JOURNAL OF VIROLOGY, May 1992, p. 2982–2989
0022-538X/92/052982-08$02.00/0

Vol. 66, No. 5

Ternary Complex Formation by Vaccinia Virus RNA Polymerase at an Early Viral Promoter: Analysis by Native Gel Electrophoresis

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Received 14 January 1992/Accepted 17 February 1992

We have resolved, by native gel electrophoresis, two intermediates in the transcription of a vaccinia virus early gene by the virus-encoded RNA polymerase. Polymerase holoenzyme containing the vaccinia virus early transcription factor (VETF) forms a complex of VETF bound to the promoter as the first step in a pathway leading to establishment of a committed ternary elongation complex. Formation of the VETF-DNA complex is stimulated by magnesium but is uninfluenced by nucleoside triphosphates. A stable binary complex of RNA polymerase bound to DNA is not detected. Assembly of a gel-stable polymerase-DNA complex depends on conditions permissive for RNA synthesis. Nucleotide omission experiments suggest that at least a tetrameric RNA must be made before a ternary complex is stabilized. RNA analysis indicates that complexes containing nascent transcripts 20 nucleotides long are stable and active. Ternary complex formation requires hydrolyzable ATP. This is consistent with an essential role for the ATPase activity of VETF at a step subsequent to DNA binding, as proposed by Broyles (S. S. Broyles, J. Biol. Chem. 266:15545–15548, 1991). The ternary complex, once formed, is resistant to dissociation by competitor DNA, as well as by salt, Sarkosyl, and heparin. The effects of these inhibitory agents on transcription complex formation suggest that they target different steps in the assembly pathway.

Vaccinia virus early mRNAs are synthesized in the cytoplasm of the host cell by a DNA-dependent RNA polymerase encapsidated within the infecting vaccinia virus particle (15). Approximately 100 different early mRNAs are transcribed from the endogenous 192-kb vaccinia virus genome. Primary transcripts are modified at the 5′ end by virus-encoded capping and methylating enzymes (22) and are 3′ polyadenylated by a virus-encoded poly(A) polymerase (11). Faithful synthesis of early mRNAs can be reconstituted in vitro on exogenous DNA templates with enzymes isolated from virions (7, 23). Purified vaccinia virus RNA polymerase is composed of nine virus-encoded subunit polypeptides (2, 19). Initiation of early transcription by the polymerase requires a virus-encoded early transcription factor (VETF) composed of 82- and 70-kDa subunits (4, 7, 12). VETF binds specifically to early promoter sequences located within a 30-bp region upstream of sites of transcription initiation; binding is manifest in the formation of a DNA-protein complex that is stable to electrophoresis through a native polyacrylamide gel (7). VETF binding to the promoter occurs in the absence of polymerase or nucleoside triphosphates (NTPs) (3, 5, 7). Binding of VETF appears to result in bending of the DNA template in the vicinity of the transcriptional start site (5). It is presumed that the VETF-promoter complex is an intermediate in transcription initiation; however, the sequence of events following VETF binding is essentially unknown.

Studies in other systems have shown that commitment of RNA polymerase to transcription of a particular template is a multistep process that is complete only when a stable ternary complex of template DNA, RNA polymerase, and nascent RNA is established. Ternary complexes are refractory to template competition and are typically resistant to dissociation by agents such as heparin, salt, and Sarkosyl. In the vaccinia virus system, it has been possible to prepare ternary elongation complexes of vaccinia virus RNA polymerase and labeled nascent RNA by using a "G-less" DNA template fused to a vaccinia virus early promoter (18). Omission of GTP from the reaction mixes leads to elongation arrest at the end of the G-less cassette, at a site 390 nucleotides downstream of the site of initiation. Upon provision of GTP and dilution of the labeled NTP precursor, the polymerase resumed elongation down the linear template. Formation of the ternary complex at +390 rendered the RNA polymerase resistant to concentrations of salt and Sarkosyl that prevented transcription initiation (18).

In order to define the intermediate stages in progression from the VETF-promoter complex to the committed ternary complex, we have studied the binding of vaccinia virus RNA polymerase to linear duplex DNA templates containing defined early promoter elements. We find that VETF binding is required for subsequent formation of a transcription complex that is stable to electrophoresis through a native polyacrylamide gel. Formation of this RNA polymerase-DNA complex requires the synthesis of at least a tetrameric nascent RNA and is dependent on ATP hydrolysis. The energy requirement is presumed to reflect an essential role in ternary complex formation of the DNA-dependent ATPase intrinsic to VETF (6).

MATERIALS AND METHODS

Enzymes. Initial fractionation of proteins extracted from vaccinia virus virions was performed as described by Broyles et al. (7). The DEAE-II-bound fraction served as the starting point for further purification of VETF and RNA polymerase. VETF was separated from RNA polymerase by sedimentation of the DEAE-II preparation through an 11-ml 15 to 35% glycerol gradient containing 0.2 M NaCl in buffer A (23). The gradient was centrifuged in a Beckman SW41 rotor at 41,000 rpm for 21.5 h at 4°C. Fractions (0.5 ml) were collected and assayed for VETF promoter binding activity as described before (7). VETF sedimented as a discrete peak at

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7.6 S (relative to the marker proteins hemoglobin and catalase centrifuged in a parallel gradient) and was resolved completely from RNA polymerase, which sedimented at 15.5 S. The DNA-protein complex formed by this glycerol gradient fraction of VETF was identical to that formed by the most highly purified VETF preparation described by Broyles and Moss (6). The extent of protein-DNA complex formation was proportional to the amount of VETF included in the binding reaction mixtures. One unit of VETF was defined as the amount sufficient to shift 1 fmol of radiolabeled DNA from the free to the protein-bound form, as measured by native gel electrophoresis under standard reaction conditions (see below).

Transcriptionally active RNA polymerase (holoenzyme containing VETF) was isolated by phosphocellulose chromatography of the DEAE-II fraction as described before (25). One unit catalyzed the incorporation of 1 nmol of UMP into acid-insoluble material under standard assay conditions with a nonspecific single-stranded DNA template (26). Specific transcription in vitro of early genes by the phosphocellulose polymerase fraction could be stimulated up to fivefold by supplementation with purified VETF (glycerol gradient fraction).

Analysis of protein-DNA complexes. A 90-bp DNA fragment containing the promoter and initial transcribed region of the vaccinia virus growth factor gene was excised from plasmid pVGF (29) with endonucleases *Hin*dIII and *Bam*HI. The DNA was radiolabeled at the 3′ end with [α-^{32}P]dCTP by using Klenow DNA polymerase. Labeled DNA was purified by electrophoresis through a nondenaturing polyacrylamide gel (5% acrylamide, 0.16% bisacrylamide) containing TBE (90 mM Tris, 90 mM borate, 2.5 mM EDTA). DNA was eluted from an excised gel slice by soaking the slice for 3 h at room temperature in a solution containing 0.5 M ammonium acetate, 10 mM $MgCl_2$, 1 mM EDTA, and 0.1% sodium dodecyl sulfate (SDS). The eluate was precipitated with ethanol and resuspended in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA.

Reaction mixtures for formation of protein-DNA complexes contained (in 20 μl) 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 5% glycerol, 2 fmol of gel-purified ^{32}P-labeled promoter-containing DNA, proteins, and additional components as specified. After incubation at 30°C, samples were applied to a nondenaturing polyacrylamide gel (4% acrylamide, 0.13% bisacrylamide) containing 6.7 mM Tris-HCl (pH 7.5), 3.3 mM sodium acetate, and 0.1 mM EDTA. Electrophoresis was performed at room temperature at 25 mA constant current until a bromphenol blue dye marker had migrated approximately 9 cm into the gel. Radiolabeled DNA was visualized by autoradiographic exposure of the dried gel.

G-less template. pG21 contains, inserted between the *Eco*RI and *Sma*I restriction sites of plasmid pBSKS+, a synthetic vaccinia virus early promoter element fused to a 20-nucleotide G-less cassette. The complete sequence of the synthetic promoter element and its specificity in directing vaccinia virus transcription in vitro have been described by Luo et al. (18). The sequence of the template in the initial transcribed region (nontemplate strand) is as follows:

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          +1
ATTCCTTTCATAACCCACTTCTATCACTAGGGG (G21)
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The site of transcription initiation is indicated by +1 at the A residue. A gel-purified *Pvu*II restriction fragment of pG21 containing the G-less transcription unit and flanking plasmid sequences was used to program in vitro transcription by vaccinia virus RNA polymerase.

RNA synthesis in vitro. Standard transcription reaction mixtures (20 μl) contained 20 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM UTP, 1 μM [α-^{32}P]CTP (1,000 Ci/mmol), 1.8 ng of DNA template (gel-purified *Pvu*II restriction fragment containing a vaccinia virus early promoter fused to a 20-nucleotide G-less cassette), vaccinia virus RNA polymerase (0.13 U; phosphocellulose fraction), and vaccinia virus capping enzyme (10 fmol). After incubation at 30°C, reactions were halted by addition of 80 μl of a solution containing 12.5 mM EDTA, 0.625% SDS, 150 μg of yeast tRNA per ml, and 5 M urea. Labeled RNA products were deproteinized by extraction with phenol-chloroform-isoamyl alcohol (50:48:2) and then recovered by ethanol precipitation. The precipitate was dissolved in 7 μl of formamide, heated at 90°C for 4 min, and then analyzed by electrophoresis through either a 12% or a 17% polyacrylamide gel (acrylamide-bisacrylamide, 20:1) containing 7 M urea in TBE. Electrophoresis was done at 60 W constant power. Reaction products were visualized by autoradiographic exposure of the gel.

Materials. Ribonucleoside triphosphates (high-pressure liquid chromatography purified) were obtained from Pharmacia. Radiolabeled nucleotides were purchased from Amersham. Restriction endonucleases and enzymes used for molecular cloning and DNA labeling were purchased from New England BioLabs and Bethesda Research Laboratories.

RESULTS

Binding of VETF and RNA polymerase to DNA containing an early viral promoter. Native gel electrophoresis has been used to great advantage in defining DNA-protein interactions during the preinitiation, initiation, and early elongation phases of transcription (8, 16, 17, 27). In the present study, we examined the interaction of vaccinia virus RNA polymerase and VETF with promoter-containing DNA. A series of experiments were performed with a radiolabeled 90-bp template containing the promoter and initial transcribed region of the early gene encoding the vaccinia virus growth factor. This transcription unit has been characterized extensively (28). The site of transcription initiation is known (30), transcription of the gene has been accomplished in vitro (21), and the binding site of VETF on the promoter has been mapped by footprinting and modification interference (5).

Labeled DNA template was incubated in vitro with either VETF or RNA polymerase (transcriptionally active holoenzyme containing VETF) under a variety of conditions, and the reaction mixes were electrophoresed through a nondenaturing polyacrylamide gel (Fig. 1). Formation of a protein-DNA complex was expected to result in the appearance of a novel labeled species of retarded electrophoretic mobility. VETF per se yielded a shifted species corresponding to the VETF-promoter complex characterized previously (Fig. 1, lane 1). VETF binding was enhanced slightly by magnesium (Fig. 1, lane 3) but was unaffected by the addition of ATP (Fig. 1, lane 5). RNA polymerase formed little or no shifted species in the absence of magnesium (Fig. 1, lane 2). Inclusion of magnesium revealed the formation of a VETF-promoter complex (Fig. 1, lane 4). VETF associated with RNA polymerase (as holoenzyme) was apparently less able to bind to DNA in the absence of a divalent cation than was free VETF. No DNA-protein complex migrating more slowly than the VETF complex was observed during incu-

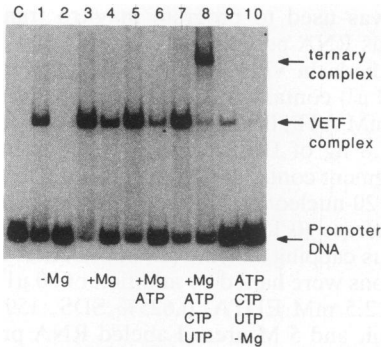

FIG. 1. Formation of protein-DNA complexes on promoter-containing DNA. Standard reaction mixtures contained either VETF (2 U; lanes 1, 3, 5, 7, and 9) or RNA polymerase (0.08 U; lanes 2, 4, 6, 8, and 10). Protein was omitted from the reaction mix in lane C. Additional components were included (where indicated below the lanes) at the following concentrations: 5 mM $MgCl_2$, 1 mM ATP, 1 mM CTP, and 1 mM UTP. Incubation was for 30 min at 30°C. An autoradiogram of the gel is shown. The positions of free DNA and of protein-DNA complexes are indicated at the right by arrows.

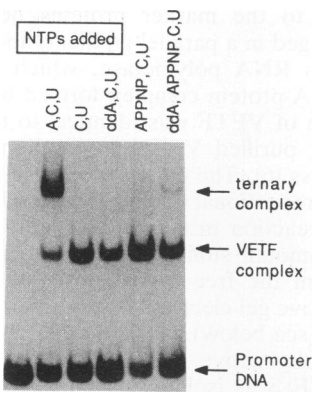

FIG. 2. Formation of the ternary complex requires hydrolyzable ATP. Reaction mixtures contained 5 mM $MgCl_2$, RNA polymerase (0.08 U), and NTPs (each at 1 mM concentration) as indicated above each lane. Polymerase was omitted from the reaction in the first lane. Adenosine analogs AMPPNP (APPNP) and ddATP (ddA) were included as indicated. Incubation was for 30 min at 30°C. An autoradiogram of the gel is shown. The positions of free DNA and of protein-DNA complexes are indicated at the right by arrows.

bation of RNA polymerase and DNA with magnesium or with magnesium and ATP. However, when ATP, CTP, and UTP were included, a novel higher-order species was formed (Fig. 1, lane 8). No higher-order complex was formed in NTP-containing reaction mixes that lacked magnesium (Fig. 1, lane 10). Insofar as the reaction conditions under which the higher-order shift was elicited were permissive for RNA synthesis, we presumed that the shifted species represented a stable ternary complex of polymerase, DNA, and nascent RNA. This was consistent with our ability to label the putative ternary complex (but not the VETF complex) in reaction mixes containing unlabeled promoter DNA, RNA polymerase, ATP, CTP, and [α-^{32}P]UTP (data not shown).

ATP requirement for ternary complex formation. If the higher-order shifted species represents a true ternary complex, the requirements for its formation should mimic those of the transcription reaction. Early transcription by viral cores or by soluble transcription systems requires ATP in hydrolyzable form (13, 20, 23, 24). Assembly of the ternary complex on the growth factor gene occurred in reaction mixes containing ATP, CTP, and UTP; omission of ATP abolished ternary complex formation without affecting VETF complex formation (Fig. 2). The nonhydrolyzable analog adenylyl(β-γ)imidotriphosphate (AMPPNP), which is a substrate for RNA polymerase (26), did not substitute for ATP in ternary complex formation. ddATP, which is hydrolyzable but is not utilized by RNA polymerase (23), also failed to support complex formation. However, addition of ddATP to reaction mixes containing AMPPNP partially restored the ability to form the ternary complex (Fig. 2). We attribute the reduced yield of ternary complex in this reaction to the lower efficiency of AMPPNP incorporation into RNA by vaccinia virus polymerase, as described previously (26).

These results were exactly reflective of the energy requirements for transcription of the growth factor gene in vitro (23). The data indicated that ATP hydrolysis was required for conversion of the VETF complex (a presumptive preinitiation intermediate) to the ternary complex, above and beyond a role for ATP as a substrate in the synthesis of nascent chains. We presume that the energy requirement reflected a role for the intrinsic DNA-dependent ATPase/dATPase activity of VETF (6).

NTP requirement for complex formation. The sequence of the initial transcribed region of the growth factor gene is 5′-AATCTTGTCATAAACACACACTGAGAAACAG (30). Inclusion of ATP, CTP, and UTP in reaction mixes containing RNA polymerase should, if initiation occurred at the correct template position, permit the synthesis of a nascent transcript at least 6 nucleotides long. Preliminary experiments to address the stage at which the ternary complex was stabilized employed a strategy of nucleotide omission (Fig. 3). The transition from VETF complex to ternary complex required ATP concentrations of $\geq$0.1 mM; the level of ternary complex appeared to plateau by 0.25 mM ATP (Fig. 3, left panel). No ternary complex was detected in reaction mixes containing ATP and UTP only (conditions permissive in theory for the synthesis of an AAU trimer). Inclusion of CTP was required to form the ternary complex; concentrations as low as 1 μM CTP supported near-saturating levels of complex formation (Fig. 3, center panel). Complex formation in the presence of ATP and CTP was strictly dependent on addition of UTP (Fig. 3, right panel). A conservative interpretation of the data is that RNA polymerase is initiating accurately and that at least a tetrameric nascent RNA (AATC) must be synthesized before a stable transcription complex is formed.

Ternary complex is committed to the template during a single cycle of transcription. Formation of the radiolabeled protein DNA complexes by RNA polymerase holoenzyme in the presence of NTPs was abrogated by inclusion of excess unlabeled promoter-containing DNA prior to the addition of enzyme (Fig. 4; compare lanes 1 and 2). Once formed, however, the putative ternary complex was refractory to dissociation by unlabeled DNA (Fig. 4, lane 3). The VETF complex, in contrast, was dissociated by addition of competitor DNA (Fig. 4, lane 3). This agreed with the finding that preformed VETF-promoter complex turns over rapidly in the presence of ATP (3). Supplementation of the preformed complexes with GTP resulted in dissociation of the radiolabeled ternary complex (Fig. 4, lane 4). This suggested that the complexes contained RNA polymerase paused stably on

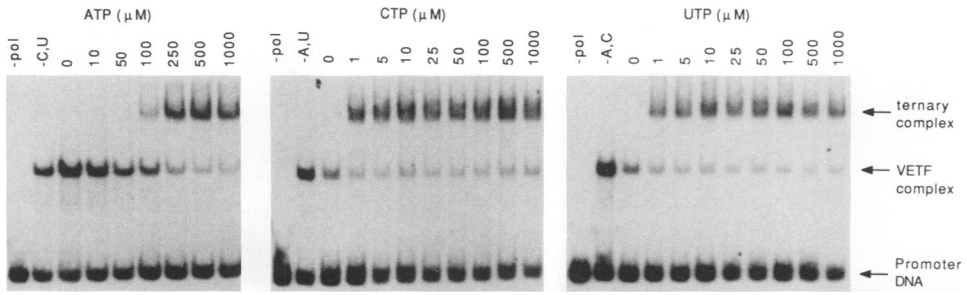

FIG. 3. NTP requirements for ternary complex formation. (Left) ATP dependence. Complete reaction mixtures contained RNA polymerase (0.08 U), 5 mM $MgCl_2$, 1 mM UTP, 1 mM CTP, and ATP at the concentration indicated above each lane. Reaction mixes lacking RNA polymerase (−pol) or lacking NTPs (−C,U) were included as controls. (Center) CTP dependence. Complete reaction mixtures contained RNA polymerase (0.08 U), 5 mM $MgCl_2$, 1 mM ATP, 1 mM UTP, and CTP at the concentration indicated above each lane. Reaction mixes lacking RNA polymerase (−pol) or lacking NTPs (−A,U) were included as controls. (Right) UTP dependence. Complete reaction mixtures contained RNA polymerase (0.08 U), 5 mM $MgCl_2$, 1 mM ATP, 10 μM CTP, and UTP at the concentration indicated above each lane. Reaction mixes lacking RNA polymerase (−pol) or lacking NTPs (−A,C) were included as controls. All incubations were for 30 min at 30°C. An autoradiogram of the gel is shown. The positions of free DNA and of protein-DNA complexes are indicated at the right by arrows.

the template because of limiting GTP concentration and that the majority of the paused complexes resumed elongation to the end of the template upon provision of GTP, at which time the polymerase could be released from the template. The labeled template would then be competing with cold DNA during subsequent rounds of complex formation. Labeled ternary complex remaining after GTP addition in the presence of unlabeled DNA either may represent dead-end elongation complexes that had not elongated in response to GTP (such complexes have been described for *Escherichia coli* RNA polymerase [1]) or may arise via reinitiation of transcription on a fraction of the labeled templates committed to more than one round of synthesis.

RNA associated with the putative ternary complex was examined by Sephadex G-50 gel filtration of reaction products programmed by the growth factor promoter DNA in the presence of RNA polymerase, ATP, CTP, and [α-^{32}P]UTP.

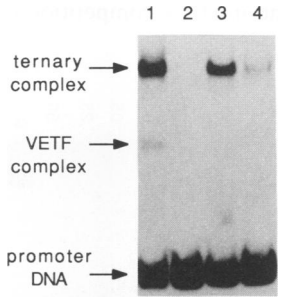

FIG. 4. Effect of competitor DNA on formation and stability of the ternary complex. Reaction mixtures contained 5 mM $MgCl_2$, 1 mM ATP, 1 mM CTP, 1 mM UTP, and RNA polymerase (0.08 U). The reaction mix in lane 1 was incubated for 40 min at 30°C with no further additions. In the reaction mix in lane 2, competitor DNA (200 fmol of unlabeled 90-bp promoter-containing DNA fragment) was included prior to the addition of RNA polymerase. This reaction mix was incubated for 40 min at 30°C. In the reaction mix in lane 3, unlabeled competitor DNA (200 fmol) was added after the radiolabeled DNA had been incubated with RNA polymerase for 30 min at 30°C. The reaction mix was then incubated at 30°C for an additional 10 min. The reaction mix in lane 4 was constituted as for lane 3 except that 1 mM GTP was added at 30 min, immediately prior to the addition of competitor DNA. The positions of reaction products are indicated at the left by arrows.

Electrophoresis of the excluded fraction through a high-resolution denaturing polyacrylamide gel revealed the presence of a cluster of labeled species approximately 22 to 24 nucleotides in length (estimated relative to DNA size markers; data not shown). This suggested that polymerase had read through the single G residue at position +7 and had paused in the vicinity of the downstream G residues at positions +22 and +24 (see the sequence in the previous section). Single-nucleotide-omission strategies for analyzing ternary complexes have often been complicated by this readthrough problem, which is attributed to trace amounts of the missing NTP contaminating even highly purified commercial preparations of the other three nucleotides (17). We have found that the major source of contamination in our experiments was the preparation of ATP, which, at a 1 mM concentration, contained sufficient GTP or ITP (generated, perhaps, by spontaneous deamination of ATP in solution) to allow readthrough of single G residues in the template (14).

Stability in the presence of salt. Initiation of vaccinia virus early transcription is sensitive to low concentrations of NaCl, while elongation of nascent chains is relatively refractory to the presence of salt (18). Formation of a ternary complex on the growth factor gene was inhibited by NaCl concentrations of ≥25 mM when salt was included prior to the addition of RNA polymerase (Fig. 5, left panel). In contrast, the VETF-promoter complex was formed under such conditions. Prior assembly of the ternary complex rendered it resistant to ≥100 mM NaCl (Fig. 5, right panel). Therefore, a salt-sensitive step occurred subsequent to VETF binding. Binding of purified VETF to the promoter (assayed in the presence of Mg and ATP) was stable in the presence of NaCl concentrations up to 200 mM but was abrogated completely by 400 mM NaCl (data not shown).

Stability in the presence of Sarkosyl. Initiation of vaccinia virus early transcription is sensitive to low concentrations of Sarkosyl, while elongation of nascent chains is relatively resistant to this detergent (18). Formation of a ternary complex on the growth factor gene was abolished by ≥0.01% Sarkosyl when the detergent was included in the reaction mixtures prior to the addition of RNA polymerase (Fig. 6). Formation of VETF complex was also inhibited at these detergent concentrations (Fig. 6). When Sarkosyl was added subsequent to RNA polymerase, the ternary complex was stable at Sarkosyl concentrations that prevented its assem-

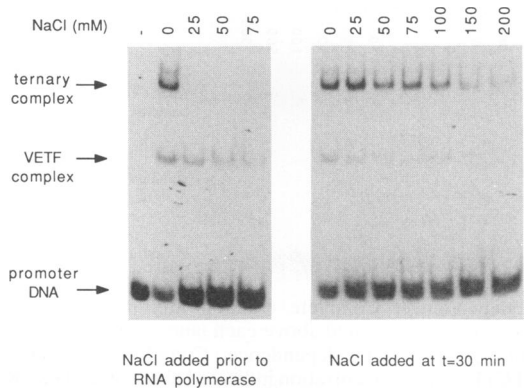

FIG. 5. Effect of salt on formation and stability of the protein-DNA complex. (Left) Reaction mixtures containing 5 mM $MgCl_2$, 1 mM ATP, 1 mM CTP, and 1 mM UTP were supplemented with NaCl (at the concentrations indicated above the lanes) prior to the addition of RNA polymerase (0.08 U). A control reaction mix lacking RNA polymerase is shown in the first lane. Incubation was for 30 min at 30°C. An autoradiogram of the gel is shown. The positions of reaction products are indicated at the left by arrows. (Right) Reaction mixtures containing 5 mM $MgCl_2$, 1 mM ATP, 1 mM CTP, 1 mM UTP, and RNA polymerase (0.08 U) were incubated for 30 min at 30°C and then supplemented with NaCl to the final concentrations indicated above each lane. Samples were incubated for an additional 2 min at 30°C before being applied to a native polyacrylamide gel.

bly. Increasing the Sarkosyl concentration from 0.01 to 0.04% effected a concentration-dependent, stepwise conversion of the original ternary complex into a discrete species of slightly more rapid electrophoretic mobility (Fig. 6, denoted by asterisk). This novel species was stable at 0.06% Sarkosyl but was eliminated at ≥0.08% Sarkosyl (data not shown). The more rapid mobility of the novel complex suggested that it arose via loss of some component present in the original ternary complex.

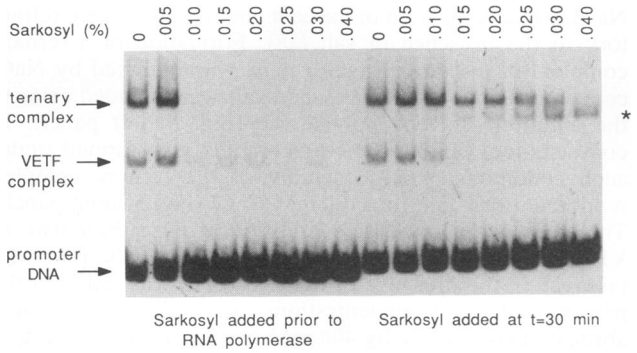

FIG. 6. Effect of Sarkosyl on formation and stability of the protein-DNA complex. Reaction mixtures containing 5 mM $MgCl_2$, 1 mM ATP, 1 mM CTP, 1 mM UTP, and RNA polymerase (0.08 U) were supplemented with Sarkosyl at the concentrations indicated above each lane. Sarkosyl was added either prior to RNA polymerase or after a 30-min incubation period in the presence of RNA polymerase (as indicated below the autoradiogram). Samples receiving Sarkosyl at 30 min were incubated for an additional 2 min at 30°C before being applied to a native polyacrylamide gel. The positions of reaction products are indicated at the left by arrows. The position of a novel complex generated in the presence of Sarkosyl is indicated at the right by an asterisk.

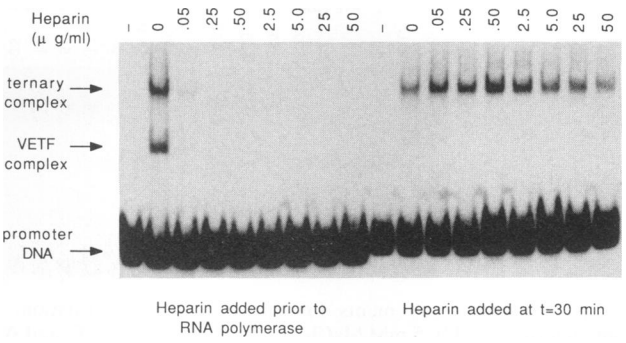

FIG. 7. Effect of heparin on formation and stability of the protein-DNA complex. Reaction mixtures containing 5 mM $MgCl_2$, 1 mM ATP, 1 mM CTP, 1 mM UTP, and RNA polymerase (0.08 U) were supplemented with heparin at the concentrations indicated above each lane. Heparin was added either prior to RNA polymerase or after a 30-min incubation period in the presence of RNA polymerase (as indicated below the autoradiogram). Samples receiving heparin at 30 min were incubated for an additional 2 min at 30°C before being applied to a native polyacrylamide gel. A control reaction mix lacking RNA polymerase is shown in lane —. The positions of reaction products are indicated at the left by arrows.

Stability in the presence of heparin. Heparin abolished formation of the ternary complex at concentrations of 0.25 μg/ml when it was included prior to addition of RNA polymerase (Fig. 7). VETF complex formation was prevented at similar concentrations. In contrast, when heparin was added subsequent to RNA polymerase, the ternary complex was stable at concentrations of heparin as high as 50 μg/ml (Fig. 7). No novel complex was generated by heparin. Binding of VETF per se to the growth factor promoter was also inhibited by prior addition of low concentrations of heparin (Fig. 8). The stability of the VETF complex in the presence of heparin was enhanced when heparin was added subsequent to protein binding (Fig. 8; compare 0.25 and 0.5 μg/ml added before or after VETF). This effect of heparin was similar to that of competitor DNA (3). As was found in DNA competition experiments (3), we

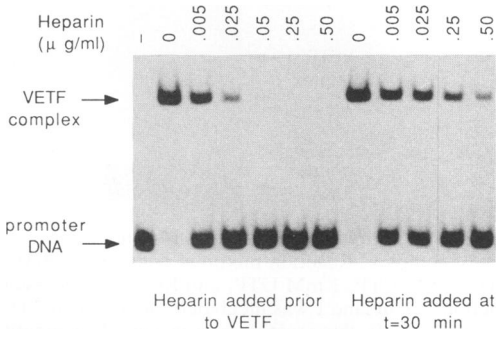

FIG. 8. Effect of heparin on formation and stability of the VETF complex. Reaction mixtures containing 5 mM $MgCl_2$ and VETF (2 U) were supplemented with heparin at the concentrations indicated above each lane. Heparin was added either prior to VETF or after a 30-min incubation period in the presence of VETF (as indicated below the autoradiogram). Samples receiving heparin at 30 min were incubated for an additional 2 min at 30°C before being applied to a native polyacrylamide gel. A control reaction lacking VETF is shown in lane —. The positions of reaction products are indicated at the left by arrows.

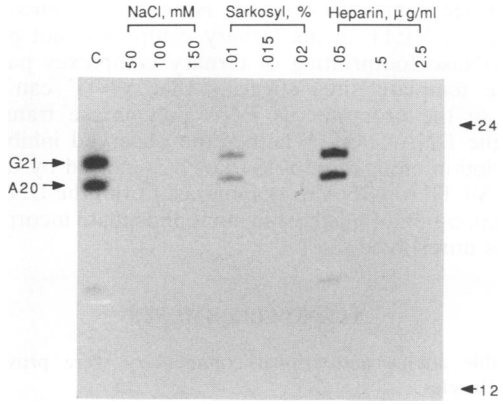

FIG. 9. Effect of salt, Sarkosyl, and heparin on the synthesis of short nascent RNAs. Reaction mixtures for in vitro transcription of the G21 template, constituted as described in Materials and Methods, were supplemented with either NaCl, Sarkosyl, or heparin (prior to the addition of RNA polymerase) at the concentrations indicated above the lanes. A standard transcription reaction mix containing no additional components is shown in lane C. Incubation was for 10 min at 30°C. CMP-labeled RNAs were analyzed by electrophoresis through a 12% polyacrylamide gel. An autoradiogram of the gel is shown. The positions and sizes (in nucleotides) of coelectrophoresed 5′-end ^{32}P-labeled single-stranded DNA oligonucleotides are indicated at the right by arrows. The major transcripts programmed by the G21 template in the absence of GTP were initiated at the +1 position of the template and elongated to either position +20A or +21G (14); these products are indicated at the left by arrows.

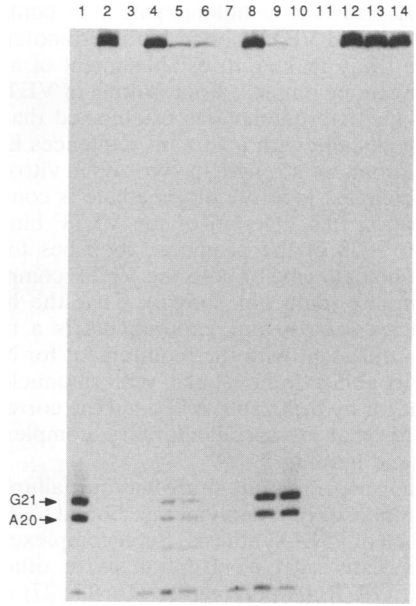

FIG. 10. Effect of salt, Sarkosyl, and heparin on elongation by paused ternary complexes. Pulse-labeled transcripts were recovered from a standard reaction mixture programmed by the G21 template after incubation for 10 min at 30°C (lane 1). An identical reaction mixture was supplemented after 10 min by addition of 1 mM CTP, 1 mM GTP, and 1 mM ATP (above that included in the original reaction mix), and incubation was continued for another 10 min, after which the products of the "chase" were recovered (lane 2). The effect of inhibitors on synthesis of nascent chains was assessed by inclusion of either 100 mM NaCl (lane 3), 0.02% Sarkosyl (lane 7), or heparin (2.5 μg/ml) (lane 11) prior to the addition of RNA polymerase. The effect of these agents on elongation by preformed ternary complexes was determined by addition of NaCl at 100 mM (lane 4), 200 mM (lane 5), or 250 mM (lane 6); of Sarkosyl at 0.02% (lane 8), 0.04% (lane 9), or 0.1% (lane 10); or of heparin at 2.5 μg/ml (lane 12), 25 μg/ml (lane 13), or 50 μg/ml (lane 14) to the reaction mixtures after the 10-min pulse-phase but prior to commencement of the chase. RNA was isolated and analyzed on a denaturing 17% polyacrylamide gel. An autoradiogram of the gel is shown. The positions of the pulse-labeled transcription products are indicated at the left by arrows.

observed that stabilization of preformed VETF complex to heparin challenge was eliminated by addition of Mg and ATP to the reaction mix (data not shown).

Elongation of nascent RNA contained within paused transcription complexes. The functional integrity of a ternary complex is defined operationally by the ability of RNA polymerase to elongate its associated transcript in a pulse-chase experiment. The above experiments with native gel analysis did not address this issue or did so indirectly (e.g., in the experiment shown in Fig. 3). In order to study complex stability directly, we have constructed a synthetic transcription unit containing a strong consensus promoter element (9) fused to a 20-nucleotide G-less cassette and flanked at the 3′ end by four consecutive G residues (described in Materials and Methods) (14). Transcription of this template in vitro by vaccinia virus RNA polymerase in reaction mixes lacking GTP yielded two predominant CMP-labeled products corresponding to transcripts initiated at +1A and having 3′ termini at either +20A or +21G (Fig. 9, lane C; Fig. 10, lane 1). A minor abortive transcript of 15 nucleotides was also evident. Synthesis of these short chains was inhibited by inclusion of ≥50 mM NaCl, 0.015% Sarkosyl, or ≥0.5 μg of heparin per ml in the reaction mixes prior to the addition of RNA polymerase (Fig. 9; also Fig. 10, lanes 3, 7, and 11). The integrity of the ternary complexes containing pulse-labeled A20 and G21 transcripts was verified by the ability of these RNAs to be elongated during a "chase" in the presence of GTP (Fig. 10). Prior to commencement of the chase, the reaction mixtures were adjusted to 1 mM CTP so as to reduce the specific activity of the radiolabeled precursor by 1,000-fold, effectively limiting the analysis to the fate of transcripts labeled during the pulse only. We observed that addition of excess GTP allowed elongation of virtually all the A20 and G21 RNAs to the end of the linear template (Fig. 10, lane 2).

The stability of the ternary complexes to challenge with salt, Sarkosyl, and heparin was examined by addition of these agents to transcription reaction mixes after the pulse phase but before the GTP chase was started (Fig. 10). Elongation of the preformed nascent chains was unaffected by 100 mM NaCl but was inhibited partially by 200 and 250 mM NaCl (Fig. 10, lanes 5 and 6). Sarkosyl at 0.02% had no effect on elongation (Fig. 10, lane 8), although concentrations of >0.04% blocked elongation completely (Fig. 10, lanes 9 and 10). It was not evident from this analysis whether salt and Sarkosyl affected elongation by inhibiting NTP incorporation, dissociating the transcript from the ternary complex, or both. Heparin had no effect on elongation by the ternary complex at concentrations of up to 50 μg/ml (Fig. 10, lanes 12 to 14).

DISCUSSION

We have resolved, by native gel electrophoresis, two intermediates in the transcription of vaccinia virus early

genes. RNA polymerase holoenzyme that contains VETF forms a complex of VETF bound to the promoter as the first step in a pathway leading to establishment of a committed ternary elongation complex. Footprinting of VETF bound to the growth factor promoter has established that the factor interacts specifically with upstream sequences known to be involved in promoter strength in vivo and in vitro (5, 9). That the VETF complex is a true intermediate is consistent with our observation that deletion of the VETF binding region from −12 to −28 of the promoter abolishes formation by polymerase holoenzyme of both the VETF complex and the ternary complex (data not shown). That the higher-order shifted species seen in our experiments is a true ternary complex is consistent with the requirement for NTPs for its formation, its ability to be labeled with ribonucleotides, the requirement for hydrolyzable ATP, and the correlation of its stability with that of specific ternary complexes defined previously and herein.

A surprising aspect of this study was our failure to observe a binary complex of RNA polymerase bound to the promoter in the absence of RNA synthesis. Such complexes have been resolved by native gel electrophoresis in other systems, including *E. coli* RNA polymerase (16, 17, 27) and eukaryotic RNA polymerase II (8, 10). Conceivably, the hypothetical binary complex of vaccinia virus RNA polymerase is not stable enough to survive the electrophoresis procedure.

The formation of a gel-stable polymerase-DNA complex clearly depends on conditions permissive for RNA synthesis. The nucleotide omission experiments suggest that at least a tetramer must be made before stabilization occurs. Conditions predicted to be permissive for the synthesis of a trimer were not sufficient to stabilize the complex (with the caveat that we have not actually demonstrated that a trimer was made under these conditions). The RNA contained within the growth factor gene ternary complex was 22 to 24 nucleotides long. Functionally stable ternary complexes contain RNAs as short as 20 nucleotides, as evinced by the experiments with the G-less cassette (Fig. 10). Radiolabeled DNA containing the G21 transcription unit is recruited into the gel-stable ternary complex in a reaction that displays the same nucleotide requirements shown for the growth factor template (14a). Gel-stable ternary complexes containing nascent chains as short as 10 nucleotides have been demonstrated by using different G-less templates (14a). The precise transition point between 4- and 10-base transcripts at which the ternary complex remains stable during native gel electrophoresis has not been determined.

The effects of inhibitory agents on transcription complex formation suggest that they target different steps in the assembly pathway. Heparin blocks binding of VETF to the promoter. Once the ternary complex is formed, it becomes completely refractory to heparin concentrations >100-fold higher than those sufficient to preclude complex assembly and/or nascent chain synthesis. Salt seems to act at the level of recruitment of RNA polymerase to the VETF complex (which is itself stable in the presence of NaCl; Fig. 5 and data not shown). Whether this is caused by interruption of contacts between VETF and polymerase or between polymerase and DNA or to prevention of RNA synthesis is not clear at present. Sarkosyl appears to act by blocking formation of the VETF-DNA complex (Fig. 6 and data not shown). The novel complex generated by Sarkosyl from the ternary complex may arise via stripping of VETF or some other component (nascent RNA or an RNA polymerase subunit) from the elongation apparatus. It should be pointed out that the protein composition of the gel-stable ternary complex has not been analyzed directly. Although evidence for the presence of VETF in the ternary complex is not provided here, DNase footprinting of ternary complexes paused at discrete template sites suggests that VETF can remain bound to the promoter as RNA polymerase translocates along the DNA (14a). Whether the observed inhibition of transcription elongation by Sarkosyl is caused by dissociation of VETF (or RNA or polymerase) from the DNA or by direct inhibition of nucleoside monophosphate incorporation remains unresolved.

ACKNOWLEDGMENTS

Valuable advice and critical commentary were provided by Steven Broyles.

This work was supported by grants GM42498 from the National Institutes of Health and JFRA-274 from the American Cancer Society. S.S. is the recipient of a Pew Scholarship in the Biomedical Sciences.

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