

Nascent RNA Cleavage by Purified Ternary Complexes of Vaccinia RNA Polymerase*

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Ternary complexes of vaccinia virus RNA polymerase containing 3'-OMeGMP-arrested transcripts were purified by native gel electrophoresis. These complexes resumed elongation *in situ* when gel slices were incubated with magnesium and NTPs. Elongation occurred in the absence of pyrophosphate, suggesting that the blocking 3'-OMeGMP residue was removed via a novel pathway. We show that purified elongation complexes contain an intrinsic nuclease activity that shortens nascent RNA from the 3'-end. RNA cleavage was absolutely dependent on a divalent cation and was stimulated by CTP. The initial 5' cleavage product remained associated with the ternary complex and could be elongated in the presence of NTPs. Multiple stepwise cleavages generated progressively shorter chains. Purified ternary complexes containing 3'-OH-terminated RNAs also displayed nuclease activity. Involvement of the vaccinia RNA polymerase subunit rpo30 in the transcript-shortening reaction is suggested based on sequence similarity of rpo30 to mammalian protein SII (TFIIS), an extrinsic transcription factor required for nascent RNA cleavage by RNA polymerase II (Reines, D. (1991) *J. Biol. Chem.* 267, 3795–3800).

Transcription elongation occurs via the sequential transfer of nucleotide monophosphates from NTPs to the 3'-OH-end of an established RNA chain, with each increment being accompanied by the release of pyrophosphate (PP_i).¹ The key intermediate in this reaction is the ternary complex of RNA polymerase, DNA template, and nascent RNA. Although the multisubunit RNA polymerases of prokaryotes and eukaryotes (the latter including nuclear RNA polymerases I, II, and III, and the RNA polymerase encoded by vaccinia virus) employ very different strategies to initiate transcription, they appear to adopt fairly similar structures in the context of their respective elongation complexes. Common features, revealed by footprinting analyses of isolated complexes, include an unwound region of template DNA near the RNA growing point (the transcription bubble), protein-DNA interactions extending ahead and behind the growing point, and a binding domain for nascent RNA within the RNA polymerase (1–11).

The ternary complex of *Escherichia coli* RNA polymerase

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¹ The abbreviations used are: PP_i, pyrophosphate; TFIIS, elongation factor SII; VETF, vaccinia early transcription factor; HPLC, high-performance liquid chromatography.

has been studied most intensively (1–5, 12–14). At any given template position, the enzyme can either rapidly incorporate the next nucleotide, pause transiently before elongating, pause indefinitely (resulting in a "dead end" complex), or dissociate from template and release the RNA (a termination reaction). Although the details of the elongation/termination decision are only beginning to be appreciated, it is clear that template sequence context is a major parameter affecting the function of the elongation complex and perhaps its structure as well (5).

A significant and most surprising property of the bacterial polymerase is the spontaneous cleavage of nascent RNA by isolated ternary complexes. In this reaction, described recently by Surrat *et al.* (15), the transcript is incised at sites up to 10 nucleotides from the growing point. The 5'-terminal fragment bearing a 3'-OH-end is retained within the ternary complex and can, in fact, be elongated upon provision of NTPs. The 3' fragment is released from the polymerase as a 5'-phosphate-terminated species. This novel cleavage reaction requires a divalent cation as cofactor. It differs clearly from previously described pyrophosphorolysis reactions mediated by ternary complexes. The latter reaction, which constitutes a true reversal of the elongation step, requires exogenous pyrophosphate and results in the release of nucleoside triphosphates from the 3'-end of the nascent RNA chain (16, 17).

Reines (18) has shown recently that ternary complexes of rat RNA polymerase II halted at a naturally occurring pause site can also shorten nascent RNA in a divalent cation-dependent, PP_i-independent reaction. Interestingly, this transcript shortening requires the elongation factor SII (TFIIS) that has been shown to promote readthrough of intrinsic pause sites by RNA polymerase II (18, 19). Removal of nucleotides from the 3'-end of the nascent chain has been invoked as a potential proofreading mechanism during mRNA synthesis (18).

This laboratory has probed the structure and catalytic properties of ternary complexes of vaccinia virus RNA polymerase halted at promoter-proximal template sites (10, 11, 20, 21). Arrest of vaccinia polymerase at unique G positions (with minimal readthrough) has been achieved using the nucleotide analog 3'-OMeGTP in lieu of GTP (21). 3'-OMeGMP-arrested nascent chains remain engaged by polymerase complexes formed in solution and can be elongated upon provision of excess GTP (10, 21). This process necessitates removal of the 3'-OMeGMP residue from the transcript. Although extension of 3'-OMeGMP-arrested chains in solution did not require exogenous PP_i, we were inclined initially to attribute 3'-nucleotide exchange to pyrophosphorolysis (21).

The present report describes a new approach to the isolation and analysis of ternary transcription complexes. Elongation complexes of vaccinia polymerase formed in solution and containing radiolabeled 3'-OMeGMP-arrested transcripts are

resolved by native gel electrophoresis; such complexes can resume elongation *in situ* when excised gel slices are provided with NTPs. Traversal of the OMeGMP block by purified complexes is independent of exogenous PP_i. We show that the ternary complex catalyzes shortening of the transcript in the presence of a divalent cation and that RNA cleavage is stimulated by CTP. This suggests that cleavage, rather than pyrophosphorolysis, accounts for the removal of chain-arresting nucleotide from the nascent chain. Amino acid sequence similarity between mammalian elongation factor SII (TFIIS) and rpo30, an intrinsic subunit of vaccinia RNA polymerase (22), suggests that rpo30 may play a role in promoting elongation and/or transcript cleavage by the viral transcription system.

EXPERIMENTAL PROCEDURES

Proteins—Transcriptionally active RNA polymerase (containing core polymerase and the vaccinia early transcription factor, VETF) was isolated by phosphocellulose chromatography of the DEAE-II-bound fraction as described (23). One unit catalyzed incorporation of 1 nmol of UMP into acid-insoluble material under standard assay conditions using a nonspecific single-strand DNA template. Capping enzyme was purified through the phosphocellulose step as described (24).

Transcription in Vitro—Standard reaction mixtures (20 μ l) contained 20 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 5% glycerol, 6 mM MgCl₂ (unless otherwise indicated), 1.8 ng of Gn DNA template (gel-purified *Pvu*II restriction fragment containing a vaccinia early promoter fused to a G-less cassette (21)), 0.13 unit of vaccinia RNA polymerase, NTPs, and other components as indicated. Reaction mixtures were incubated for 10 min at 30 °C. RNA synthesis was halted by addition of SDS-containing buffer, and the mixtures were processed for denaturing gel electrophoresis as described (21).

Isolation of Ternary Complexes Containing Radiolabeled Nascent RNA—Standard transcription reaction mixtures were incubated for 10 min at 30 °C and then applied directly to a native polyacrylamide gel (4% acrylamide, 0.13% bisacrylamide; 0.4-mm thickness) containing 0.25 \times TBE (22.5 mM Tris, 22.5 mM borate, 0.25 mM EDTA). Electrophoresis was performed at room temperature at 15 mA until a bromphenol blue dye marker had migrated approximately 13 cm. The gel was transferred to a sheet of Whatman 3MM filter paper, and radiolabeled ternary complexes were located by autoradiographic exposure of the gel for 1 h at room temperature. Slices containing labeled ternary complexes were excised from the gel and placed in 0.1 ml of a solution containing 20 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, and other components as indicated. RNA was isolated from the slice by the addition of 400 μ l of RNazol (Cinna-Biotex Laboratories) supplemented with 1 mM EDTA and 12.5 μ g/ml of yeast tRNA. After vigorous vortexing, the liquid was removed from the polyacrylamide and then mixed with chloroform (40 μ l). The mixture was vortexed, and the phases were separated by centrifugation. The labeled RNA was then recovered from the aqueous phase by precipitation with isopropanol. Precipitates were dissolved in 7 μ l of formamide, heated at 90 °C for 4 min, and then analyzed by electrophoresis through a 17% polyacrylamide gel (acrylamide:bisacrylamide = 20:1) containing 7 M urea in TBE (90 mM Tris, 90 mM borate, 1 mM EDTA). Electrophoresis was at 60 watts of constant power. Reaction products were visualized by autoradiographic exposure of the gel.

Materials—Ribonucleoside triphosphates (high-pressure liquid chromatography-purified) were obtained from Pharmacia LKB Biotechnology Inc. Radiolabeled nucleotides were purchased from Amersham. Restriction endonucleases were purchased from New England Biolabs. Divalent metal salts were purchased from Fisher.

RESULTS

Rationale—Analysis of transcription elongation intermediates necessitates the isolation of homogeneous populations of ternary complexes paused at unique template positions. To accomplish this, advantage can be taken of the propensity of RNA polymerase to pause *in vitro* at naturally occurring template sites. Alternatively, stable arrest can be induced by manipulation of *in vitro* reaction conditions, e.g. via nucleotide omission or incorporation of chain-arresting nucleotide ana-

logs. Various methods have been employed to separate ternary complexes from unincorporated NTPs and unengaged transcription factors; these include gel filtration (12), DNA affinity purification using biotinylated template (25), and RNA affinity purification using monoclonal anti-RNA antibody (26). In each case, it has been possible to verify the integrity of the isolated complexes by their ability to resume elongation upon provision of NTPs.

We have shown that elongation complexes of vaccinia RNA polymerase can be formed in solution (with virtually quantitative template utilization) on linear duplex templates containing a viral early promoter fused to a G-less cassette (11). Transcripts programmed by our G21 template in the presence of ATP, UTP, [α -³²P]CTP and 3'-OMeGTP included a predominant species of a size expected for a 3'-OMeGMP-arrested 21-mer (Fig. 1, lane 1). This RNA was engaged by RNA

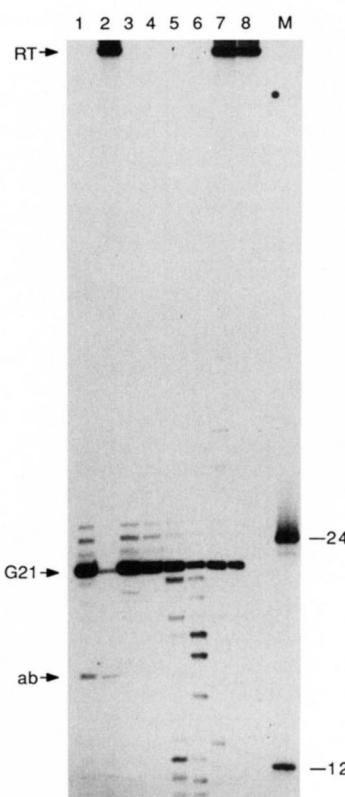


FIG. 1. Transcription *in situ* and RNA shortening by purified ternary complexes. Arrested ternary transcription complexes were formed in the presence of G21 template DNA, 1 mM ATP, 0.1 mM UTP, 1 μ M [α -³²P]CTP (1000 Ci/mmol), and 0.1 mM 3'-OMeGTP. Reactions were incubated for 10' at 30 °C. RNA from control reactions was either isolated immediately (lane 1) or chased by addition of 1 mM ATP, 1 mM UTP, 1 mM CTP, and 1 mM GTP with further incubation for 10 min at 30 °C prior to isolation (lane 2). Ternary complexes from all other reactions (lanes 3-8) were gel-purified as described under "Experimental Procedures." Gel slices containing ternary complexes were incubated in buffer as described under "Experimental Procedures" with no additions (lane 3) or in buffer supplemented with the following: Lane 4, 20 mM EDTA; lane 5, 6 mM MgCl₂; lane 6, 6 mM MgCl₂ and 1 mM sodium pyrophosphate; lane 7, 6 mM MgCl₂ and 1 mM ATP, 1 mM UTP, 1 mM CTP, and 1 mM GTP; or lane 8, 6 mM MgCl₂, 1 mM sodium pyrophosphate, and 1 mM each of ATP, UTP, CTP, and GTP. RNA was recovered from the gel slice and analyzed by gel electrophoresis. An autoradiograph of the gel is shown. Arrows at the left indicate the positions of pulse-labeled 3'-OMeG21 RNA (G21), shorter abortive transcripts (ab), and full-length runoff transcripts (RT). Arrows on the right indicate the positions and sizes (in nucleotides) of coelectrophoresed 5'-end-labeled single-stranded DNA oligonucleotide markers (lane M).

polymerase within an active transcription complex insofar as it could be elongated in solution upon supplementation of the reaction mixture with excess unlabeled ATP, CTP, UTP, and GTP (Fig. 1, lane 2). A minor RNA species (approximately 15 nt long) that did not elongate represented an abortive transcript (Fig. 1, lanes 1 and 2, indicated by "ab"). We have also demonstrated that the radiolabeled nascent chain remains associated with the ternary complex during electrophoresis through a polyacrylamide gel under nondenaturing conditions (11). RNA extracted from a gel slice containing the ternary complex was essentially identical to the product of reactions in solution, the exception being that the minor abortive transcript was not associated with the ternary complex (Fig. 1, lane 3). Thus, electrophoresis of transcription reaction mixtures provided an easy, one-step method to obtain RNA-labeled ternary complexes well resolved from nucleotides, free DNA, and other protein-DNA complexes. Initial experiments addressed whether these purified complexes were transcriptionally active.

Transcription Elongation *In Situ*—Upon transfer of an intact gel slice containing nascent 3'-OMeG21 RNA to a solution containing $MgCl_2$, ATP, UTP, CTP, and GTP, the majority of the transcripts could be elongated to the end of the DNA template, as evinced by the appearance of a labeled runoff transcript ("RT" in Fig. 1, lane 7). Clearly, ternary complexes of vaccinia polymerase retained catalytic activity even after electrophoretic isolation and autoradiography (procedures routinely entailing some 3 h of manipulation at room temperature). The proportion of gel-purified ternary complexes undergoing elongation during the chase was somewhat less than that of complexes formed in solution (Fig. 1, compare lanes 7 with 1). Transcription elongation *in situ* did not require exogenous PP_i, nor was the proportion of elongated complexes affected by its inclusion (Fig. 1, compare lane 8 with 7). This suggested that the removal of the arresting 3'-OMeGMP group, a prerequisite for the observed elongation, did not occur via pyrophosphorolysis.

Transcript Shortening—No elongation of nascent RNA was seen when gel slices were incubated in the absence of NTPs and Mg²⁺, nor was there any change in the size distribution of the transcripts under these conditions (Fig. 1, lanes 3 and 4). In contrast, ternary complexes incubated in the presence of Mg²⁺ only gave rise to multiple novel RNA species shorter than the initial 21-mer nascent chain (Fig. 1, lane 5). This RNA shortening reaction did not require exogenous PP_i. In the presence of Mg²⁺ and PP_i (absent NTPs) the ternary complexes generated additional shortened products (Fig. 1, lane 6); the species unique to the PP_i-containing reaction were presumed to arise via pyrophosphorolysis of the 21-mer, as demonstrated earlier for vaccinia ternary complexes assembled in solution (21). The PP_i-independent reaction was presumed to occur via RNA cleavage, as shown directly for *E. coli* RNA polymerase (15) and suggested for RNA polymerase II (18, 19).

The elongation of nascent RNA by isolated ternary complex in the presence of Mg²⁺ and NTPs was blocked by exposure of the gel slice to 0.1% SDS (Fig. 2, lane 1 vs. 2), indicating that the elongation complex had been disrupted by detergent treatment. Production of shortened RNA products in the presence of Mg²⁺ was similarly abrogated by SDS (lane 3 vs. 5). To eliminate the possibility that the RNA cleavage observed in these reactions was caused by a Mg²⁺-dependent ribonuclease extraneous to the ternary complex (*i.e.* as a contaminant of the reagents), a gel slice containing isolated ternary complex was incubated in the presence of magnesium and purified exogenous radiolabeled RNA (G34 transcript

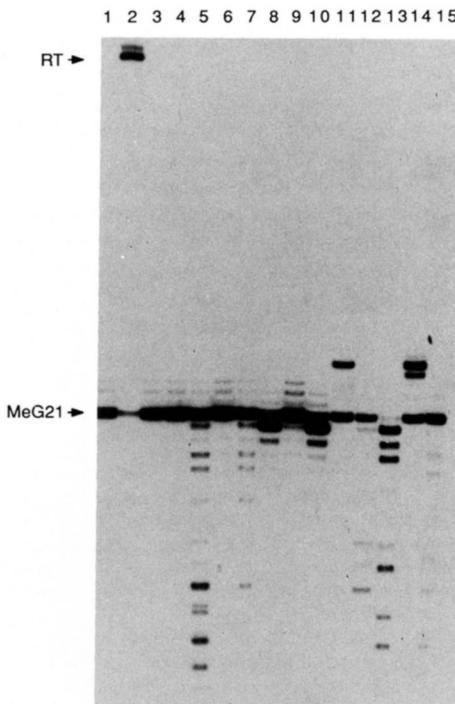


FIG. 2. Effect of divalent cations and nucleotides on RNA cleavage. Ternary complexes containing 3'-OMeG21 transcript were formed and gel-purified as described in the legend to Fig. 1. Gel slices were incubated for 15 min in buffer with the following additions: 6 mM MgCl₂, 0.1% SDS, and 1 mM each of ATP, UTP, CTP, and GTP (lane 1); 6 mM MgCl₂, and 1 mM each of ATP, UTP, CTP and GTP (lane 2); 6 mM MgCl₂, 0.1% SDS (lane 3); no addition (lane 4); 6 mM MgCl₂ (lane 5); 6 mM CaCl₂ (lane 6); 6 mM MnCl₂ (lane 7); 6 mM CoCl₂ (lane 8); 6 mM CuSO₄ (lane 9); 6 mM ZnCl₂ (lane 10); 6 mM MgCl₂ and 1 mM ATP (lane 11); 6 mM MgCl₂ and 1 mM UTP (lane 12); 6 mM MgCl₂ and 1 mM CTP (lane 13); 6 mM MgCl₂ and 1 mM GTP (lane 14); 6 mM MgCl₂ and 1 mM 3'-OMeGTP (lane 15). The positions of pulse-labeled 3'-OMeG21 RNA (MeG21) and runoff transcripts (RT) are indicated at the left of the autoradiogram by arrows.

synthesized by vaccinia RNA polymerase). The exogenous RNA remained completely intact during such incubation (data not shown), suggesting that cleavage occurred in *cis*, *i.e.* only on the nascent RNA contained within an elongation complex.

Divalent Cation Requirement for Transcript Cleavage—RNA shortening by the vaccinia ternary complex required a divalent cation; optimal shortening in the presence of Mg²⁺ occurred between 6 and 17 mM concentration (not shown). Other divalent cations at 6 mM concentration could substitute for Mg²⁺ in supporting transcript cleavage. The distribution of cleavage products in the presence of Mn²⁺ was similar to that seen with magnesium, although there was a lower yield of the smallest species (Fig. 2, lane 7). In contrast, cleavage effected by Co²⁺ or Zn²⁺ yielded transcripts that were shortened by only one or two nucleotides (Fig. 2, lanes 8 and 10). Incubation with 6 mM Ca (lane 6) or Cu (lane 9) resulted in no RNA cleavage. Other concentration of these metals were not evaluated.

The divalent cation specificity for RNA cleavage by the purified ternary complexes was compared to that for chain elongation (Fig. 3B). Extension of nascent 3'-OMeG21 RNA to the end of the linear template occurred in the presence of Mg²⁺, Mn²⁺, or Co. Nearly all nascent chains were elongated *in situ* in this experiment. Discrete elongation products of intermediate size were seen with Mn²⁺ and Co²⁺ that were not apparent in Mg²⁺-containing reactions (Fig. 3B). These

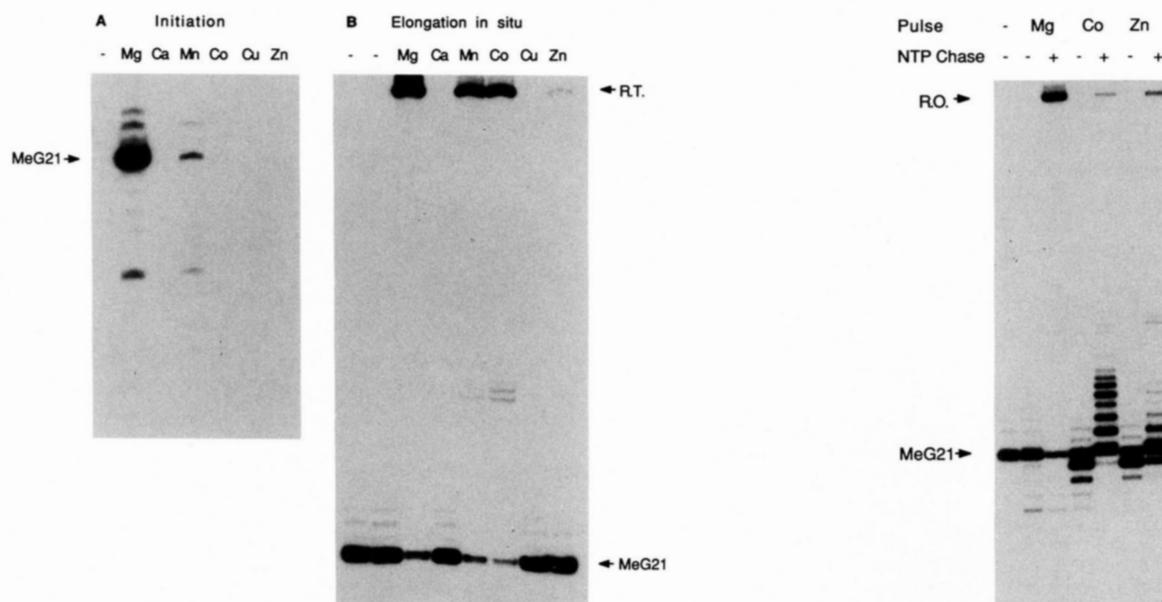


FIG. 3. Divalent cation requirement for transcription initiation and elongation. *A*, pulse-labeling reactions were performed in the presence of G21 template DNA, 1 mM ATP, 0.1 mM UTP, 1 μ M [α - 32 P]CTP (1000 Ci/mmole), 0.1 mM 3'-OMeGTP, and 6 mM concentration of either MgCl₂, CaCl₂, MnCl₂, CoCl₂, CuSO₄, or ZnCl₂ as indicated. A control reaction (−) contained no divalent cation. Incubation was for 10 min at 30 °C. RNA was purified and analyzed by gel electrophoresis. The position of pulse-labeled 3'-OMeG21 RNA (*MeG21*) is indicated at the left of the autoradiogram by the arrow. *B*, gel-purified ternary transcription complexes halted at G21 (isolated as described in the legend to Fig. 1) were incubated in buffer containing 1 mM each of ATP, UTP, CTP, and GTP, and 6 mM concentration of either MgCl₂, CaCl₂, MnCl₂, CoCl₂, CuSO₄, or ZnCl₂ as indicated. Control reactions (−) contained no divalent cation. Incubation was for 15 min at 30 °C. The positions of pulse-labeled 3'-OMeG21 RNA (*MeG21*) and runoff transcripts (*RT*) are indicated at the right of the autoradiogram by arrows.

may arise via pausing or premature termination. Zn²⁺ supported synthesis of only a low level of readthrough product. Although some intermediate-sized chains were apparent on longer exposure, it was obvious that the majority of polymerases did not elongate in the presence of Zn²⁺. Ca²⁺ and Cu²⁺ supported no RNA elongation in this assay. Thus, the ability to elongate the 3'-OMeG21 RNA in the presence of these metals correlated with their potential to support transcript cleavage.

Metal effects on initiation and early elongation were assayed by the amount of 3'-OMeG21 synthesized in a 10-min pulse-labeling reaction in solution (Fig. 3A). Synthesis in the presence of Mg²⁺ was significantly more efficient than with Mn²⁺. Ca²⁺, Co²⁺, Cu²⁺, or Zn²⁺ did not support detectable synthesis (Fig. 3A). The narrower cation specificity in initiation/elongation *versus* "pure" elongation may reflect the requirement during the initiation step for the DNA-dependent ATPase of the vaccinia early transcription factor (VETF), which is activated by Mg²⁺ or Mn²⁺, but not by Zn²⁺ or Co²⁺ (27). Ca²⁺ can activate VETF ATPase (27), but, as shown in Fig. 3B, is unable to serve as a cofactor for chain elongation by the polymerase. Because synthesis of the 21-mer requires initiation and elongation, we would attribute the Ca²⁺ effect seen in Fig. 3A to its inability to support elongation.

Shortened Transcripts Are Elongated *In Situ*—Gel-purified ternary complexes were incubated with either Mg²⁺, Co²⁺, or Zn²⁺ for 15 min (cleavage phase), then either extracted immediately or else supplemented with NTPs and incubated for an additional 15 min (NTP chase, Fig. 4). Mg²⁺-dependent

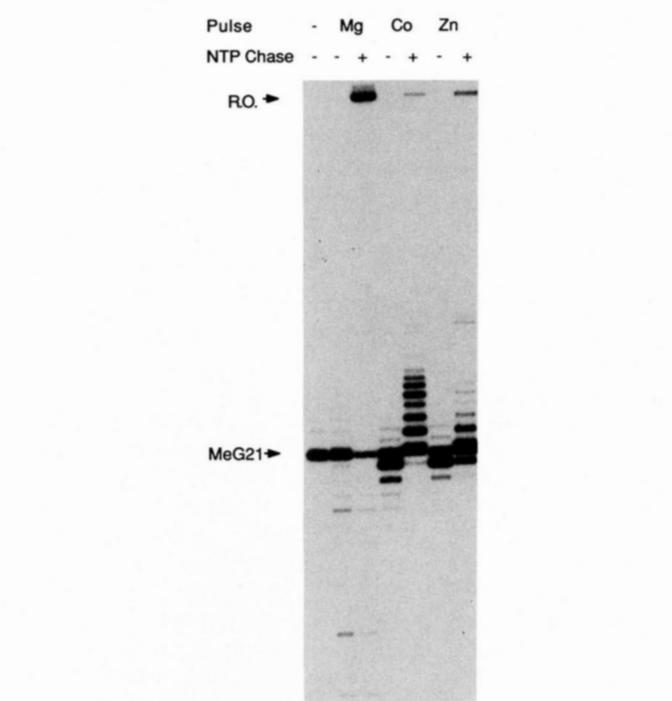


FIG. 4. Cleaved transcripts are elongated *in situ*. Gel-purified G21 ternary complexes were induced to cleave nascent RNA during a "pulse" phase by incubation for 15 min at 30 °C in the presence of 6 mM divalent cation (either Mg²⁺, Co²⁺, or Zn²⁺) as indicated. A control reaction (*Pulse* −) was incubated in the absence of divalent cation. RNA was either isolated from the gel slice immediately after the pulse phase (*NTP Chase* −) or after the reaction mixture had been subjected to a chase phase by addition of 1 mM each of ATP, UTP, CTP, and GTP with further incubation for 15 min at 30 °C (*NTP Chase* +). The positions of 3'-OMeG21 RNA (*MeG21*) and runoff RNA (*R.O.*) are indicated at the left of the autoradiogram by arrows.

RNA cleavage products included species with apparent chain lengths of 20, 19, 18, 17, 15, 11, and 8 nucleotides (Fig. 4). The RNAs 18–20 nucleotides long were not evident after the chase, indicating that they (along with a portion of the RNAs not yet cleaved during the first phase) were elongated during the second phase to yield a runoff transcript (Fig. 4, compare Mg²⁺ lanes − and +). Few of the 17-mer and 11-mer cleavage products were elongated during the chase; no elongation of the 15-mer and 8-mer species was evident.

Cleavage products generated in the presence of Co²⁺ included major species 20 and 19 nucleotides long and minor RNAs of 18 and 17 nucleotides. These transcripts were elongated virtually quantitatively during the NTP chase. However, the extent of elongation during the Co²⁺ chase was, for the majority of the transcripts, limited to the addition of 2–10 nucleotides. Only a minority of the chains were elongated to the end of the template. Comparison of the Co²⁺-dependent elongation products of the pulse-chase experiment (Fig. 4) with those seen after one-step elongation *in situ* (Fig. 3B) suggested that extended incubation of the ternary complex in solutions containing Co²⁺ served to diminish the processivity of the template-engaged RNA polymerase. Cleavage products of 20 and 19 nucleotides generated by incubation with Zn²⁺ were elongated during NTP chase, but only by the addition of 2–10 bases, with few chains being extended to the end of the template.

These data showed that most of the products of metal-induced shortening of the 3'-OMeG21 RNA, particularly those 18 nucleotides or greater in length, contain a 3'-hydroxyl terminus and remain associated with the RNA polym-

erase elongation complex. Because elongation required prior removal of the blocking 3'-OMeGMP residue, shortening of these transcripts must have occurred (at least in part) from the 3'-end.

Effect of Nucleotides on RNA Cleavage—Gel-purified ternary complexes were incubated in the presence of magnesium and 1 mM concentration of each of the four individual nucleoside triphosphates. Because the DNA sequence (nontemplate strand) of the region immediately surrounding G21 is C₁₈TAG₂₁GGGAT₂₆, it was to be expected that, in the presence of GTP only, the removal of the blocking 3'-OMeGMP moiety might be followed by elongation of the chain to position G24. Elongation to G24 was indeed observed upon addition of GTP, however the majority of transcripts that had elongated were halted one base downstream at position A25 (Fig. 2, lane 14). We attributed this to contamination of the GTP preparation with ATP. Likewise, incubation of ternary complexes with ATP alone resulted in readthrough to position A25 (Fig. 2, lane 11). This was likely caused by contamination of the ATP stock solution with either GTP or inosine triphosphate (21). NTP cross-contamination (even with HPLC-purified NTPs) with unwanted transcriptional readthrough is an oft-encountered problem with nucleotide omission experiments (12, 21). Contamination of purine NTPs with pyrimidines appeared insignificant insofar as elongation past A25 was not seen during incubation with ATP or GTP. The pyrimidine NTPs were apparently not contaminated with GTP because no elongation past G21 occurred during incubation with UTP alone (lane 12). Incubation of the gel-purified complexes with a combination of GTP, ATP, and UTP allowed elongation of RNA chains to position U26 (not shown).

When G21 ternary complexes were able to elongate in the presence of GTP or ATP, the accumulation of shortened RNAs was greatly suppressed (Fig. 2, compare lanes 11 and 14 with lane 5), suggesting that whatever chains were shortened could be "walked" to the next arrest site upon provision of the appropriate NTPs. The sizes of the walked transcripts indicated that shortening had occurred exclusively from the 3'-end of the nascent RNA; had there been removal of nucleotides from the 5'-end, the walked RNAs should be smaller than the 25-mer and more heterogeneous in distribution. Also, it was inferred that the first cleavage event from the 3'-end of the transcript must have occurred within two nucleotides of the 3'-OMeGMP residue, because any strand scission further upstream would have mandated incorporation of pyrimidines during the walk. This implied that the shortest cleavage products arose via several steps of nucleotide removal, rather than a single endonucleolytic event.

The inclusion of CTP with Mg²⁺ stimulated the cleavage reaction, resulting in nearly complete conversion of 3'-OMeG21 RNA into shorter products (Fig. 2, lane 13; Fig. 5). No such stimulation was observed with UTP (Fig. 2, lane 12) or 3'-OMeGTP (Fig. 2, lane 15). The size distribution of the CTP-dependent cleavage products differed from that observed with Mg²⁺ alone; the majority were 18–20 nucleotides in length, indicative of shortening by 1, 2, or 3 bases from the 3'-end. Note that the high yield of the 20-mer species could not be attributed to incorporation of CMP subsequent to cleavage, insofar as the first C residue was at position 18.

Various cytidine nucleotides (at 1 mM concentration) were examined for their effect on transcript shortening. The cleavage reaction was stimulated specifically by CTP, but not by CMP (Fig. 5). CDP did not affect the extent of the reaction (in terms of the proportion of RNAs cleaved), but did alter slightly the distribution of reaction products of <18 nucleo-

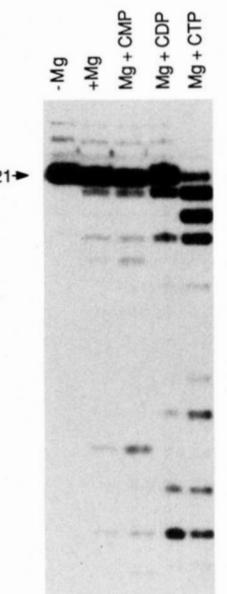


FIG. 5. Effect of cytidine nucleotides on RNA cleavage. Gel-purified G21 ternary complexes were incubated in the presence of 6 mM MgCl₂ and the indicated cytidine nucleotide (either CTP, CDP, or CMP) at 1 mM concentration. A control reaction lacked MgCl₂ (-Mg). Incubation was for 15 min at 30 °C. The position of labeled 3'-OMeG21 RNA (MeG21) is indicated at the left of the autoradiogram. The sequence of the G21 transcript is displayed alongside the ladder of RNA cleavage products at the right so as to indicate the presumptive 3'-terminal base of each labeled species.

tides toward that observed with CTP (Fig. 5). Incubation with dCTP or ddCTP had no effect on the cleavage reaction (data not shown). Titration of CTP in 10-fold increments from 0.1 μM to 1 mM concentration showed that the increase in the proportion of transcripts shortened (with accumulation of 18-, 19-, and 20-mers) occurred at 1 mM concentration, whereas a more subtle change in the distribution of the smaller cleavage products (identical to that seen with CDP in Fig. 5) with no increase in the generation of the larger cleaved transcripts was manifest even at 0.1 μM CTP (data not shown). Reference to the nucleotide sequence of the transcript (shown at the side of Fig. 5) suggested that the shift in distribution of the smaller cleavage products was caused by incorporation of CMP at the 3'-end of the cleaved nascent chain (e.g. note that the transcripts shortened to positions A17, U15, and U11 in the presence of Mg²⁺ alone were not evident in the presence of CTP; instead there was an increase in, or new appearance of, chains one base longer and ending at C18, C16, and C12). We suspect then that the subtle effect of 1 mM CDP (Fig. 5) may be caused by contamination of the CDP with low levels of CTP.

Time Course of Transcript Shortening—Ternary complexes synthesized on a G34 template and containing predominantly 3'-OMeG34 RNA (Fig. 6, lane 0) were gel-purified and then incubated for various times in the presence of Mg²⁺. Shortened RNA species were evident within 30 s. Cleaved RNAs 18 to 33 nucleotides long that accumulated at early time points (up to 2 min) clearly underwent further shortening as incubation was continued (to 5 min and beyond), yielding multiple species of 13 or fewer bases in length. (Note that the yield of labeled cleavage products shorter than 7 nucleotides might not be reflected accurately by this experiment because a precipitation step was used during recovery of the RNA). Although certain cleavage intermediates accumulated preferentially, it was evident that a ladder had been generated, suggesting that all internucleotide bonds were able to be

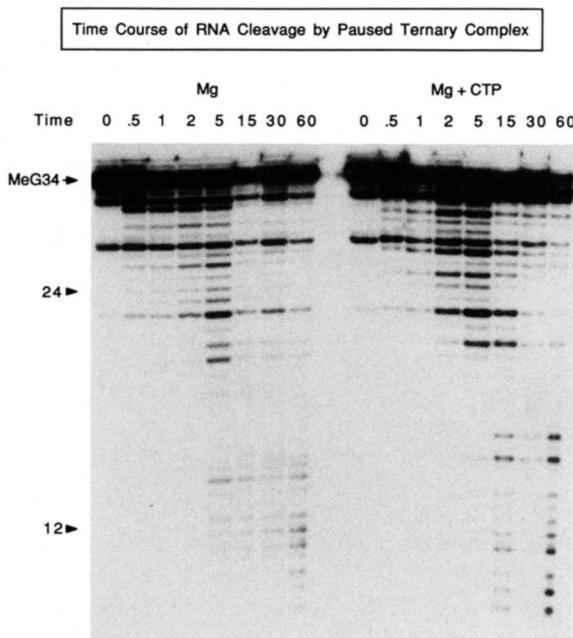


FIG. 6. Kinetics of RNA shortening. Arrested ternary complexes were formed in the presence of G34 template DNA, 6 mM MgCl₂, 1 mM ATP, 0.1 mM UTP, 1 μM [α -³²P]CTP (1000 Ci/mmol), 0.1 mM 3'-OMeGTP, and 50 μM AdoMet, and then gel-purified. Individual gel slices were incubated for various times in the presence of 6 mM MgCl₂ (left) or 6 mM MgCl₂ plus 1 mM CTP (right). Reaction times were 0, 0.5, 1, 2, 5, 15, 30, or 60 min as indicated above the lanes. RNA was isolated and analyzed by gel electrophoresis. The positions of labeled 3'-OMeG34 RNA (MeG34) and 5'-end-labeled 24- and 12-mer DNA markers are indicated at the left of the autoradiogram.

incised. A parallel time course indicated that CTP did not greatly influence the rate of chain shortening or the timing of the appearance of the first shortened RNA. Instead, the primary effect seemed to be to increase the proportion of ternary complexes that cleaved the nascent chain (this was more obvious on lighter autoradiographic exposures of the gel). During the period between 5 and 60 min, two distinct classes of cleaved RNAs were produced in the presence of CTP, these being longer RNAs 31 to 32 nucleotides in length and a mixture of shorter RNAs less than 13 nucleotides in length. The CTP-dependent accumulation of RNAs shortened by only a few nucleotides was apparently independent of the length of the starting nascent chain (compare G21 in Figs. 3 and 4 with G34 in Fig. 5).

Stability of Shortened Transcripts Within the Ternary Complex—Radiolabeled nascent chains contained within G34 ternary complexes (Fig. 7, lanes 1 and 2) were elongated nearly quantitatively to the end of the template upon incubation of the gel slice in the presence Mg²⁺ and the four NTPs (lane 3). Incubation with Mg²⁺ alone generated a ladder of cleavage products (lane 4). When complexes that had been incubated initially with Mg²⁺ alone were supplemented with NTPs, it was seen that those cleavage products 19 nucleotides or longer were efficiently chased to the end of the template (lane 5). Comparison of lanes 4 and 5 suggested that some of the 18-, 17-, and 11-mer transcripts were also elongated. Shorter species arising from Mg²⁺-induced cleavage were not prone to elongation, indicating that they had either dissociated from RNA polymerase or were engaged by the enzyme within a "dead-end" complex. (Note that these estimates of chain length were based on the spacing of the RNA ladder, not on mobility relative to the 5'-labeled 24- and 12-mer DNAs used

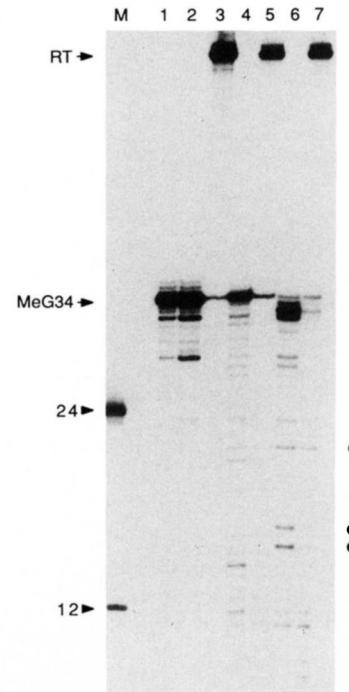


FIG. 7. Elongation of cleaved RNAs by polymerase engaged on G34 template. Arrested ternary complexes were generated on G34 template DNA as described in Fig. 6. RNA was isolated directly from a control reaction (lane 1). Gel-purified ternary complexes were incubated with no addition (lane 2) or in the presence of 6 mM MgCl₂ plus 1 mM each of ATP, UTP, CTP, and GTP (lane 3) for 15 min at 30 °C. A second set of purified ternary complexes was incubated with 6 mM MgCl₂ (lanes 4 and 5) or 6 mM MgCl₂ plus 1 mM CTP (lanes 6 and 7) for 15' at 30 °C. RNA was either isolated immediately (lanes 4 and 6) or after the samples were subjected to a chase phase by addition of 1 mM each of ATP, UTP, CTP, and GTP with further incubation for 15 min at 30 °C (lanes 5 and 7). The positions of labeled 3'-OMeG34 RNA (MeG34), runoff transcript (RT), and 5'-end-labeled 24- and 12-mer DNA markers are indicated at the left of the autoradiogram. Transcripts denoted by filled and open circles at right are discussed in the text.

to establish a size range of the electrophoretic analysis.)

Cleavage products generated in the presence of CTP (lane 6) could also be elongated efficiently upon provision of NTPs (lane 7). These included the major cleavage products 31–33 nucleotides long and multiple other cleavage products with apparent chain lengths as short as 12 or 13 nucleotides (denoted by filled circles in Fig. 7). Cleaved RNAs less than 12 nucleotides were not elongated (lane 7). The ability to extend the shortened transcripts was not determined solely by the size of the cleaved chain insofar as the 18-mer cleavage product seen in the presence of CTP (denoted by open circle) was not elongated, whereas chains of smaller size were chased (lane 7). These findings suggested that the stability of the ternary complex during transcript shortening was influenced by transcript size and template sequence context in much the same way as during establishment of the elongation complex after chain initiation (21).

Shortening of Cap-labeled Nascent Chains—RNAs synthesized by vaccinia RNA polymerase can be uniquely 5' cap-labeled *in vitro* when transcription reactions are performed in the presence of [α -³²P]dGTP, AdoMet, and vaccinia capping enzyme (10, 20). Such cap-labeled transcripts were contained with the G34 ternary complexes isolated by native gel electrophoresis (Fig. 8, lane 1). Incubation of the gel slice with Mg²⁺ and CTP resulted in transcript shortening (lane 2). The fact that the 5' cap structure was retained within the shortened

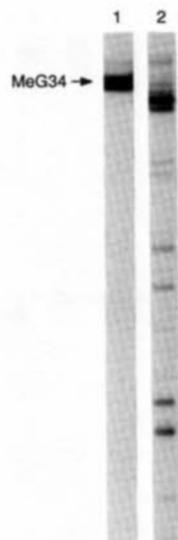


FIG. 8. Shortening of 5' cap-labeled nascent RNA. Cap-labeled nascent chains were synthesized in reaction mixtures (30 μ l) containing G34 template DNA (2.7 ng), 0.1 mM ATP, 0.1 mM UTP, 1 μ M CTP (lanes 1–3), 0.6 μ M [α -³²P]dGTP (3000 Ci/mmol), 50 μ M AdoMet, 150 fmol of capping enzyme, 6 units of VETF (glycerol gradient fraction as described (32)), and 0.2 unit of RNA polymerase. After incubation for 10 min at 30 °C, the mixtures were supplemented with 0.1 mM 3'-OMeGTP and incubated for another 2 min, at which time the labeled ternary complexes were gel-purified. Gel slices were incubated for 15 min at 30 °C in buffer with no additions (lane 1), or with 6 mM MgCl₂ plus 1 mM CTP (lane 2). RNA was isolated and analyzed by gel electrophoresis. The position of the labeled 3'-OMeG34 transcript (MeG34) is indicated at the left.

RNAs argued strongly that cleavage by the ternary complex had occurred from the 3'-end.

Shortening of RNAs Containing 3'-OH Termini—To exclude the possibility that transcript cleavage was either induced by or dependent upon the presence of the 3'-O-methyl terminus in the nascent chain, we gel-purified ternary complexes formed on the G21 template in the presence of ATP, CTP, and UTP. As shown previously (21), the two major RNAs synthesized in solution under these conditions were halted at template positions A20 and G21. These same species were present in gel-purified ternary complexes (Fig. 9, lane 1) and could be elongated quantitatively upon incubation of the gel slice with Mg²⁺ and the four NTPs (lane 2). Incubation of the gel slice in the presence of Mg²⁺ alone resulted in the appearance of shortened RNAs (lane 3). Inclusion of CTP stimulated cleavage (in terms of the proportion of nascent transcripts shortened). As noted previously, most of the CTP-dependent cleavage products had been shortened by only one to three nucleotides (lane 5). These shortened transcripts remained stably engaged by RNA polymerase, as evinced by their ability to be elongated during a chase in the presence of the four NTPs (Fig. 9, lanes 4 and 6).

DISCUSSION

An enzymatic activity capable of removing nucleotides from the 3'-end of nascent RNA is associated with the ternary complex of vaccinia virus RNA polymerase. The transcript shortening reaction is absolutely dependent on a divalent cation, is stimulated by CTP, and proceeds stepwise. Although the 3' product of the first cleavage step has not been analyzed directly, it is suggested that chains are shortened in increments of one or two nucleotides. The initial 5' cleavage product remains associated with the ternary complex, as evinced by its ability to be elongated in the presence of NTPs.



FIG. 9. Shortening of nascent RNAs containing 3'-OH termini. Transcription complexes were formed on the G21 DNA template in reactions containing 0.1 mM ATP, 0.1 mM UTP, and 1 μ M [α -³²P]CTP, but no added guanine nucleotide. Gel-purified complexes were incubated for 15 min in buffer with the following components: no addition (control); 6 mM MgCl₂; 6 mM MgCl₂ plus 1 mM CTP. RNA was either isolated immediately (Control, Mg²⁺, and Mg²⁺ + CTP) or after the samples were subjected to a chase phase by addition of 1 mM each of ATP, UTP, CTP, and GTP with further incubation for 15 min at 30 °C (Mg²⁺→Chase and Mg²⁺+CTP→Chase). The positions of the labeled G21 and A20 nascent transcripts are indicated at the left by arrows.

The findings are consistent with simple hydrolysis of the sugar phosphate backbone to yield a 3'-hydroxyl-terminated 5' fragment and a 5'-phosphate-terminated mono- or oligonucleotide.

Demonstration of the RNA cleavage reaction illuminates how ternary complexes halted by incorporation of a chain-arresting nucleotide analog (e.g. 3'-OMeGTP) are able to resume elongation when provided with the standard NTP (GTP). The pathway of pyrophosphorylative nucleotide exchange demonstrated by Kassavetis *et al.* (28) appears not to apply to the present system for the following reasons: (i) gel purification separates the ternary complexes from small molecules either included in the original transcription reaction mixture or generated during RNA synthesis, (ii) traversal of the 3'-OMeG block requires no exogenous PP_i, and (iii) addition of PP_i does not enhance traversal of the block.

It has been suggested, by analogy to DNA polymerases, that the nuclease activity of the RNA polymerase ternary complex may provide a proofreading function during mRNA synthesis (18). Exonucleolytic proofreading by DNA polymerases ensures fidelity through the removal of misincorporated bases. It is not completely clear, however, to what extent DNA-dependent RNA polymerases misincorporate NTPs, because published misincorporation frequencies vary considerably (discussed in Ref. 30). Blank *et al.* (30) found that *E. coli* RNA polymerase was extremely faithful, with misincorpora-

tion of GMP for AMP at a frequency of about 1/10⁵. They argued that the polymerase itself enhanced fidelity by 100-fold above the level expected based solely on considerations of base complementarity. A presynthetic proofreading mechanism is described for the bacterial enzyme that entails recognition and hydrolysis of noncognate NTPs by the template-bound polymerase before they can be misincorporated into RNA (31). In the present context, 3'-OMeGMP is more aptly described as "malincorporated," i.e. its addition to the RNA chain, while of pernicious consequence for the elongation complex, is specified appropriately by a complementary C residue in the template DNA strand. The vaccinia ternary complex can shorten transcripts containing a conventional 3'-hydroxyl terminus with much the same efficiency as those with blocked 3'-ends. Whether vaccinia polymerase (or any other RNA polymerase) preferentially shortens chains with a mispaired 3'-end remains to be addressed.

Multiple cycles of cleavage by the vaccinia G34 ternary complex can remove more than 25 nucleotides from the 3'-end of nascent RNA. Most cleaved RNAs \geq 18 nucleotides long, as well as some cleavage products as short as 11, 12, or 13 nucleotides, can be elongated when NTPs are provided. Thus, the catalytic center of the template-engaged polymerase must be able to translocate backward as shortening occurs. Failure to elongate cleavage products of \leq 10 bases suggests that the shortest RNAs had been released from the transcription complex. We have shown that ternary complex stability at promoter-proximal sites depends on the length of the nascent RNA and on the template sequence (21). This now appears to be the case whether the position of RNA polymerase on the template is achieved by movement in the 5' direction (elongation) or the 3' direction (via transcript shortening).

The vaccinia RNA shortening reaction is similar in some respects to the cleavage reactions described for ternary complexes of *E. coli* RNA polymerase or RNA polymerase II (15, 18, 19). Unifying themes include a strict requirement for a divalent cation and retention of the 5' fragment of the incised RNA strand within a functional elongation complex. Mechanistic differences do apply, however. Cleavage by the bacterial polymerase is endonucleolytic and can occur as far as 10 nucleotides upstream of the chain growing point (15). In contrast, human RNA polymerase II cleaves within two nucleotides of the 3' terminus (19). Our data suggest that the vaccinia polymerase may resemble RNA polymerase II in this respect. One notable distinction between the vaccinia and cellular systems is the need for accessory factors to promote transcript shortening. Rat liver and human polymerase II complexes require the transcription elongation factor SII, which is apparently not associated tightly with paused or

arrested elongation complexes assembled *in vitro* (18, 19). The gel-purified vaccinia RNA polymerase ternary complex is competent *per se* in transcript cleavage. Therefore, if nuclease activity requires a protein component other than the known polymerase subunits, this component must remain stably associated with the ternary complex during electrophoretic purification. Of the eight gene products that constitute the vaccinia RNA polymerase core enzyme (29), three (rpo147, rpo135, and rpo7) are viral homologs of RNA polymerase II subunits (*i.e.* yeast subunits rpoB1, rpoB2, and rpoB10, respectively), and one (rpo30) is homologous to the RNA polymerase II elongation factor SII (22). We suggest that whatever role SII plays in facilitating RNA cleavage by polymerase II is likely to be mediated in the vaccinia system by rpo30 as an intrinsic (*i.e.* nondissociable) component of the viral RNA polymerase. The effect of CTP on transcript shortening, which is unique thus far to the vaccinia ternary complex, suggests that the RNA cleavage reaction may be subject to allosteric activation.

REFERENCES

1. Straney, D. C., and Crothers, D. M. (1985) *Cell* **43**, 449-459
2. Krummel, B., and Chamberlin, M. J. (1989) *Biochemistry* **28**, 7829-7842
3. Carpousis, A. J., and Gralla, J. D. (1985) *J. Mol. Biol.* **183**, 165-177
4. Metzger, W., Schickor, P., and Heumann, H. (1989) *EMBO J.* **8**, 2745-2754
5. Krummel, B., and Chamberlin, M. J. (1992) *J. Mol. Biol.* **225**, 239-250
6. Linn, S. C., and Luse, D. S. (1991) *Mol. Cell. Biol.* **11**, 1508-1522
7. Rice, G. A., Kane, C. M., and Chamberlin, M. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 4245-4249
8. Bateman, E., and Paule, M. R. (1988) *Mol. Cell. Biol.* **8**, 1940-1946
9. Kassavetis, G. A., Braun, B. R., Nguyen, L. H., and Geiduschek, P. E. (1990) *Cell* **60**, 235-245
10. Hagler, J., and Shuman, S. (1992) *Science* **255**, 983-986
11. Hagler, J., and Shuman, S. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 10099-10103
12. Levin, J. R., Krummel, B., and Chamberlin, M. J. (1987) *J. Mol. Biol.* **196**, 85-100
13. Arndt, K. M., and Chamberlin, M. J. (1990) *J. Mol. Biol.* **213**, 79-108
14. Kainz, M., and Roberts, J. (1992) *Science* **255**, 838-841
15. Surrat, C. K., Milan, S. C., and Chamberlin, M. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 7983-7987
16. Maitra, U., and Hurwitz, J. (1967) *J. Biol. Chem.* **242**, 4897-4907
17. Krakow, J. S., and Fronk, E. (1969) *J. Biol. Chem.* **244**, 5988-5993
18. Reines, D. (1992) *J. Biol. Chem.* **267**, 3795-3800
19. Izban, M. G., and Luse, D. S. (1992) *Genes & Dev.* **6**, 1342-1356
20. Luo, Y., Hagler, J., and Shuman, S. (1991) *J. Biol. Chem.* **266**, 13303-13310
21. Hagler, J., and Shuman, S. (1992) *J. Biol. Chem.* **267**, 7644-7654
22. Ahn, B.-Y., Gershon, P. D., Jones, E. V., and Moss, B. (1990) *Mol. Cell. Biol.* **10**, 5433-5441
23. Shuman, S., Surks, M., Furneaux, H., and Hurwitz, J. (1980) *J. Biol. Chem.* **255**, 11588-11598
24. Shuman, S., Broyles, S. S., and Moss, B. (1987) *J. Biol. Chem.* **262**, 12372-12380
25. Marshall, N. F., and Price, D. H. (1992) *Mol. Cell. Biol.* **12**, 2078-2090
26. Reines, D. (1991) *J. Biol. Chem.* **266**, 10510-10517
27. Broyles, S. S., and Moss, B. (1988) *J. Biol. Chem.* **263**, 10761-10765
28. Kassavetis, G. A., Zentner, P. G., and Geiduschek, E. P. (1986) *J. Biol. Chem.* **261**, 14256-14265
29. Moss, B., Ahn, B.-Y., Amegadzie, B., Gershon, P. D., and Keck, J. G. (1991) *J. Biol. Chem.* **266**, 1355-1358
30. Blank, A., Gallant, J. A., Burgess, R. R., and Loeb, L. A. (1986) *Biochemistry* **25**, 5920-5928
31. Libby, R. T., Nelson, J. L., Calvo, J. M., and Gallant, J. A. (1989) *EMBO J.* **8**, 3153-3158
32. Hagler, J., and Shuman, S. (1992) *J. Virol.* **66**, 2982-2989