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Selective Transcription of Vaccinia Virus Genes in Template Dependent Soluble Extracts of Infected Cells

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Summary

A soluble system that specifically and accurately initiates transcription on defined vaccinia virus templates has been obtained from lysates of infected cells. The required regulatory signals are contained within a DNA segment extending about 230 bp upstream and 30 bp downstream of the RNA start site. Transcription is resistant to α-amanitin and inhibited by antibodies to the viral RNA polymerase. Whole cell extracts from uninfected cells cannot accurately transcribe vaccinia DNA. Conversely, extracts prepared at 2 hr or later after vaccinia infection no longer transcribe RNA polymerase II templates but retain the ability to transcribe RNA polymerase III templates as well as vaccinia virus DNA. These profound changes in transcriptional specificity may contribute to the selective expression of viral genes following vaccinia infection.

Introduction

Poxviruses are large DNA viruses that encode biosynthetic enzymes, enabling them to replicate in the cytoplasm of eucaryotic cells. Nevertheless, there are many similarities between poxvirus and eucaryotic transcription. RNA synthesized in vitro by infectious vaccinia virus particles is polyadenylated, capped, and methylated, and can be accurately translated in reticulocyte cell-free systems (Kates and Beeson, 1970; Wei and Moss, 1975; Cooper and Moss, 1978). Moreover, comparisons of the individual capping and methylating enzymes of vaccinia virus and HeLa cells suggest similar specificities and mechanisms of action (Ensinger and Moss, 1976; Langberg and Moss, 1981; Venkatesan and Moss, 1982). Like its eucaryotic counterpart, the viral RNA polymerase is a complex enzyme with a molecular weight of about 500,000 daltons and contains two large polypeptides greater than 100,000 daltons as well as several smaller ones (Nevins and Joklik, 1977; Baroudy and Moss, 1980; Spencer et al., 1980). After purification, neither eucaryotic RNA polymerase II nor vaccinia RNA polymerase can initiate transcription accurately and both enzymes require single-stranded DNA templates and Mn^{2+} for optimal activity in vitro. Some of the other enzymes that have been isolated from vaccinia virus particles might be involved in initiation and in later steps of transcription. This list of enzymes includes two nucleic acid dependent nucleoside triphosphatases (Paoletti and Moss, 1974), poly (A) polymerase (Moss et al., 1975), protein kinase (Kleiman and Moss, 1975), topoisomerase (Bauer et al., 1977), 5′-phosphate polynucleotide kinase (Spencer et al., 1978), endoribonuclease (Paoletti and Lipinskas, 1978), and deoxyribonucleases (Rosemond-Hornbeak and Moss, 1974; Pogo and O'Shea, 1978).

Although the unique features of vaccinia virus can greatly facilitate genetic and biochemical studies of RNA synthesis, the absence of a soluble transcription system dependent on an exogenous double-stranded template has retarded progress. The development of RNA polymerase II eucaryotic transcription systems followed the availability of defined templates and the design of specific assays that focused on the initiation step in the complex process (Weil et al., 1979; Manley et al., 1980). Recent advances in the transcriptional mapping and sequencing of the vaccinia virus genome revealed that the nucleotides upstream of several early vaccinia virus genes are extremely rich in adenine and thymine residues and differ significantly from both procaryotic and eucaryotic consensus sequences (Venkatesan et al., 1981, 1982; Weir and Moss, 1983). Evidence that this region contains transcriptional regulatory signals comes from studies using vaccinia virus as a vector for foreign genes (Mackett et al., 1982; Smith et al., 1983). A segment of DNA, including the RNA start site and about 230 bp upstream and 30 bp downstream, promoted expression of several different coding sequences.

Here, we show that RNA synthesis is accurately initiated on defined vaccinia DNA templates by a transcription system prepared from cells at two to six hr after virus infection. Significantly, transcription is resistant to α-amanitin and inhibited by antibodies to the viral RNA polymerase. Furthermore, whole cell extracts prepared at two hr or later after infection no longer transcribe templates ordinarily recognized by RNA polymerase II but retain the ability to transcribe RNA polymerase III templates. These changes in transcriptional specificity may contribute to the selective expression of viral genes following infection.

Results

Transcription of Truncated Vaccinia Virus DNA Templates In Vitro

Whole cell extracts were prepared according to the procedure of Manley and Gefter (1981) from HeLa cells at various times after infection with vaccinia virus. For a template, we chose a segment of vaccinia virus DNA that was transcriptionally mapped (Wittek et al., 1980; Cooper et al., 1981), sequenced (Venkatesan et al., 1981), and shown to contain signals needed for gene expression in vivo (Mackett et al., 1982). Since the 1 kb RNA encoded by this DNA is translatable in vitro to give a polypeptide of 7500 daltons, the name 7.5K gene has been used. It has been classified as an immediate early gene because large amounts of the mRNA are made in vitro by virus cores and in vivo in the presence of inhibitors of protein synthesis. The plasmid pAG4 (Venkatesan et al., 1982) contains about 230 bp of DNA upstream of the RNA start site and

about 730 bp downstream but does not include termination regions. At the bottom of Figure 1, the solid and interrupted lines represent DNA of vaccinia and plasmid origin respectively, and the position corresponding to the 5′ end of the mRNA is indicated. When pAG4 is cut with restriction endonucleases Pvu I, Sal I, and Nru I, correctly initiated run-off transcripts would be 626, 730, and 1051 nucleotides respectively. In extracts of cells prepared at 6 hr after vaccinia infection, α^{32}P-UTP labeled RNA species close to the predicted sizes were synthesized from these truncated templates (Figure 1). The minor size discrepancies may be attributed to the use of DNA molecular weight standards (Luse and Roeder, 1980). In succeeding sections of this paper, evidence will be presented that accurate initiation of transcription on a vaccinia template is dependent on viral enzymes.

In addition to specific transcripts, higher molecular weight bands are present in all lanes (Figure 1). These products are labeled during in vitro transcription reactions performed with infected or uninfected extracts in the presence or absence of added template. Previous investigators have treated such bands as artifacts because of end-labeling or other enzymatic reactions of the crude lysates (Manley and Gefter, 1981). Accordingly, no further references will be made to such bands.

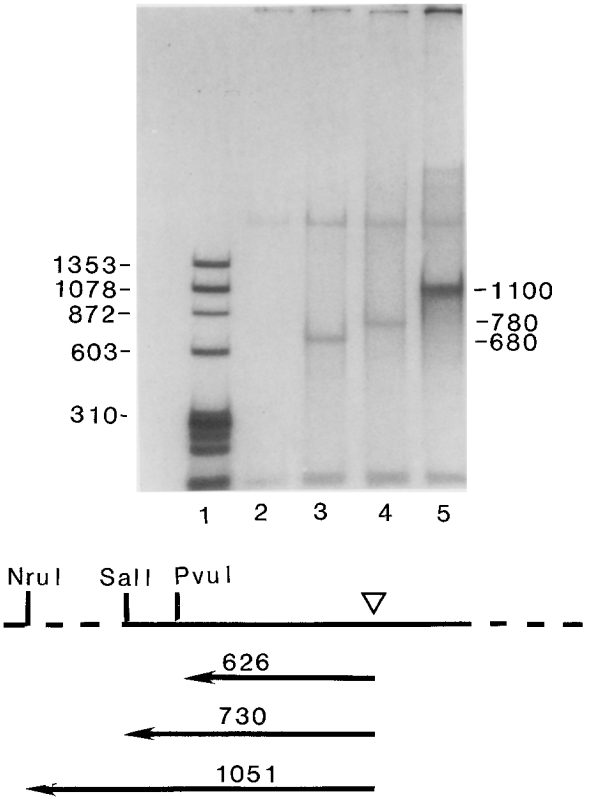

Figure 1. Transcription of Truncated Vaccinia Virus DNA Templates In Vitro

A segment of the plasmid pAG4 is depicted near the bottom. Continuous and interrupted lines represent vaccinia and plasmid DNA sequences respectively. The apex of the triangle points to the in vivo site of initiation of RNA synthesis. Arrows indicate the lengths of predicted run-off transcripts obtained after cleavage of the plasmid with the indicated restriction enzymes. The numbers above the arrows indicate the lengths of the predicted RNAs in nucleotides. An autoradiograph is shown in the upper part of the figure. One microgram of pAG4 cleaved with Pvu I (lane 3), Sal I (lane 4), or Nru I (lane 5) was added to the standard in vitro transcription mixture. No DNA was added to one mixture (lane 2). RNA labeled with α-^{32}P-UTP was purified, glyoxylated, and analyzed by agarose gel electrophoresis. The size in nucleotides of standard glyoxylated DNA markers (lane 1) is shown at the left and the estimated sizes of RNAs at the right.

Isolation of Transcriptional Regulatory Sequences and RNA Start Site

To better define the DNA sequence required for initiation of RNA synthesis, a 265 bp Hinc II–Rsa I fragment containing the region upstream and 32 bp downstream of the in vivo start site was inserted into the Hinc II site of plasmid pUC9 (Mackett et al., submitted). The latter plasmid, named pGS15, has two Ava II sites, one of which is 1080 bp downstream of the in vivo RNA start site and another about 1695 bp upstream. With Ava II cleaved pGS15 as template, a predominant run-off transcript close to the predicted size was obtained (Figure 2, lane 3). In contrast, only minor bands of different size were detected when Ava II cleaved pUC9 was used as template (Figure 2, lane 4). To confirm the in vitro start sites, pGS15 was cleaved with Bam HI and Ava II. The absence of a detectable

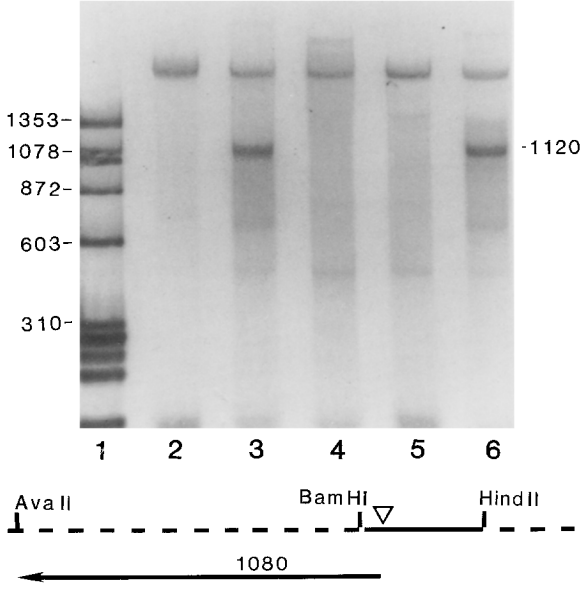

Figure 2. Transcription of a Plasmid Containing Isolated Transcriptional Regulatory Sequences and RNA Start Site

A portion of the plasmid pGS15 is shown at the bottom of the figure. The continuous line represents the 265 bp vaccinia DNA segment and the interrupted lines plasmid DNA. The in vivo RNA start site is indicated by the triangle. The predicted size of the run-off transcript is 1080 nucleotides. An autoradiograph is shown in the upper part of the figure. No DNA (lane 2), 1 μg of pGS15 cleaved with Ava II (lane 3), 1 μg of pUC9 cleaved with Ava II (lane 4), 1 μg of pGS15 cleaved with Ava II and Bam HI (lane 5), 1 μg of pGS15 cleaved with Ava II and Hind II (lane 6) were added to standard transcription reactions. Lane 1 contains DNA size markers. The sizes in nucleotides of markers and transcripts are shown on the left and right respectively.

transcription product was consistent with the location of the Bam HI site only 32 bp downstream of the RNA start site (Figure 2, lane 5). Furthermore, cleavage of pGS15 at a Hinc II site located just upstream of the vaccinia DNA segment and with Ava II did not alter the size of the run-off transcript obtained after cleavage with the latter alone (Figure 2, lane 6). Thus the 265 bp vaccinia DNA fragment appeared to be sufficient for in vitro transcription and intragenic sequences beyond 32 bp were not required.

Determination of the In Vitro Transcriptional Initiation Site by Nuclease S1 Analysis

A more precise determination of the in vitro RNA start site was obtained by nuclease S1 analysis. In one variation of the procedure, a DNA fragment labeled at one 5′ end is hybridized to RNA (Weaver and Weissman, 1979). After nuclease S1 digestion, the protected DNA fragment is analyzed by electrophoresis next to a sequence ladder prepared with the same 5′ labeled DNA. Our preliminary experiments established that viral mRNA was present in the infected cell extracts used for transcription. Moreover, this endogenous RNA hybridized to ^{32}P-labeled vaccinia DNA to give a nuclease S1 protected fragment. To circumvent this problem, Ava II cleaved pGS15 was used as template for in vitro RNA synthesis because only the first 32 nucleotides of the 1050 nucleotide run-off product are complementary to vaccinia DNA. The remainder of the transcript is complementary to plasmid DNA. Therefore, a 5′ ^{32}P-labeled plasmid fragment would be protected by in vitro synthesized RNA but not by the endogenous viral mRNA. A Hind II–Pvu II fragment of 394 bp labeled at the Pvu II 5′ end was used both for hybridization and for preparation of a sequence ladder (Figure 3). The major nuclease S1 protected bands corresponded precisely with the RNA start sites determined previously with in vivo synthesized RNA (Venkatesan et al., 1981). Although not shown in Figure 3, no nuclease S1 protected bands were detected by hybridizing RNA from mock transcription reactions incubated without added template.

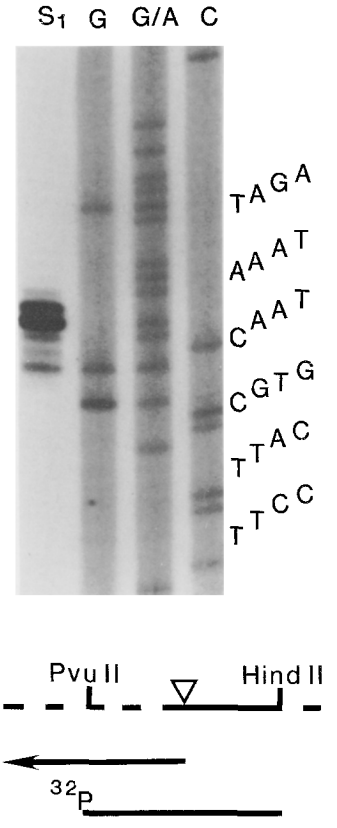

Figure 3. Determination of the In Vitro Transcriptional Initiation Site by Nuclease S1 Analysis

The 265 bp vaccinia DNA segment of pGS15 is shown with a continuous line and plasmid sequences with an interrupted line. The in vivo RNA start site is indicated with a triangle. A run-off transcript depicted by an arrow was hybridized to the indicated 5′ ^{32}P-labeled Pvu II–Hind II fragment. After nuclease S1 digestion, the protected DNA was electrophoresed alongside partial sequencing reactions on a 8% polyacrylamide gel. The previously determined (Venkatesan et al., 1981) DNA sequence is shown on the right. The symbols at the top refer to the S1 digestion products (S1) and to Maxam–Gilbert sequence reactions (G, G/A, C).

Requirement for Upstream Sequences

Experiments described in the previous sections indicated that no more than 32 bp downstream of the RNA start site are required for in vitro transcription. To gain preliminary information regarding the requirement for upstream sequences, the pAG4 template was cut with the restriction enzymes indicated in the bottom of Figure 4. In each case, the DNA fragments were analyzed by agarose gel electrophoresis to check completeness of digestions. Lane 5 shows that the predicted size transcript is produced when 233 bp of upstream sequence is present. When Hpa II and Hph I are used, the upstream sequence is reduced to 100 bp and 66 bp respectively. Although significant reduction in band intensity was noted in three independent experiments, transcripts of the appropriate size were detected (Figure 4, lanes 3 and 4). However, when the RNA start site and upstream sequences were completely removed by cleavage with Sph I, only a faint band possibly generated by end-to-end transcription of the template was generated (Figure 4, lane 2). From these data, we conclude that less than 100 and possibly less than 66 nucleotides upstream of the RNA start site are essential for in vitro transcription.

Specificity of Infected Cell Extracts for Vaccinia Templates

When a vaccinia template was added to a whole cell extract made from uninfected HeLa cells, the predicted size run-off transcripts were not detected (Figure 5). Some correct initiation was obtained with extracts prepared at 2 hr after infection but higher levels were obtained at 4 to 6 hr. In parallel, we checked the template activity of a Bam HI cleaved plasmid, pϕ4, which contains the adenovirus 2 late promoter (Hu and Manley, 1981). This promoter was chosen because it is well characterized and is one of the

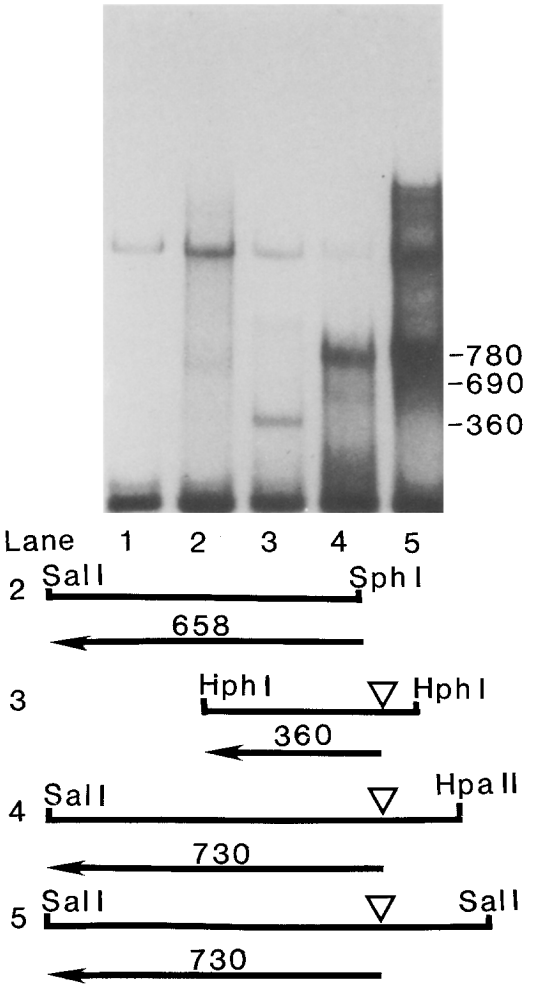

Figure 4. Requirement for Upstream Sequences

pAG4 was digested with one or two restriction enzymes as shown at the bottom. The predicted run-off transcripts are shown in lines numbered 3 to 5. The size of an end-to-end transcript is shown in line 2. Autoradiographs show the transcription products obtained with no added DNA (lane 1) or with cleaved pAG4 DNA (lanes 2–5). The numbers below lanes 2 through 5 correspond to the numbers in the diagram.

most active for in vitro transcription in uninfected HeLa cell extracts. As shown in Figure 5, the appropriate run-off product of about 400 nucleotides was obtained using extracts of uninfected HeLa cells. However, this band could not be detected at all, even after long autoradiographic exposure when extracts were prepared from cells at 2 hr or later after vaccinia infection. Similar results were also obtained using an SV40 template (not shown).

The ability of extracts made from whole infected cells to transcribe DNA recognized by RNA polymerase III was tested using the plasmid pBal M (Janik et al., 1981) which contains adenovirus VA genes. Transcription of this gene by RNA polymerase III in crude extracts of mammalian cells results in a product of about 160 nucleotides (Wu, 1978; Weil et al., 1979). The synthesis of VA RNA by HeLa cells extracts is shown in Figure 6, lane 2. VA RNA is also made in extracts prepared from cells infected with vaccinia virus, although there is some reduction in intensity of the band (Figure 6, lane 3). Thus there is preferential inhibition of RNA polymerase II mediated transcription.

Resistance of Transcription to α-Amanitin

The vaccinia virus RNA polymerase can be distinguished from eucaryotic RNA polymerase II by its resistance to α-amanitin (Nevins and Joklik, 1977; Baroudy and Moss, 1980; Spencer et al., 1980). The latter studies were carried out with purified viral enzyme under conditions in which correct initiation did not occur. Resistance to α-amanitin was also found in extracts of infected cells that initiate transcription correctly on vaccinia templates (Figure 7, lanes 6 and 7). At the same 1 μg/ml concentration of α-amanitin, transcription of a template containing the late adenovirus promoter was completely inhibited in uninfected cell extracts (Figure 7, lanes 2 and 4). The other lanes in Figure 7 reproduce the specificity of uninfected cell and infected cell extracts for adenovirus and vaccinia promoters respectively.

Inhibition of Transcription by Antibodies Made against Purified Vaccinia Virus RNA Polymerase

Further evidence that vaccinia RNA polymerase, present in extracts of infected cells, is involved in transcription of viral templates was obtained through the use of specific antibodies. RNA polymerase was purified from vaccinia virus particles by successive chromatography on DEAE-cellulose, DEAE-Biogel, phosphocellulose, and aminopentyl agarose columns followed by glycerol gradient centrifugation (Baroudy and Moss, 1980). The purified enzyme was then used to immunize rabbits and the antiserum was shown to inhibit purified vaccinia RNA polymerase and to specifically immunoprecipitate ^{35}S-methionine labeled enzyme from crude extracts (E. Jones, personal communication). The IgG fractions of preimmune and immune sera were purified over a Staphylococcus A protein column (Ey et al., 1978), dialyzed and then incubated with extracts from uninfected and infected cells for 3 hr at 4°C. When these extracts were used in transcription assays, the results shown in Figure 8 were obtained. Antibodies to the vaccinia RNA polymease did not inhibit transcription by uninfected cell extracts of DNA containing the adenovirus late promoter, but completely inhibited transcription by infected cell extracts of vaccinia sequences. Antibodies to the vaccinia RNA polymerase also did not inhibit transcription of the adenovirus VA genes by either uninfected or infected cell extracts (not shown). The latter result implies that the VA gene is still transcribed by eucaryotic RNA polymerase III in infected cell extracts and not by the vaccinia RNA polymerase.

Discussion

A soluble cell-free system that specifically and accurately initiates transcription of added vaccinia virus DNA is

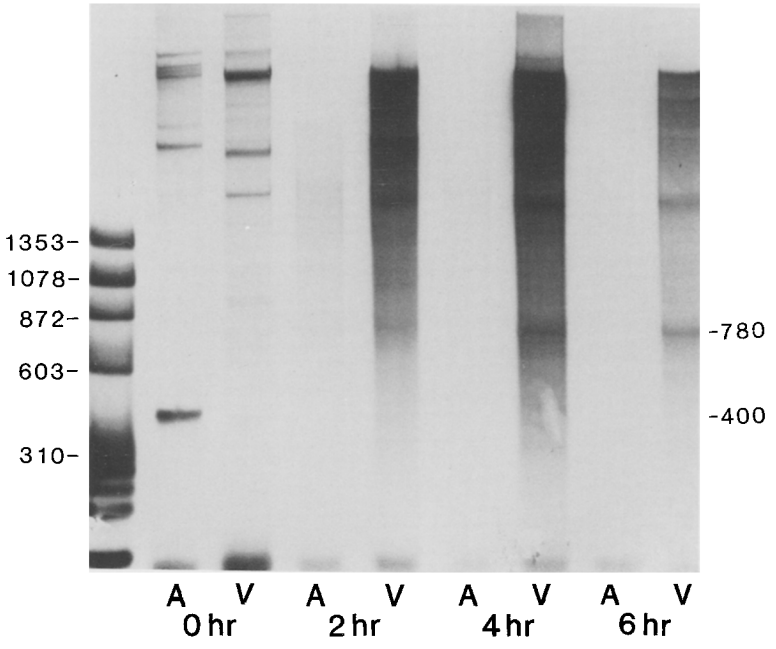

Figure 5. Specificity of Infected Cell Extracts for Vaccinia Templates

At 2, 4, and 6 hr after infection with vaccinia virus, whole cell extracts were prepared. One portion of cells was not infected and used to prepare the "0 hr" extract. Either 1 μg of Bam HI cleaved pϕ4 (A) or Sal I cleaved pAG4(V) was added to transcription mixtures. The sizes in nucleotides of glyoxylated DNA markers (left) and glyoxylated RNA species (right) are shown.

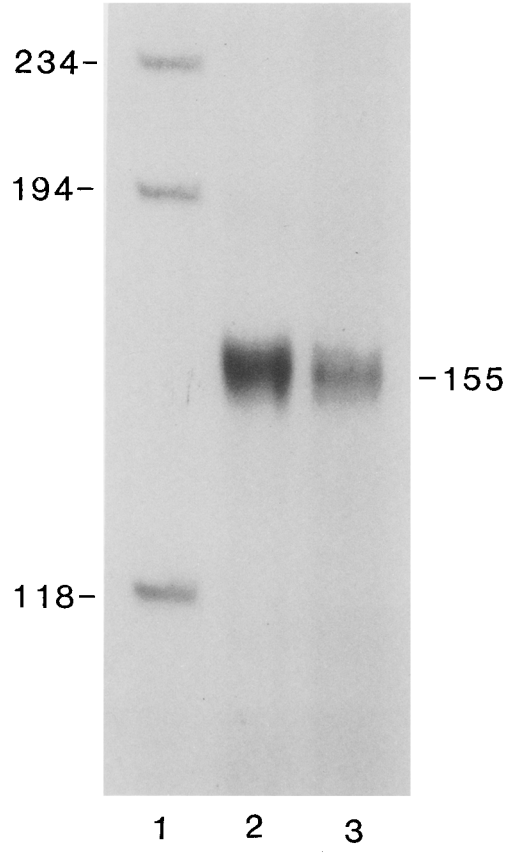

Figure 6. Transcription of Adenovirus VA Genes by Uninfected and Infected Cell Extracts

An autoradiograph of transcription products made with uninfected (lane 2) or infected (lane 3) extracts using intact pBal M as template. The sizes of DNA markers (lane 1) are indicated in nucleotides.

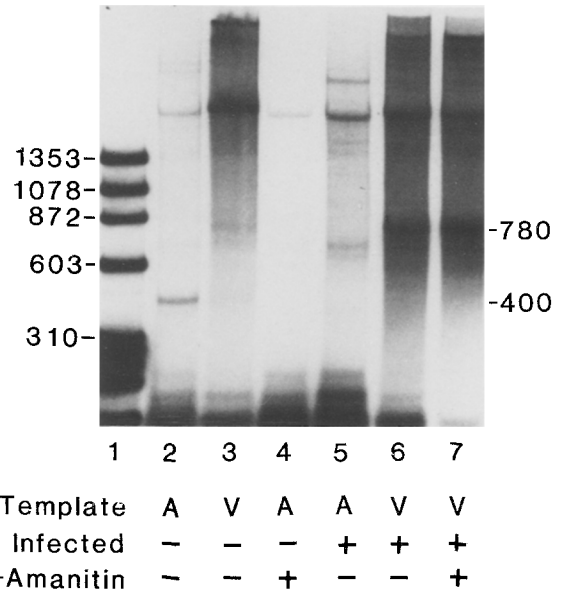

Figure 7. Resistance of Transcription to α-Amanitin

Transcription reactions contained extracts from uninfected or infected cells. Lanes 2–7 contain products of transcription reactions performed with Bam HI cleaved pϕ4 (A) or Sal I cleaved pAG4 (V) as template. Zero (–) or 1 (+) μg of α-amanitin was added to the transcription reaction. The transcription conditions used are indicated by appropriate symbols below each lane. Above are the autoradiographs of the glyoxylated RNAs.

needed to determine the nucleotide sequence requirements of the template and to identify the protein factors involved. Probably for technical reasons related to the use of strong detergents, such a system has not yet been obtained from disrupted virus particles. However, evidence presented here and by Foglesong and Hurwitz (personal

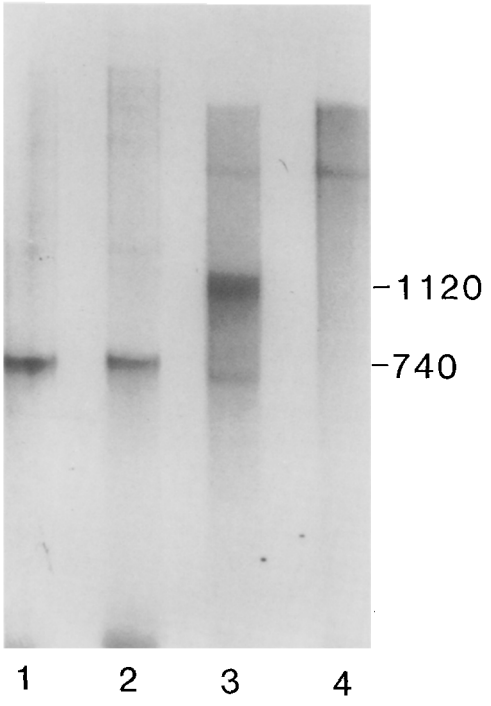

Figure 8. Inhibition of Transcription by Antibodies Made against Purified RNA Polymerase

Extracts prepared from uninfected and infected cells were incubated with preimmune or immune IgG. Bam HI cleaved pϕ4 and Sal I cleaed PAG4 were used as templates with uninfected and infected extracts respectively. Lanes correspond to (1) uninfected extract, preimmune IgG; (2) uninfected extract; immune IgG; (3) infected extract, preimmune IgG; (4) infected extract, immune IgG.

communication) indicates that transcription can be obtained with crude dialyzed and concentrated extracts of whole vaccinia infected cells. The first step in developing a transcription system was to find a suitable template. An early gene coding for a 7.5K polypeptide was selected because it was shown that the transcriptional start sites coincide with the 5′ ends of the mature capped mRNA (Venkatesan and Moss, 1981) and that a 265 bp DNA segment promotes expression of ligated foreign protein coding sequences (Mackett et al., 1982). Although vaccinia virus transcription occurs in the cytoplasm of infected cells, we decided to use established procedures for the preparation of whole cell extracts (Sugden and Keller, 1973; Manley et al., 1980). To focus on the initiation step in transcription, truncated DNA templates were used in a run-off assay. With these protocols, we found that a plasmid containing 230 bp of vaccinia DNA upstream of the RNA start site and 32 bp downstream served as template. Moreover, nuclease S1 analysis indicated that initiation occurred at precisely the sites used in vivo.

Several lines of evidence indicate that this system is specific for vaccinia virus templates. First of all, extracts of uninfected cells did not accurately transcribe the 7.5K gene promoter. In addition, transcription of vaccinia DNA in infected extracts was resistant to α-amanitin, a potent inhibitor of eucaryotic RNA polymerase II, but was inhibited by antibodies to purified vaccinia RNA polymerase. The additional finding that neither RNA polymerase II nor III transcription was inhibited by these antibodies suggests they do not share highly antigenic epitopes with the viral enzyme. This result may have occurred because of little sequence homology between the viral and mammalian enzymes or because immune tolerance prevented the rabbit from producing antibodies to shared sequences.

Remarkably, extracts of cells prepared 2 hr or later after infection no longer transcribed eucaryotic RNA polymerase II templates. However, an RNA polymerase III template, the adenovirus VA gene, was still transcribed. Transcription of the VA gene was not the result of the viral RNA polymerase as it was resistant to antibodies to the latter enzyme. The selectiveness of this inhibition is consistent with the continued synthesis of host tRNA throughout vaccinia infection (Oda and Joklik, 1967). Reductions in the synthesis of high molecular weight RNA as well as a block in cytoplasmic transport have been reported (Salzman et al., 1964; Becker and Joklik, 1964; Jefferts and Holowczak, 1971) as well as greatly decreased levels of cellular mRNA in the cytoplasm following vaccinia infection (Boone and Moss, 1978; Cooper and Moss, 1979).

Extracts prepared from poliovirus (Crawford et al., 1981) and adenovirus (Fire et al., 1981) infected cells also exhibit altered transcriptional specificity. Poliovirus, like vaccinia virus, preferentially inhibits RNA polymerase II transcription relative to that of RNA polymerase III. Perhaps because these two dissimilar viruses replicate in the cytoplasm and use their own transcription systems, they have evolved analogous mechanisms to inhibit host gene expression. It will be interesting to determine whether the same targets are affected by both viruses. Adenovirus, which needs RNA polymerase II and III for expression of its own genes, preferentially inhibits RNA polymerase I transcription.

In vitro deletion and mutagenesis of the sequences upstream of the RNA start site will be needed to further analyze the transcriptional specificity of the in vitro system. As a preliminary experiment, we noted that restriction enzyme digestions that left 100 and 66 bp of upstream sequences still allowed accurate initiation, although at lower levels. Several other early and one late vaccinia virus genes have also been tested in the in vitro system. Although run-off transcripts of the approximate size were detected, none were more efficient than the 7.5K gene promoter.

An important use of the in vitro template dependent system will be to isolate the factors required for transcription. Initial experiments indicate that all of the factors are present in the supernatant remaining after 18,000 × g centrifugation of cytoplasmic fractions. The ability to isolate enzymes from purified vaccinia virus particles and to obtain viral mutants should facilitate biochemical and genetic approaches to the study of transcription.

Experimental Procedures

Plasmids

Plasmids were purified by alkaline sodium dodecyl sulfate extraction (Maniatis et al., 1982), ethidium bromide–CsCl buoyant density gradient centrif-

ugation and either gel filtration through Sephacryl 1000 (Pharmacia) or chromatography on NACS 52 (Bethesda Research Laboratories). Restriction enzymes were purchased from New England Biolabs, Bethesda Research Laboratories, or Boehringer Mannheim and used as described by Maniatis et al., 1982. Completeness of digestions was monitored by agarose gel electrophoresis and ethidium bromide staining. DNA was extracted with phenol:chloroform:isoamyl alcohol (48:48:4 v/v/v) and chloroform:isoamyl alcohol (24:1 v/v) and ethanol precipitated.

Preparation of Whole Cell Extracts

All extracts were prepared from HeLa S3 cells grown in spinner culture with Eagle's medium containing 5% horse serum. Cells were concentrated to a density of 5×10^6 cells/ml and infected for 30 min and then diluted 10-fold. Sucrose gradient purified vaccinia virus WR was used at a multiplicity of 30 plaque forming units per cell. At appropriate times, whole cell extracts were prepared as described by Manley and Gefter (1981). After dialysis, samples were stored at −70°C. Protein concentrations were approximately 3 mg/ml.

In Vitro Transcription Conditions

Standard reactions were performed at 30°C for 60 min with 30 μl of dialyzed cell extract and final concentrations of 12 mM Hepes (pH 7.9), 7 mM $MgCl_2$, 60 mM KCl, 0.2 mM EDTA, 1.2 mM dithiothreitol, 10% glycerol, 400 μM each of ATP, GTP, and CTP and 40 μM UTP, and 10 μCi of α-^{32}P-UTP (400 Ci/mmole), and from 20 μg/ml to 60 μg/ml of plasmid template in a total volume of 50 μl. The amount of plasmid template used was determined for each cell extract preparation so as to optimize transcription. Reactions were terminated by addition of 50 μl of 0.3 M Tris-HCl (pH 7.5) 0.3 M NaCl, 25 mM EDTA and 2% sodium dodecyl sulfate. 25 μg of yeast tRNA (Boehringer-Mannheim) was added as carrier and the mixture was digested with Proteinase K (Beckman) at a concentration of 200 μg/ml for 15 min at 37°C. Following this, 0.4 ml of 9 M urea, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 mM NaCl was added and the mixture was extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (48:48:4 v/v/v) and once with an equal volume of chloroform:isoamyl alcohol (24:1 v/v). RNA was precipitated three times with 70% ethanol and the pellet was washed once with 95% ethanol and dried in vacuo. Samples were then dissolved in 28 μl of 1 mM EDTA (pH 8.0), 0.2% Sarkosyl and one fourth was glyoxylated by a modification of the procedure of McMaster and Carmichael (1981). Glyoxylated RNA and marker DNA samples were analyzed by electrophoresis on 1.5% agarose or 5% polyacrylamide gels in 10 mM NaH_2PO_4 (pH 6.8) buffer.

Mapping the 5′ Ends of Transcripts by Nuclease S1 Protection

Transcription reactions were scaled up 5-fold and RNA was purified as described above. After the second ethanol precipitation, RNA samples were digested with 100 μg/ml of DNAase I (RNAase-free, Worthington) in 0.1 ml of 10 mM Tris-HCl (pH 8.0), 100 mM $MgCl_2$, 100 mM NaCl for 10 min at 37°C. Samples were then extracted as above with equal volumes of phenol:chloroform:isoamyl alcohol and chloroform:isoamyl alcohol and then ethanol precipitated. RNA combined with 5′ ^{32}P end-labeled DNA was dissolved in 10 μl of 80% formamide, 40 mM Pipes (pH 6.4), 400 mM NaCl, 1 mM EDTA and heated at 68°C for 10 min and then at 42°C for 3 hr. Nuclease S1 (PL Biochemicals) digestion was carried out in 0.1 ml of 280 mM NaCl, 30 mM sodium acetate, 1 mM $ZnSO_4$ (pH 4.4), 480 U/ml of nuclease at 20°C for 1 hr.

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