

# Factor-dependent Release of Nascent RNA by Ternary Complexes of Vaccinia RNA Polymerase\*

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Liang Deng, Jeremiah Hagler, and Stewart Shuman‡

From the Molecular Biology Program, Sloan-Kettering Institute, New York, New York 10021

**Factor-dependent transcription termination during synthesis of vaccinia early mRNAs occurs at heterogeneous sites downstream of a UUUUUNU signal in the nascent transcript. The choice of termination site is flexible and is determined by a kinetic balance between nascent chain elongation and the transmission of the RNA signal to the polymerase. To eliminate ongoing elongation as a variable, we have established a system to study transcript release by purified ternary complexes halted at a defined template position 50-nucleotides 3' of the first U residue of the termination signal. Release of the nascent RNA depends on the vaccinia termination factor (VTF) and an ATP cofactor. Transcript release is blocked by BrUMP substitution within the termination signal of the nascent RNA. In these respects, the release reaction faithfully mimics the properties of the termination event. We demonstrate that ternary complexes are refractory to VTF-mediated transcript release when the first U of the UUUUUNU signal is situated 20 nucleotides from the growing point of the nascent chain. Ribonuclease footprinting of the arrested ternary complexes defines a nascent RNA binding site on the polymerase elongation complex that encompasses a 16–21 nucleotide RNA segment extending proximally from the 3' end of the chain. We surmise that access of VTF to the signal sequence is prevented when UUUUUNU is bound within the nascent RNA binding site. Hence, physical not kinetic constraints determine the minimal distance between the signal and potential sites of 3' end formation.**

Vaccinia virus RNA polymerase is a multisubunit enzyme devoted exclusively to the synthesis of mRNA (1). Specificity for transcription of vaccinia early genes is conferred upon the RNA polymerase by an early transcription factor (ETF),<sup>1</sup> a heterodimer of 82- and 70-kDa subunits that binds to the early promoter and recruits RNA polymerase to the template (2–6). An essential 94-kDa polypeptide associated with the RNA polymerase (variously named RAP94, H4, or rpo94) is required for early promoter-specific transcription (7–11). rpo94 is believed to act as molecular bridge between the RNA polymerase and ETF bound at the promoter. Termination of early transcription occurs at heterogeneous sites downstream of a simple termination signal TTTTNT in the nontemplate DNA strand

(12). The signal is appreciated at the RNA level as UUUUUNU (13). A separate vaccinia termination factor (VTF) is required to transduce the UUUUUNU signal to the elongating polymerase (14). VTF is identical to the vaccinia capping enzyme, a heterodimer of 95- and 33-kDa subunits required for 5' capping and methylation of the nascent RNA chain (14–16).

VTF-dependent termination is a dynamic, energy-requiring process. The termination event is coupled to the hydrolysis of ATP (17). Termination site choice and the overall efficiency of termination are determined by a kinetic balance between the rate of signaling and the rate of polymerase movement (17). Slowing elongation rate (*e.g.* by analog-induced pausing) results in termination at template positions closer to the UUUUUNU element. In contrast, slowing the rate of signal transduction (*e.g.* by lowering the ATP concentration) shifts the distribution of termination sites further away from the termination signal (17).

Mechanistic studies of the termination reaction are complicated by the ongoing process of chain elongation, *i.e.* the target for the termination factor is constantly in flux as the polymerase changes template position. Under these circumstances, two events are required to achieve bona fide termination: (i) polymerase must elect not to incorporate the next NTP and (ii) the nascent RNA must be released from the ternary complex. Our goal in the present study was to focus strictly on the transcript release step of the termination reaction by removing elongation as a variable. Our strategy was to purify homogeneous populations of RNA-labeled ternary complexes paused at a unique template position located upstream (5') of a termination signal, then to "walk" the RNA polymerase to a defined template position downstream of the termination signal, and then test for the release of the RNA from the ternary complex in response to VTF/capping enzyme. Purification of the elongation complexes was simplified by the use of early promoter-containing DNA templates bound to a solid support (17). With this approach, we have now demonstrated that factor-dependent transcript release occurs in the absence of concomitant elongation and that the release reaction is ATP-dependent. Additional experiments establish that the UUUUUNU sequence must be situated beyond a threshold distance from the active site of the polymerase in order to signal transcript release.

## EXPERIMENTAL PROCEDURES

**Immobilized DNA Templates**—The pBS-based G21 plasmid containing a vaccinia early promoter fused to a G-less cassette has been described (18). Construction of the G21(TER29)A78 plasmid involved replacement of the sequence between the *Bam*HI and *Xba*I sites of the G21 plasmid with a synthetic A-less cassette by using standard molecular cloning techniques. The insert included a TTTTTTTT termination signal (refer to Fig. 1). A *Pvu*II fragment of G21(TER29)A78 containing the vaccinia transcription unit was then inserted into pUC19. Construction of plasmid G21(TER59)A78 plasmid entailed replacement of the segment between the *Ngo*MI and *Xba*I sites of the pUC19-G21(TER29)A78 plasmid with a different synthetic A-less cassette (see Fig. 1). The G21(TER29)A78 and G21(TER59)A78 plasmids were linearized with *Acc*65-1, which cleaved the DNA template upstream of the

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‡ To whom correspondence should be addressed: Molecular Biology Program, Sloan-Kettering Institute, 2175 York Ave., New York, NY 10021. Tel.: 212-639-7143; Fax: 212-717-3623.

<sup>1</sup> The abbreviations used are: ETF, early transcription factor; VTF, vaccinia termination factor; OMe, methoxy; CE, capping enzyme; BrUMP, bromo-UMP.

vaccinia early promoter region. Biotinylated dATP was incorporated at the 3' ends using Klenow DNA polymerase. The biotinylated DNAs were then digested with *Pvu*II, and the restriction fragment containing the transcription cassette was isolated by preparative agarose gel electrophoresis. Purified DNA fragments were attached to streptavidin-coated magnetic beads (Dynabeads M280; Dynal) by incubating the DNA with beads in 0.1 ml of TE buffer (10 mM Tris HCl (pH 8.0), 1 mM EDTA) for 30 min at room temperature. The beads were then concentrated using a horseshoe magnet and washed with 0.1 ml of TE. After three cycles of washing, the immobilized templates were resuspended in TE and stored at 4 °C for use in *in vitro* transcription reactions.

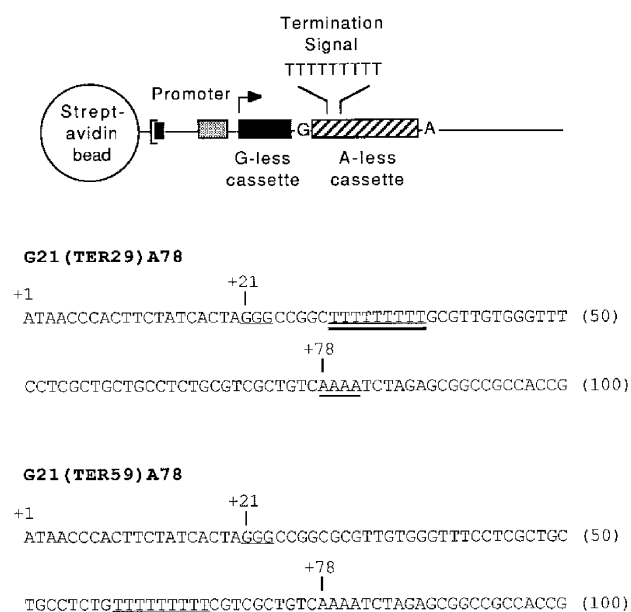
**Enzyme Purification**—Vaccinia RNA polymerase was extracted from virion cores with deoxycholate (14), and a transcriptionally active preparation containing the ETF was purified by sequential DEAE-5PW, SP-5PW, and heparin-agarose column chromatography steps using a Waters 650 chromatography system and chromatography columns purchased from Waters. Transcription activity during purification was assayed as described elsewhere (24) in reactions programmed by a *Sma*I-cut pSB24 template, which contains a vaccinia early promoter fused to a 382-nucleotide G-less cassette (24). Capping enzyme was purified from virion cores as described previously (14). The phosphocellulose fraction was used in the present study; the molar concentration of active capping enzyme was determined by enzyme-GMP complex formation (14).

**Transcription *in Vitro***—Ternary transcription complexes were formed in standard reaction mixtures containing (per 20- $\mu$ l reaction volume) 20 mM Tris-HCl (pH 8.0), 6 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 1 mM ATP, 0.1 mM UTP, 1  $\mu$ M [ $\alpha$ -<sup>32</sup>P]CTP (1000 Ci/mmol), 0.1 mM 3'-OMeGTP, bead-linked DNA (~100 fmol), and vaccinia RNA polymerase. Reaction mixtures were incubated at 30 °C for 10 min, then concentrated by microcentrifugation for 15 s. The beads were held in place by application of an external horseshoe magnet while the supernatant was removed and replaced with 0.1 ml of 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol. The beads were resuspended and subjected to two further cycles of concentration and washing; after the third wash, the beads were resuspended in a small volume of the wash buffer, and aliquots were distributed into individual reaction tubes to achieve approximately the same concentration of template as that used in the pulse-labeling phase. Elongation reactions were performed as specified in the figure legends.

## RESULTS

**Walking RNA Polymerase Past the Termination Signal**—Transcription *in vitro* by vaccinia RNA polymerase was programmed by linear templates linked to streptavidin-coated paramagnetic beads (Fig. 1). The prototype G21(TER29)A78 transcription unit consisted of a synthetic early promoter fused to a 20-nucleotide G-less cassette, which was flanked by a run of three G residues at positions +21 to +23. Downstream of the G-less cassette was inserted a 57-nucleotide A-less cassette flanked at its 3' end by a run of four A residues at positions +78 to +81. Placed within the A-less cassette was a termination signal, TTTTTTTT, spanning positions +29 to +37 (Fig. 1). Pulse-labeling transcription reactions contained ATP, UTP, [ $\alpha$ -<sup>32</sup>P]CTP and 3'-OMeGTP. The reaction products (template-engaged ternary complexes containing radiolabeled nascent RNA) were recovered by centrifugation and concentration of the beads with an externally applied magnet, followed by washing the beads with buffer lacking nucleotides and magnesium (17). The major pulse-labeled nascent chain was a 3'-OMeGMP-arrested 21-mer, as expected (Fig. 2, lane 1). Minor 22-, 23-, and 24-mer RNA species were also detected; these are 3' co-terminal transcripts that initiated from upstream template positions -1C, -2U, and -3U (18).

The integrity of the isolated ternary complexes was verified by their ability to resume elongation of the pulse-labeled RNA upon provision of unlabeled NTPs and magnesium. Omission of ATP from the elongation reaction and inclusion of the chain-terminating nucleotide 3'-dATP (cordycepin triphosphate) allowed us to "walk" the ternary complexes through the A-less cassette from G21 to A78 (Fig. 2, lane 2). All polymerase molecules that elongated past G21 were arrested at A78; we detected no read through past the +78A position under these

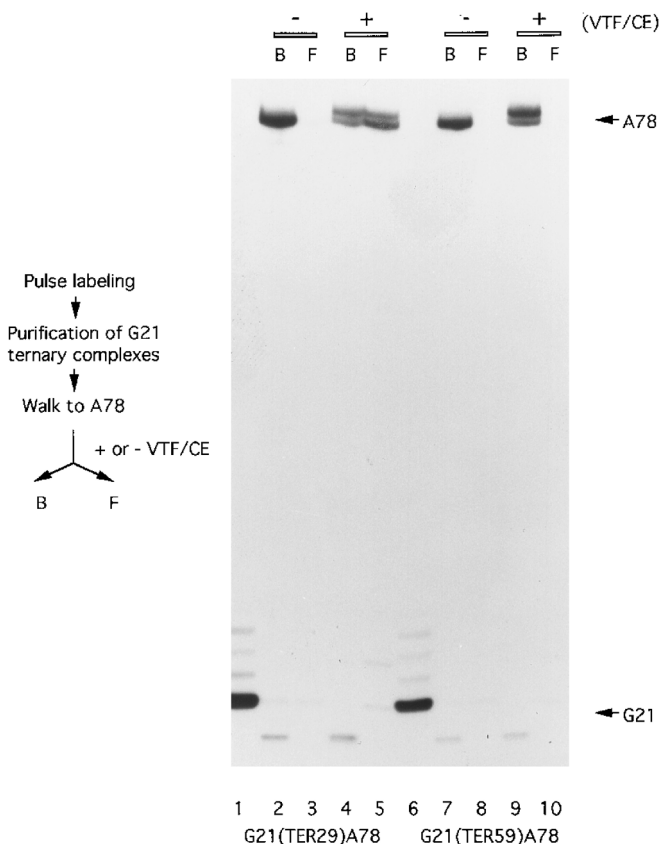


**FIG. 1. Immobilized DNA templates for assay of transcript release.** The architecture of the G21(TER)A78 DNA templates is shown in schematic form. The DNA contains a biotinylated nucleotide incorporated uniquely at the 3' end of the template DNA strand, which anchors the DNA to a streptavidin-coated magnetic bead (17). A viral early promoter element specifies the initiation of transcription at position +1 of a 20-nucleotide G-less cassette. A 57-nucleotide A-less cassette is situated downstream of the G-less cassette. The nucleotide sequences of the transcribed regions (non-template strand only) of the TER29 and TER59 templates are shown; template positions are numbered from the major site of transcription initiation at +1A to +100 of the transcription unit. The termination signal TTTTTTTTT is underlined.

reaction conditions.

**Single-nucleotide Step Increment of Nascent Chain Cleavage**—Elongation of the nascent chains beyond the arrest site at G21 depended upon removal of the blocking 3'-OMeGMP moiety by a hydrolytic activity intrinsic to the vaccinia RNA polymerase elongation complex (19). The sequence of the RNA chain at the site of pulse arrest was 5'-CTAG<sub>OMe</sub>. The distribution of RNAs after the elongation in the presence of 3'-dATP was very instructive with respect to the step increment of nascent chain cleavage. In the experiment in Fig. 2, most of the OMeG21 transcripts were elongated to the next templated A position at +78. However, a minor fraction was converted to a shorter dA20 species. The dA20 RNA was formed by incorporation of 3'-dAMP into chains that had been shortened by 2 nucleotides to T20. The fact that very few of the G21 chains were trapped at A20 argues strongly that the predominant initial RNA cleavage event was a single nucleotide step to A20, followed by elongation to A78. We cannot tell whether chains trapped at A20 were generated by two sequential one-nucleotide cleavages or by initial removal of a dinucleotide. However, the results of the cordycepin trap confirm an earlier suggestion (19) that the vaccinia ternary complex cleaves in mononucleotide increments. The viral enzyme is clearly different in this respect from cellular RNA polymerases II and III, which shorten nascent chains primarily in dinucleotide increments when polymerase II or polymerase III elongation complexes are arrested by nucleotide omission (20–22).

**VTF-dependent Transcript Release**—The use of bead-bound DNA templates provided a convenient method to assay transcript release by centrifugal/magnetic separation of template-engaged RNA products (bead-bound) from released transcripts (recovered in the supernatant). The labeled RNAs that had been walked to A78 were recovered in the template-bound



**FIG. 2. VTF-dependent transcript release.** Pulse-labeling reactions were programmed by bead-linked G21(TER29)A78 (lanes 1–5) and G21(TER59)A78 templates (lanes 6–10) in reaction mixture containing (per 20  $\mu$ l) 20 mM Tris HCl, pH 8.0, 6 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 1 mM ATP, 0.1 mM UTP, 1  $\mu$ M [ $\alpha$ -<sup>32</sup>P]CTP (1000 Ci/mmol), 0.1 mM 3'-OMeGTP, and vaccinia RNA polymerase. After incubation for 10 min at 30 °C, the bead-bound ternary complexes were purified as described (17) and resuspended in 20 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol. The bead-bound pulse-labeled RNA is shown in lanes 1 and 6. Purified G21 ternary complexes were walked to A78 during a 5 min chase in the presence of 6 mM MgCl<sub>2</sub> and 1 mM each of 3'-dATP, GTP, CTP, and UTP. The mixtures were then incubated for an additional 5 min at 30 °C with 36 fmol of VTF/CE purified from vaccinia virions. Control reactions were incubated without added VTF. The bead-bound RNA (B) was separated from released RNA (F, free) by microcentrifugation of the reaction mixtures. RNA was then recovered from the pellet and supernatant fractions by phenol extraction and ethanol precipitation. The transcription products were analyzed by electrophoresis through a 17% polyacrylamide gel containing 7 M urea in TBE (90 mM Tris, 90 mM borate, 2.5 mM EDTA). An autoradiogram of the gel is shown with the positions of the pulse-labeled OMeG21 RNA (G21) and the walked A78 transcript indicated by arrows at the right.

fraction (Fig. 2, lane 2) and very little A78 RNA was free in the supernatant (Fig. 2, lane 3). Addition of VTF/capping enzyme to the arrested A78 elongation complexes resulted a release of 60–70% of the total RNA from the beads into the supernatant (Fig. 2, lanes 4 and 5). Thus, complexes that had already synthesized the UUUUUNU signal could be induced to release the RNA chain in the absence of ongoing elongation. Note that the addition of VTF/capping enzyme to the A78 complexes resulted in capping of the 5' end of the A78 RNA. The mixed population of guanylated and unguanylated A78 chains migrated as a doublet (Fig. 2, lanes 4 and 5). Note also that the trapped dA20 transcripts remained stably associated with the template and were not released in response to VTF/capping enzyme (Fig. 2, lanes 2 and 4).

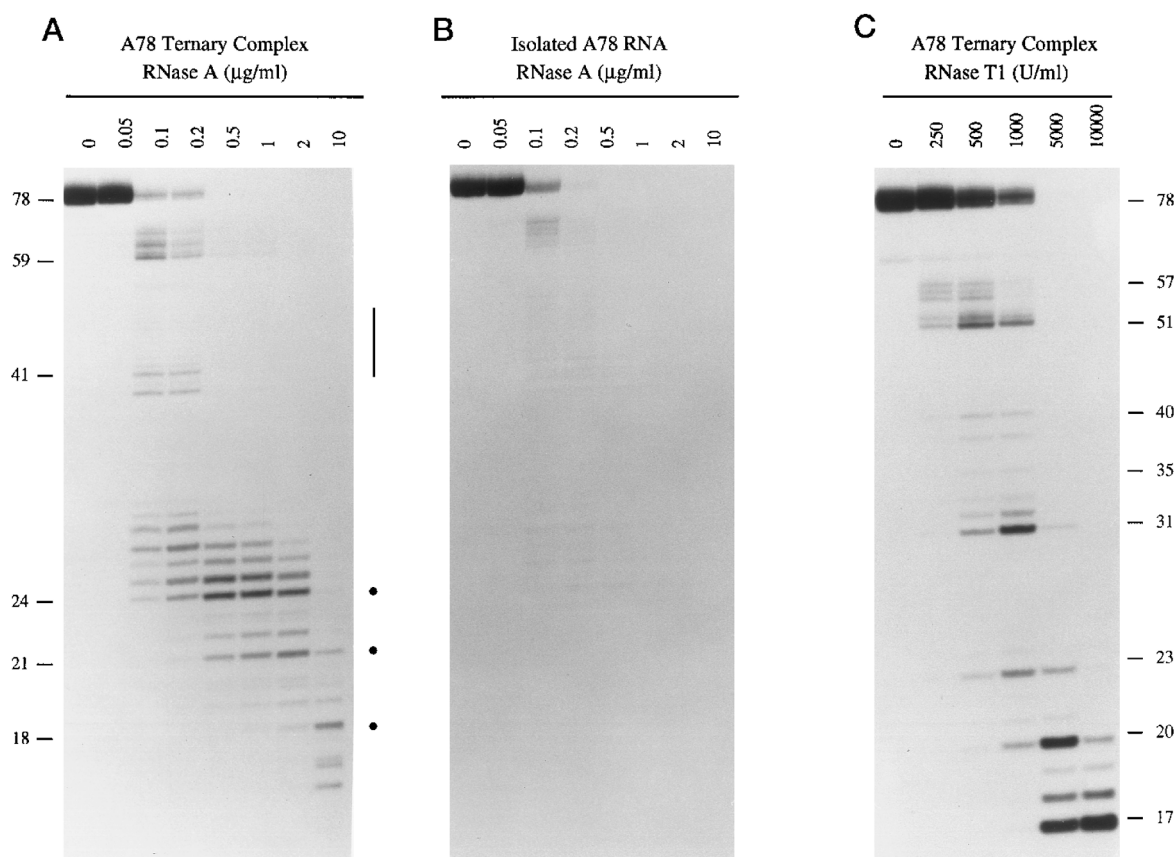
*Transcript Release Requires Extrusion of the UUUUUNU Signal Out of the RNA Binding Site on the Polymerase Elongation Complex*—A tenet of the kinetic coupling model for

VTF-dependent termination is that a slowing chain elongation rate will cause termination to occur at sites closer to the RNA signal; and, indeed, this has been observed (17). Yet there appeared to be some constraint on the system such that termination was limited to sites ~30 nucleotides or more downstream of the first U of the UUUUUNU signal (17). It was not clear whether this phenomenon represented a physical or a kinetic constraint. We were able to address the question using the pulse-walk-release assay by manipulating the physical distance between the termination signal and the downstream site of elongation arrest. In the G21(TER59)A78 transcription unit, the TTTTTTTT terminator element was shifted distally such that the first T was at position +59 (Fig. 1).

When pulse-labeled OMeG21 chains were walked to A78 on the G21(TER59)A78 template, the transcripts remained template-engaged (Fig. 2, lanes 7 and 8). However, these ternary complexes were refractory to VTF-mediated transcript release, *i.e.* all RNA remained template-associated after incubation with VTF/capping enzyme (Fig. 2, lanes 9 and 10). The conversion of the template-engaged A78 transcripts into a doublet of capped and uncapped RNAs provided assurance that capping enzyme had access to the nascent RNA. Note that the size of the RNA chain is not a variable in this experiment and that the sequence of the 11 nucleotides at the 3' end of the nascent chain is the same in the VTF-responsive TER29 and VTF-refractory TER59 complexes. Because the salient variable is distance from the signal to the 3' end of the chain (50 nucleotides in the case of TER29 *versus* 20 nucleotides for TER59), and because elongation rate is not an issue here, we surmise that there is a threshold distance between the terminator and the termination site that is dictated by physical constraints on the access of VTF/capping enzyme to the UUUUUNU sequence.

We propose that this physical constraint arises because the UUUUUNU signal is shielded from trans-acting proteins so long as it remains within the RNA binding pocket of the polymerase elongation complex. The dimensions of the RNA binding site were defined previously by RNase protection analysis of the nascent chains engaged within OMeGMP-arrested ternary complexes (23). An 18-nucleotide region of the transcript extending back from the 3' growing point of the chain was protected from RNase digestion; the size of the protected fragment did not change as the polymerase elongation complex moved away from the promoter. This suggests that the termination signal ought to be shielded on the TER59 template, but not on TER29. However, because the earlier RNase protection analysis involved transcription complexes paused within a different template sequence context from that of the A78 complexes, we felt it was important to determine the dimensions of the polymerase-RNA interface for the A78 complexes on the G21/A78 templates.

To accomplish this, we prepared ternary complexes containing A78 RNA labeled uniquely at the 3' end. Transcription was initiated on the G21(TER29)A78 template in the presence of unlabeled ATP, CTP, UTP, and 3'-OMeGTP; the bead-bound OMeG21 ternary complexes were purified and then walked to A78 in reactions containing GTP, CTP, UTP, and 3'-[ $\alpha$ -<sup>32</sup>P]dATP. The labeled cordycepin monophosphate moiety was incorporated exclusively at the 3' end of the A78 chain. The walked complexes were digested with increasing amounts of RNase A (Fig. 3). A ladder of digestion products was generated at low concentrations of RNase A (Fig. 3A; 0.1–0.2  $\mu$ g/ml). The sizes of the individual cleavage products reflected the distance of the RNase cleavage sites from the 3' end of the nascent chain. Purine gaps within the pyrimidine cleavage ladder provided landmarks for aligning the 3' end-labeled digestion prod-



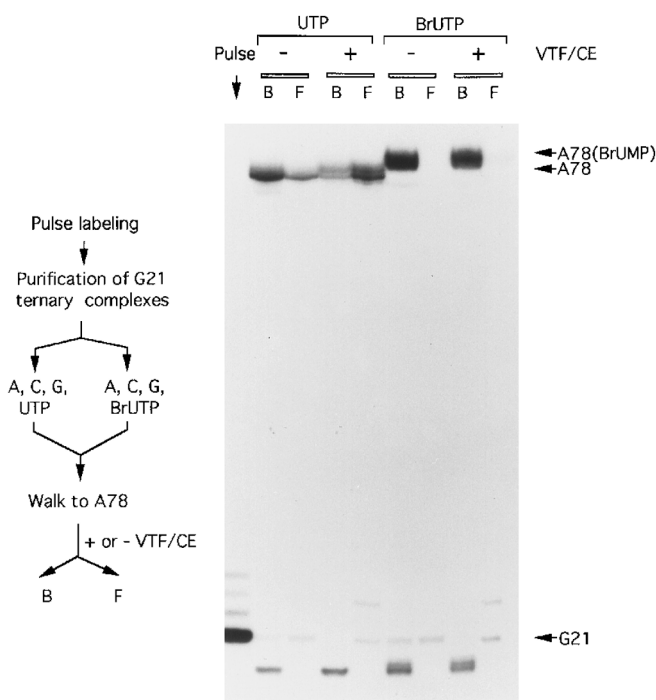
**FIG. 3. An RNA binding site on the A78 ternary complex protects the 3' end-labeled nascent RNA from nuclease digestion.** Synthesis of the OMeG21 RNA on the bead-bound G21(TER29)A78 template was performed in reactions containing unlabeled nucleotides: 1 mM ATP, and 0.1 mM UTP, CTP, and 3'-OMeGTP. The complexes were purified, washed, and then chased for 10 min at 30 °C in reaction mixtures containing (per 20 µl) 1 mM CTP, 0.1 mM UTP, 0.1 mM GTP, and 0.2 µM 3'-[ $\alpha$ - $^{32}$ P]dATP (2500 Ci/mmol). The 3'-labeled A78 RNA-containing ternary complexes were then digested with either RNase A (panel A) or RNase T1 (panel C) as follows. Panel A, ternary complexes were incubated for 2 min at 22 °C with 0.05, 0.1, 0.2, 0.2, 1, 2, or 10 µg/ml of RNase A, as indicated above the lanes. The reactions were halted by addition of a stop solution containing 10 mM EDTA, 0.5% SDS, 125 µg/ml yeast tRNA, 4 M urea. The samples were extracted with phenol and labeled RNA was recovered by ethanol precipitation. The digestion products were analyzed by denaturing gel electrophoresis. The sizes (in nucleotides) of 3'-labeled cleavage products are indicated on the left. Panel B, a parallel set of RNase A digestions was performed on isolated 3'-[ $^{32}$ P]dAMP-labeled A78 RNA, which was extracted from the A78 ternary complexes after denaturation in stop solution, then recovered by ethanol precipitation and resuspension in transcription buffer. Panel C, ternary complexes were incubated for 15 min at 30 °C with RNase T1 (Boehringer Mannheim) added to a concentration of 250, 500, 1000, 2500, or 10000 units/ml, as indicated above the lanes. The sizes (in nucleotides) of 3'-labeled G-specific cleavage products derived from the nascent A78 RNA are indicated on the right.

ucts within the predicted RNA sequence (Fig. 3). The digestion products were shortened with increasing RNase (Fig. 3A). A limit size was achieved at 0.5 µg/ml nuclease and this did not change substantially at 1–2 µg/ml RNase. The protection of 21–25 nucleotides at 2 µg/ml RNase suggested an upper limit of the size of the RNA binding site. Increasing RNase to 10 µg/ml trimmed the protected transcripts to 16–18 nucleotides, but still did not fully degrade the transcripts (Fig. 3A). Control digests were performed using 3'-[ $^{32}$ P]dAMP-labeled A78 RNA that was synthesized in the vaccinia *in vitro* system, then recovered by phenol extraction and ethanol precipitation (Fig. 3B). This free RNA was digested to completion at RNase levels to which the 3' segment of A78 RNA in the ternary complex was resistant (Fig. 3B).

A readable ladder of G-specific cleavage products was generated when 3'-dAMP-labeled A78 ternary complexes were probed with RNase T1. Increasing the amount of input nuclease resulted in shortening of the cleavage products to a limit size of 17 nucleotides (Fig. 3C). Shorter digestion products were not detected (not shown). We conclude from these experiments that the ternary complex strongly protects a 16–21-nucleotide 3' segment of the nascent A78 chain from nuclease digestion *in trans*. Whereas the upstream RNA segment that includes the UUUUUUUU termination signal was fully accessible in the

G21(TER29)A78 transcription complex (this segment is demarcated by the vertical bar in Fig. 3A), the termination signal would be protected in the context of the TER59 complex. To verify this, RNase A digests were also performed on 3'-[ $^{32}$ P]dAMP-labeled A78 RNA contained within the transcription complexes formed on the G21(TER59)A78 template. Transcripts 18–23 nucleotides long were protected (not shown).

**Inhibition of Transcript Release by BrUMP Substitution in the Nascent RNA**—Incorporation of bromo-UMP or iodo-UMP in lieu of UMP during synthesis of the UUUUUNU signal blocks transcription termination (13, 24, 25). This effect is unique to substituted uracil bases and is not observed when bromo-CMP or iodo-CMP replace CMP in the nascent chain (13). It was hypothesized that recognition of the uracil moieties, either by VTF or another component of the elongation complex, is essential for termination signal transduction. To eliminate elongation effects as a variable, we tested the ability of VTF to induce the release of BrUMP-substituted A78 RNA. Pulse-labeled G21 complexes were walked to A78 on the G21(TER29)A78 template in chase reactions containing either UTP or bromo-UTP (Fig. 4). (The BrUMP-substituted A78 RNA migrated more slowly than the UMP-containing transcript during gel electrophoresis.) The arrested complexes were then challenged with VTF and the bound and free RNAs were sep-

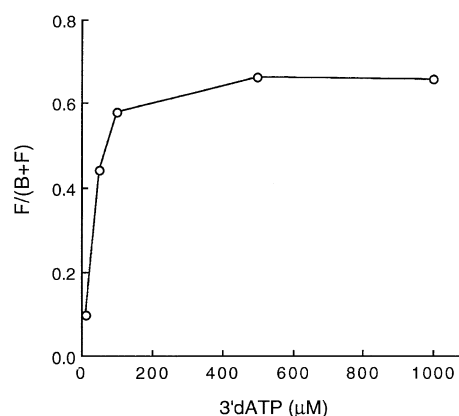


**FIG. 4. BrUMP substitution in the nascent RNA prevents transcript release.** Pulse-labeled G21 complexes were formed on the bead-linked G21(TER29)A78 template as detailed in Fig. 2, purified, and then walked to A78 in the presence of 1 mM concentration each of 3'-dATP, GTP, CTP, and either UTP or bromo-UTP, as indicated. The reaction mixtures were then incubated for 5 min at 30 °C with or without VTF/CE (36 fmol) as indicated. The bead-bound (B) and released (F) RNAs were recovered and analyzed by polyacrylamide gel electrophoresis. The positions of the pulse-labeled OMeG21 RNA (G21) and the walked A78 and BrUMP-substituted A78 transcripts are indicated by arrows at the right.

arated. VTF stimulated release of the control A78 RNA. (In this experiment, a low background of free RNA was seen in the absence of added VTF. The background free RNA varied in different experiments, but did not exceed 10% of the total RNA.) In contrast, the bromouridine-substituted A78 transcript was completely refractory to VTF-mediated release from the ternary complex (Fig. 4).

**Transcript Release Is Adenine Nucleoside Triphosphate-dependent**—VTF-dependent termination requires significantly higher concentrations of ATP than are necessary to support RNA chain elongation by the vaccinia polymerase (17). We used the pulse-walk-release approach to determine whether ATP plays a role in transcript release. It was established previously that deoxyadenosine nucleotides could substitute for ATP in transcription termination (17). Hence, we assessed the ATP requirement for RNA release simply by adjusting the concentration of 3'-dATP after the complexes had been walked to A78. (We could not use ATP in these experiments for the obvious reason that their inclusion in the reaction would permit resumption of elongation beyond the arrest site at +78A. The same applies to nonhydrolyzable analogs of ATP which are used as substrates for chain elongation by the vaccinia polymerase).

Control experiments confirmed that 10  $\mu$ M 3'-dATP was sufficient to arrest RNA polymerase at A78. Yet, when reactions containing 10  $\mu$ M 3'-dATP were challenged with VTF, the extent of RNA release was low (10% of total RNA) (Fig. 5). RNA release increased to 45% as 3'-dATP concentration was raised to 50  $\mu$ M and increased further to ~60% at 100  $\mu$ M. The release reaction plateaued at ~60–65% at 0.1–1 mM 3'-dATP (Fig. 5). We conclude that RNA release is ATP-dependent in the ab-



**FIG. 5. 3'-dATP dependence of transcript release.** Pulse-labeled G21 complexes were formed on the bead-linked G21(TER29)A78 template, purified, and then walked to A78 in the presence of 1 mM CTP, 0.1 mM UTP, 0.1 mM GTP and 10 mM 3'-dATP. Aliquots of the A78 complexes were distributed to new tubes and supplemented with VTF/CE (36 fmol); the 3'-dATP concentration was either maintained at 10  $\mu$ M or adjusted to 50, 100, 500, or 1000  $\mu$ M. The release reaction mixtures were incubated for 5 min at 30 °C, after which bound and free RNAs were separated and analyzed by gel electrophoresis. The distribution of bound and free A78 RNA in each reaction was quantitated by scanning the gel with a FUJIX BAS1000 Bio-Imaging Analyzer. The percent RNA released [ $F/(B + F)$ ] is plotted as a function of 3'-dATP concentration.

sence of concomitant elongation.

It is instructive to compare these findings with previous studies of termination by G21 transcription complexes that were pulse-labeled and then chased through a TTTTTTTT termination signal. Hagler *et al.* (17) found that optimal termination efficiency was achieved at 0.5 mM ATP and that lowering the ATP concentration to 0.1 mM caused a shift in termination sites to more distal template positions. At 50  $\mu$ M ATP, termination was barely detectable. From the present data, it appears that lower concentrations of adenosine nucleotide are needed to release transcripts from arrested transcription complexes than to elicit termination by elongating complexes. This is in keeping with the kinetic coupling model that posits ATP concentration as a determinant of signaling rate (17). In the case of transcription termination by elongating RNA polymerase, there is a window of opportunity during which VTF must transduce the signal, or else the polymerase will run off the end of the linear template. Hence signaling must be relatively rapid for the investigator to detect the termination event in this assay. In the pulse-walk-release assay, the polymerase is arrested at +78A, thereby providing an unrestricted temporal window for VTF to effect transcript release.

**Ternary Complexes That Do Not Release RNA in Response to VTF Are Refractory to Repeat Challenge with VTF**—Even under optimal conditions, not all of the A78 transcripts were released upon incubation with VTF/capping enzyme. Did this reflect an equilibrium between VTF-responsive and VTF-unresponsive states of the ternary complexes or were the residual template-engaged transcripts inherently refractory to VTF-mediated release? We addressed the question as shown in Fig. 6. Pulse-labeled transcripts were walked to A78 and then incubated with VTF; this resulted in the release of 60–70% of the transcripts into the supernatant (Fig. 6, first round). The pelleted beads were resuspended in transcription buffer containing nucleotides and 3'-dATP. This material was then incubated with or without VTF/capping enzyme and the bound and free RNAs were recovered. We found that RNAs that were not released during the first incubation with VTF were refractory to repeated challenge with VTF. Thus, these ternary complexes appeared to be inherently VTF-resistant. It was not simply the case that these were catalytically inert "dead-end" complexes,

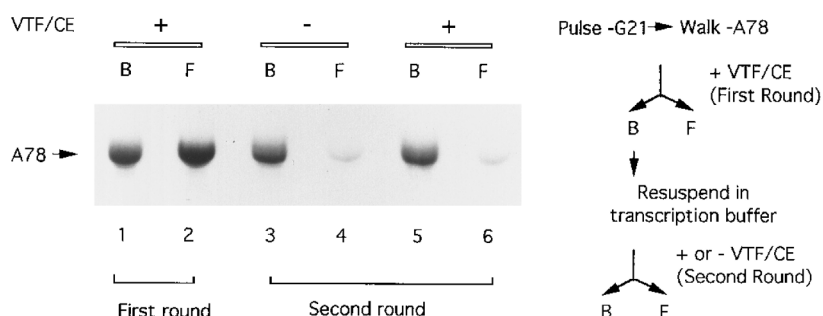


FIG. 6. **VTF-unresponsive ternary complexes are resistant to repeat challenge with VTF.** Purified G21 ternary complexes assembled on the TER29 template were walked to A78 in the presence of 1 mM concentration each of 3'-dATP, GTP, CTP, and UTP. The mixture were supplemented with 36 fmol of VTF/CE and incubated for an additional 5 min at 30 °C. The mixture was separated by centrifugation and the bound and free A78 RNAs were analyzed by gel electrophoresis (*First round*). Pellet beads containing RNA that had not been released during the first round of incubation with VTF were resuspended in 20 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 6 mM MgCl<sub>2</sub>, and 1 mM each of 3'-dATP, GTP, CTP, and UTP. These mixtures were incubated for 5 min at 30 °C with or without VTF/CE (36 fmol) as indicated. The bead-bound and free RNA fractions were recovered and resolved by gel electrophoresis (*Second round*). The portion of the autoradiogram containing the A78 transcript is shown.

because the majority of the RNAs could be elongated to the end to the linear template when provided with ATP to overcome the elongation block at +78A (data not shown).

#### DISCUSSION

DNA templates containing tandem G-less and A-less cassettes have been exploited to study factor-dependent release of the nascent RNA chain from purified elongation complexes of vaccinia RNA polymerase halted at unique template positions downstream of a TTTTNT transcription termination signal. The strategy of focusing on RNA release allows direct assessment of the signaling phase of the termination reaction, by eliminating ongoing elongation as a confounding variable. The principal findings are: (i) transcript release depends on an accessible UUUUUNU signal in the nascent RNA, (ii) the signal is inaccessible when it is contained within the nascent RNA binding site on the polymerase elongation complex, (iii) bromouridine base substitution within the signal interferes with factor-dependent release, and (iv) release depends on an ATP cofactor. The aforementioned properties and requirements for transcript release by halted polymerase complexes are consistent with all that is known about the composite termination reaction occurring during vaccinia early mRNA synthesis.

Several novel insights emerged from the experiments presented above that supplement our understanding of the relationship between elongation and signaling. For example, we provide evidence that a physical constraint to termination site-choice is imposed by the need to extrude the UUUUUNU signal out of the RNA binding site on the polymerase elongation complex. This finding has clear mechanistic implications, *i.e.* that the signal itself must be accessible to some component of the termination-competent transcription complex. The most plausible scenario is that UUUUUNU is bound directly by VTF/capping enzyme and that this is a prerequisite for transcript release. It is certainly not the case that the UUUUUNU signal must be extruded in order to for VTF to be recruited to the elongation complex, as we have shown that this occurs via signal-independent binding of VTF to the nascent RNA once a critical chain length is extruded from the polymerase (17). The length of the extruded chain on the A78 complexes is about 55 nucleotides (taking a conservative value of 23 nucleotides for the size of the RNA region protected by the polymerase), which we have shown is long enough to recruit VTF (17). Thus, VTF-unresponsiveness of the TER59 complex can be ascribed to lack of signaling, rather than lack of VTF binding. The same is true of the TER29 complex containing bromouridine-substituted RNA, *i.e.* the signal is exposed, but cannot be read by VTF. Bromouridine-substitution does not affect binding of

VTF/capping enzyme to the ternary complex; indeed, VTF/capping enzyme can be efficiently UV cross-linked to bromouridine-substituted RNA within arrested elongation complexes (17). The implication is that binding of VTF to the unperturbed UUUUUNU signal elicits a conformational change in the elongation complex that precipitates transcript release. Defining the nature of the conformational change, and its connection to ATP hydrolysis, is the next mechanistic challenge.

An interesting and potentially instructive finding was that a fraction of the arrested complexes was refractory to VTF-induced RNA release and remained unresponsive after repeat challenge with VTF. We considered two models to account for this. One model posits that the vaccinia elongation complex can adopt VTF-responsive and VTF-unresponsive conformations. Conformational fluctuations of the elongation complex have been described in other polymerase systems (26, 27). Such conformational differences determine responsiveness to factor-induced RNA cleavage (27, 28). In the case of the vaccinia transcription complex, one might postulate that the polymerase active site must be appropriately positioned relative to the 3' end of the chain in order to release the chain in response to VTF, or that subtle differences in RNA-protein contacts within the RNA-binding site of the polymerase dictate VTF responsiveness.

A second model holds that VTF-responsive and VTF-refractory complexes differ not in conformation but in their protein composition. According to this view, VTF-dependent termination might require one or more additional protein cofactors that are present on some, but not all, of the A78 transcription complexes. (Alternatively, a polypeptide constituent might be differentially modified in a subfraction of the elongation complexes.) This would be consistent with the notion that VTF has either a partner or a direct target on the elongation complex besides the UUUUUNU signal. Candidates for this partner/target role would necessarily be polypeptides that are not strictly essential for chain elongation, otherwise the VTF-unresponsive complexes would not have been able to walk down the template to the end of the A-less cassette. The idea that a second component might cooperate with VTF to bring about transcription termination owes much to recent studies of the role of NusG during rho-dependent termination by *Escherichia coli* RNA polymerase (29–31). We discussed previously (17) the mechanistic parallels between VTF and rho, both of which transduce RNA signals to the elongating polymerase in an NTP-hydrolysis dependent, kinetically coupled reaction. Identification of a NusG-like function in the vaccinia system would consolidate the notion that RNA-based termination mecha-

nisms are fundamentally conserved in bacteria and eukaryotes. In the future, we will seek to test the second model by analyzing the polypeptide composition of the A78 complexes. This will entail the preparation of immunological probes against all known transcription components, an effort that is currently under way.

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