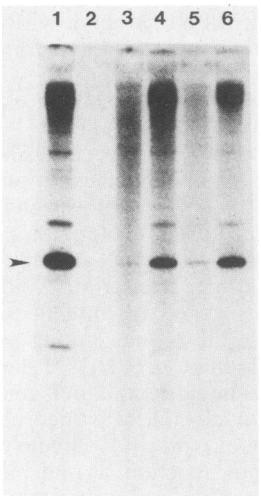

FIG. 5. Reconstruction of transcription activity in fractionated soluble extract. RNA was synthesized in transcription reactions containing 1 μg of *Hin*dIII-cleaved pHpaF DNA and the following: lane 1, 10 μg of unfractionated soluble extract (complete system); lane 2, 5 μl of the postpolymerase fraction obtained by glycerol gradient centrifugation of crude extract; lane 3, 5 μl of the RNA polymerase peak from the glycerol gradient; lane 4, 5 μl of gradient-purified RNA polymerase and 5 μl of the postpolymerase fraction; lane 5, 15 μl of RNA polymerase purified by DEAE-cellulose and phosphocellulose chromatography; lane 6, 15 μl of chromatographically purified RNA polymerase and 5 μl of the postpolymerase glycerol gradient fraction. RNA was analyzed by electrophoresis in a 4% acrylamide gel. The position of the 309-nucleotide runoff transcript is indicated to the left of the autoradiograph.

compare the bound material (lane 3) with both the unfractionated reaction mixture (lane 1) and the RNAs not bound by the oligodeoxythymidylic acid column (lane 2). These latter samples contained the major 955-nucleotide runoff transcript from the 38K promoter. Ten percent of the total RNA applied to the column was bound and recovered. The bound RNAs were heterogeneous in size and larger than the 955-nucleotide transcript. S1 nuclease digestion of the bound fraction revealed that this size difference was due to the presence of polynucleotides 100 to 200 bases in length that were not encoded by the template. When the material in this bound fraction was hybridized back to pHpaF DNA and then treated with S1 nuclease, a sharp band was generated that comigrated with the 955 nucleotide RNA species of the unbound fraction (Fig. 9, lane 4). These results indicate that the size heterogeneity of the bound RNAs is due to polyadenylation of the runoff transcripts.

DISCUSSION

By modifying a sodium deoxycholate procedure used previously to solubilize enzymes in vaccinia virions (27), we have prepared a soluble transcription system composed entirely of viral proteins. Other extraction procedures described before this report include nonionic detergents such as Triton X-100 and Nonidet P-40 in addition to sodium deoxycholate for the preparation of crude extracts of virus particles (1, 32). Our findings indicate that nonionic detergents (but not sodium deoxycholate, which is rapidly removed by ion-exchange chromatography) appear to irreversibly inactivate the accurate transcription of double-stranded DNA templates by these extracts. Presumably the nonionic detergent affects some element(s) required for proper transcription, but does not affect the ability of the viral polymerase to transcribe single-stranded DNA templates. These detrimental effects of nonionic detergent manifest themselves only in the soluble system since intact viral cores prepared in the presence of such detergents are capable of extensive and accurate transcription in vitro of the endogenous viral genome.

Under the standard conditions defined in this study, a 50-μl reaction mixture was programmed with 5×10^{10} molecules of linearized template DNA containing a single, truncated vaccinia virus transcription unit. Solubilized virion proteins from the equivalent of 10^{10} virus particles was added. Since there are an estimated 100 to 200 polymerase molecules per virion, it was determined that 20 to 40 polymerase molecules per DNA template were present in this assay. Under these conditions of excess polymerase specifically initiated transcripts were produced from each template, at nearly a 1:1 ratio. Initiation was limited to the first 5 to 10 min of the reaction. We also observed several distinct, weaker initiation sites in the pBR322 sequence of the vector. Preliminary experiments indicate that selective initiation at vaccinia virus promoter sites, relative to these weaker sites, is increased as the ratio of extract to template is decreased. These stoichiometric considerations indicate that the synthesis of specific transcripts by the extract is fairly efficient and that quantities of vaccinia virus mRNA suitable for in vitro translation studies should be readily obtained.

It has been reported that double-stranded DNAs are poor templates for purified preparations of vaccinia virus DNA-dependent RNA polymerase, whereas single-stranded templates are transcribed 50 to 100 times more actively (1, 26, 32). In our crude soluble extract, single-stranded and dou-

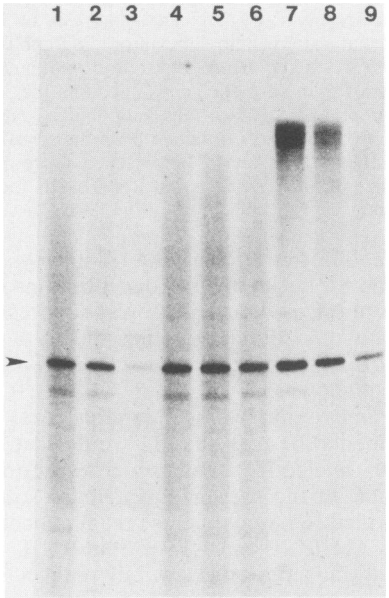

FIG. 6. Effects of inhibitory drugs on transcription in soluble extract. RNAs synthesized in standard transcription reactions containing 1 μg of *Hin*dIII-cleaved pHpaF DNA were analyzed by electrophoresis in a 4% polyacrylamide gel. Incubation mixtures were supplemented with the following: lane 1, no addition; lanes 2 and 3, 1 and 5 μg of actinomycin D per ml, respectively; lane 4, 300 μg of α-amanitin per ml; lane 5, 100 μg of rifampin per ml; lanes 6 to 9, 0.25, 0.5, 0.75, and 1.0 mM novobiocin, respectively. The position of the 309-nucleotide runoff transcript is indicated to the left of the autoradiograph.

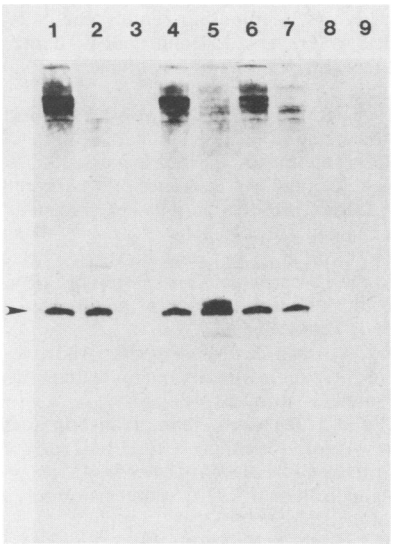

FIG. 7. Effects of nucleotide analogs on transcription in soluble extract. Trancription reactions directed by *Hin*dIII-cleaved pHpaF DNA and labeled with [^{3}H]UTP were carried out under standard conditions, except for the following nucleotide changes: lanes 1 and 6, no substitutions (standard system); lane 2, GTP was replaced with 1 mM GMPPNP; lane 3, ATP was replaced with 1 mM AMPPNP; lane 4, both 1 mM AMPPNP and 1 mM ATP were added; lane 5, both 1 mM AMPPNP and 1 mM dATP were added; lane 7, GTP was replaced with 1 mM GTPγS; lane 8, ATP was replaced with 1 mM ATPγS; lane 9, both 1 mM ATPγS and 1 mM ATP were added. RNAs were analyzed on a 4% polyacrylamide gel. To the left of the fluorograph the position of the 309-nucleotide runoff transcript is indicated.

ble-stranded templates support nearly equal levels of RNA synthesis. Furthermore, we have demonstrated that DNA-dependent RNA polymerase that has been only partially purified not only shows a strong preference for single-stranded DNA, but also is incapable of accurately initiating and transcribing well-defined double-stranded templates containing vaccinia virus early genes. The addition of other virion proteins, themselves lacking polymerase activity, to the partially purified polymerase restored the capacity for proper transcription. These observations are consistent with the idea that vaccinia virus polymerase requires additional viral proteins or factors to correctly synthesize viral mRNA. We found that such transcription factors were separated from the vaccinia virus RNA polymerase during the early stages of polymerase purification. The early loss of these factors is consistent with the behavior of other eucaryotic RNA polymerases during purification (28) and distinguishes the vaccinia virus enzyme from many procaryotic polymerases (5). Because our soluble transcription system consists entirely of viral components, it should be a powerful tool for identifying individual, required elements that work in conjunction with transcriptase to synthesize viral RNA.

The parallel inhibition of topoisomerase activity and transcription with increasing levels of novobiocin and coumermycin A (7) suggests the possible involvement of vaccinia virus topoisomerase in the transcription of early genes by virion cores. As reported above, transcription of native templates in soluble extracts is also sensitive to novobiocin, although with a different dose-response curve from that of cores. Whereas a nonspecific inhibition of transcription by novobiocin cannot be ruled out, the observed lack of effect of the drug on RNA polymerase activity with single-stranded templates is consistent with the idea that topoisomerase is involved in transcription. Soluble extracts show a more stringent requirement for an unmodified β-γ phosphodiester bond in adenosine nucleotides than that found for RNA polymerase. This observation suggests a critical ATP-utilizing activity in transcription, distinguished by its ability to use dATP, but not ATPγS, as a substrate. At least one virion enzyme, the nucleic acid-dependent phosphohydrolase I, is known to possess such properties (30).

The enzymes that mediate post-transcriptional modification of RNA occur in vaccinia virus extracts. Therefore, it is not surprising that the RNAs synthesized in vitro by our system are modified in this manner. The methylation (and presumptive capping) of RNAs is significant, since it suggests the possibility of analyzing such RNAs by in vitro translation. Information obtained by translation studies would greatly facilitate the mapping of polypeptides on specific regions of the viral genome. The polyadenylation of runoff transcripts is also noteworthy in that the 3′-proximal regions of these synthetic RNAs lack vaccinia virus-specific sequences. Polyadenylation of transcripts generated in whole mammalian cell extracts requires a specific recognition signal near the RNA 3′-terminus (17). In contrast, Gershowitz and Moss previously found that abortive transcripts produced in vitro and in vivo by UV-irradiated vaccinia virus particles were polyadenylated (9). Their observation and our results suggest that the vaccinia virus enzyme is less specific. In support of this concept is the fact that the late transcripts from a given gene are extremely heterogeneous in size. This heterogeneity is due to an apparently random termination at the 3′ ends of the RNAs, yet all size classes of transcripts are extensively polyadenylated.

A potentially useful property of the soluble extract is its ability to discriminate quantitatively in the level of initiation

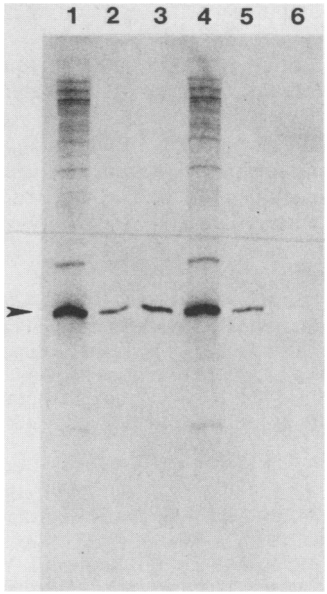

FIG. 8. Methylation of runoff transcripts synthesized in soluble extract. RNAs synthesized in standard transcription reactions containing *Hin*dIII-cleaved pHpaF DNA were labeled with the following: lanes 1 and 4, [α-^{32}P]GTP; lanes 2 and 5, [^{3}H]UTP; and lanes 3 and 6, [^{3}H]*S*-adenosylmethionine. The reactions shown in lanes 4, 5, and 6 were also supplemented with 0.1 mM *S*-adenosylhomocysteine. A fluorograph of the electrophoretic analysis in a 4% polyacrylamide gel is shown.

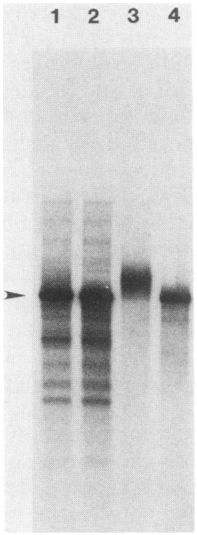

FIG. 9. Polyadenylation of runoff transcripts in soluble extract. RNA synthesized in soluble extract templated with *Sal*I-cleaved pHpaF DNA was fractionated by oligodeoxythymidylic acid-cellulose chromatography. A sample of retained RNA was hybridized back to *Sal*I-cleaved pHpaF DNA in 80% formamide buffer at 39°C before digestion with nuclease S1. Lane 1, total RNA; lane 2, RNA not bound to column; lane 3, RNA retained on oligodeoxythymidylic acid-cellulose; lane 4, oligodeoxythymidylic acid-retained RNA after S1 digestion. RNAs were analyzed by electrophoresis in 1.5% agarose after glyoxal denaturation. The position of the 955-nucleotide transcript resulting from initiation at the 38K promoter is indicated to the left of the autoradiograph.

observed with different vaccinia virus promoters. Based on the limited set of early genes we have examined, the relative level of transcription in the soluble system generally reflects the relative abundance of the respective mRNAs synthesized by cores in vitro and in infected cells. It may therefore be possible to optimize the in vitro system for obtaining accurate measurements of promoter strengths. Our system would then be applicable to studies with in vitro mutagenesis to modify promoter sequences. Such studies are necessary to define the parameters that regulate the level of gene expression in vaccinia virus and to construct artificial promoters for use with poxvirus expression vectors.

ACKNOWLEDGMENTS

We extend special thanks to Ame Holden for her excellent technical assistance, and we are grateful to Jacqueline Siegel for her critical review of this manuscript and helpful suggestions.

This research was supported in part by Public Health Service research grant AI 15941 from the National Institute of Allergy and Infectious Diseases awarded to J.R.K.

LITERATURE CITED

1. **Baroudy, B. M., and B. Moss.** 1980. Purification and characterization of a DNA-dependent polymerase from vaccinia virions. J. Biol. Chem. **255**:4372–4380.
2. **Bauer, W. R., E. C. Ressner, J. Kates, and J. V. Patzke.** 1977. A DNA nicking-closing enzyme encapsidated in vaccinia virus: partial purification and properties. Proc. Natl. Acad. Sci. U.S.A. **74**:1841–1845.
3. **Belle Isle, H., S. Venkatesan, and B. Moss.** 1981. Cell-free translation of early and late mRNAs selected by hybridization to cloned DNA fragments derived from the left 14 million to 72 million daltons of the vaccinia virus genome. J. Virol. **112**:306–317.
4. **Bolivar, F., and K. Backman.** 1979. Plasmids of *Escherichia coli* as cloning vectors. Methods Enzymol. **68**:245–267.
5. **Burgess, R., A. Travers, J. Dunn, and E. Bautz.** 1969. Factor stimulating transcription by RNA polymerase. Nature (London) **211**:43.
6. **Cooper, J. A., R. Wittek, and J. Moss.** 1981. Extension of the transcriptional and translational map of the left end of the vaccinia virus genome to 21 kilobase pairs. J. Virol. **39**:733–745.
7. **Foglesong, P. D., and W. R. Bauer.** 1984. Effects of ATP and inhibitory factors on the activity of vaccinia virus type I topoisomerase. J. Virol. **49**:1–8.
8. **Gershowitz, A., R. F. Boone, and B. Moss.** 1978. Multiple roles for ATP in the synthesis and processing of mRNA by vaccinia virus: specific inhibitory effects of adenosine (β,γ-imido) triphosphate. J. Virol. **27**:399–408.
9. **Gershowitz, A., and B. Moss.** 1979. Abortive transcription products of vaccinia virus are guanylyated, methylated, and polyadenylated. J. Virol. **31**:849–853.
10. **Golini, F., and J. R. Kates.** 1984. Transcriptional and translational analysis of a strongly expressed early region of the vaccinia virus genome. J. Virol. **49**:459–470.
11. **Kates, J., and J. Beeson.** 1970. Synthesis of poly A by vaccinia cores. J. Mol. Biol. **50**:19–33.
12. **Kates, J. R., and B. R. McAuslan.** 1967. Poxvirus DNA-dependent RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. **58**:134–141.
13. **Mackett, M., G. L. Smith, and B. Moss.** 1982. Vaccinia virus: A selectable eukaryotic cloning and expression vector. Proc. Natl. Acad. Sci. U.S.A. **79**:7415–7419.
14. **Mahr, A., and B. E. Roberts.** 1984. Organization of six early transcripts synthesized from a vaccinia virus *Eco*RI DNA fragment. J. Virol. **49**:497–509.
15. **Mahr, A., and B. E. Roberts.** 1984. Arrangement of late RNAs transcribed from a 7.1-kilobase *Eco*RI vaccinia virus DNA fragment. J. Virol. **49**:510–520.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Manley, J. L.** 1983. Accurate and specific polyadenylation of mRNA precursors in a soluble whole-cell lysate. Cell **33**:595–605.
18. **Manley, J. L., A. Fire, A. Cano, P. A. Sharp, and M. L. Gefter.** 1980. DNA-dependent transcription of adenovirus genes in a soluble whole cell extract. Proc. Natl. Acad. Sci. U.S.A. **77**:3855–3859.
19. **Martin, S. A., and B. Moss.** 1975. Modification of RNA by mRNA guanylyl-transferase and mRNA (guanine-7) methyltransferase from vaccinia virus. J. Biol. Chem. **250**:9330–9335.
20. **Martin, S. A., E. Paoletti, and B. Moss.** 1975. Purification of mRNA guanylyltransferase and mRNA (guanine-7) methyltransferase from vaccinia virions. J. Biol. Chem. **250**:9322–9329.
21. **McMaster, G. K., and G. C. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. U.S.A. **74**:4835–4838.
22. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101**:20–77.
23. **Moss, B., A. Gershowitz, W. C. Wei, and R. Boone.** 1976. Formation of the guanylated and methylated 5′-terminus of vaccinia virus mRNA. Virology **72**:341–351.
24. **Moss, B., E. N. Rosenblum, and E. Paoletti.** 1973. Polyadenylate polymerase from vaccinia virus. Nature (London) New Biol. **245**:59–62.
25. **Munyon, W., E. Paoletti, and J. T. Grace.** 1967. RNA polymerase activity in purified infectious vaccinia virus. Proc. Natl. Acad. Sci. U.S.A. **58**:2280–2287.
26. **Nevins, J. R., and W. K. Joklik.** 1977. Isolation and properties of the vaccinia virus DNA-dependent RNA polymerase. J. Biol. Chem. **252**:6930–6938.
27. **Paoletti, E., H. Rosemond-Hornbeak, and B. Moss.** 1974. Two nucleic acid-dependent nucleoside triphosphate phosphohydrolases from vaccinia virus. J. Biol. Chem. **249**:3273–3280.
28. **Parker, C. S., J. A. Jaehning, and R. G. Roeder.** 1977. Faithful gene transcription by eukaryotic RNA polymerases in reconstructed systems. Cold Spring Harbor Symp. Quant. Biol. **42**:577–588.

29. **Puckett, C., and B. Moss.** 1983. Selective transcription of vaccinia virus genes in template dependent soluble extracts of infected cells. Cell **35**:441–448.
30. **Shuman, S., E. Spencer, H. Furneaux, and J. Hurwitz.** 1980a. The role of ATP in *in vitro* vaccinia virus RNA synthesis. J. Biol. Chem. **255**:5396–5403.
31. **Shuman, S., M. Surks, H. Furneaux, and J. Hurwitz.** 1980b. Purification and characterization of a GTP-pyrophosphate exchange activity from vaccinia virions. J. Biol. Chem. **255**: 11588–11598.
32. **Spencer, E., S. Shuman, and J. Hurwitz.** 1980. Purification and properties of vaccinia virus DNA-dependent RNA polymerase. J. Biol. Chem. **255**:5388–5395.
33. **Traktman, P., P. Sridhar, R. C. Condit, and B. E. Roberts.** 1984. Transcriptional mapping of the DNA polymerase gene of vaccinia virus. J. Virol. **49**:125–131.
34. **Venkatesan, S., B. M. Baroudy, and B. Moss.** 1981. Distinctive nucleotide sequences adjacent to multiple initiation and termination sites of an early vaccinia virus gene. Cell **125**:805–813.
35. **Venkatesan, S., and B. Moss.** 1981. In vitro transcription of the inverted terminal repetition of the vaccinia genome: correspondence of initiation and cap sites. J. Virol. **37**:738–747.
36. **Wei, C. M. and B. Moss.** 1975. Methylated nucleotides block 5′-terminus of vaccinia virus messenger RNA. Proc. Natl. Acad. Sci. U.S.A. **72**:318–322.
37. **Weil, P. A., D. S. Luse, J. Segall, and R. G. Roeder.** 1979. Selective and accurate initiation of transcription at the Ad2 major late promoter in a soluble system dependent on purified RNA polymerase II and DNA. Cell **18**:469–484.
38. **Wittek, R., J. A. Cooper, E. Barbosa, and B. Moss.** 1980. Expression of the vaccinia virus genome: analysis and mapping of mRNAs encoded within the inverted terminal repetition. Cell **21**:487–493.
39. **Wittek, R., M. Hanggi, and G. Hiller.** 1984. Mapping of a gene coding for a major late structural polypeptide on the vaccinia virus genome. J. Virol. **49**:371–378.