Transcription Termination by Vaccinia RNA Polymerase Entails Recognition of Specific Phosphates in the Nascent RNA*

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Vaccinia virus RNA polymerase terminates transcription downstream of a UUUUUNU signal in the nascent RNA. Transduction of the RNA signal to the elongating polymerase requires a termination factor (vaccinia termination factor/capping enzyme) and is coupled to the hydrolysis of ATP. It was shown previously that incorporation of 5-bromouracil or 5-iodouracil within the UUUUUNU element abolishes termination by preventing factor-dependent release of the nascent chain from the polymerase elongation complex. Here, we report that termination is prevented by phosphorothioate substitution at UMP residues in the nascent RNA. In contrast, phosphorothioate substitution at AMP, CMP, and GMP nucleotides does not inhibit termination. Thus, the action of a eukaryotic termination factor entails recognition of the nucleotide bases and the phosphate groups of the target sequence in nascent RNA.

The 3' ends of vaccinia virus early mRNAs are formed by transcription termination, which is dictated by a cis-acting heptamer sequence UUUUUNU in the nascent RNA chain (1). The termination event occurs at heterogeneous sites downstream of the signal. Site choice is determined by a kinetic balance between the rate of signaling and the rate of chain elongation (2). Vaccinia RNA polymerase by itself is not responsive to the signal but requires the vaccinia termination factor, VTF/capping enzyme (3).

To understand how information in the nascent RNA is transmitted to an elongating RNA polymerase, the essential structural features of the signal must be delineated. The small size and limited sequence complexity of the vaccinia UUUUUNU motif provides a unique opportunity to accomplish this. A requirement for UMP at every position except the N base was demonstrated initially by mutating the DNA template (4). We have since used RNA modification interference to obtain a more refined view of the signal. The approach is to introduce nucleotide analogs into the nascent RNA and to assess their impact on VTF-dependent termination in vitro. It was shown previously that incorporation of 5-bromouracil or 5-iodouracil within the nascent RNA abolishes VTF-dependent termination, whereas incorporation of 5-bromocytosine or 5-iodocytosine has no effect (1). This suggested that termination signaling involves recognition of the uracil bases within the UUUUUNU element. In this study, we probe the role of the phosphate moieties of the termination signal by incorporating ribonucleoside phosphorothioates during transcription elongation.

**EXPERIMENTAL PROCEDURES**

Ternary transcription complexes containing 32P-labeled nascent RNA were formed as described (2) in reaction mixtures containing (per 20 μl) 20 mM Tris-HCl (pH 8.0), 6 mM MgCl2, 2 mM dithiothreitol, 1 mM ATP, 0.1 mM UTP, 1 μM [α-32P]CTP (1000 Ci/mmol), 0.1 mM 3'-OMeGTP, vaccinia RNA polymerase (heparin agarose fraction containing the vaccinia early transcription initiation factor), and bead-linked G21(TER) or G21(TER29)A78 DNA. Reaction mixtures were incubated at 30 °C for 10 min. The beads were concentrated by microcentrifugation for 15 s and then held in place with a magnet while the supernatant was removed and replaced with 0.1 μl 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 (chase phase) were performed in reaction mixtures containing 20 mM Tris-HCl (pH 8.0), 6 mM MgCl2, 2 mM dithiothreitol, and standard NTPs or α-thio-NTPs as specified in the figure legends. The reactions were halted by the addition of a stop solution containing 10 mM EDTA, 0.5% SDS, 125 μg/ml yeast tRNA, and 4 m urea. The samples were extracted with phenol/chloroform and labeled RNA was recovered by ethanol precipitation. Transcription products were analyzed by electrophoresis through a 17% denaturing polyacrylamide gel and radiolabeled transcripts were visualized by autoradiography. GTPoS, ATPoS, UTPoS, and CTPoS (Sp diastereomers) were purchased from Amersham Corp.

**RESULTS AND DISCUSSION**

An initial series of experiments was conducted to test whether vaccinia RNA polymerase was capable of utilizing α-thio-NTPs as substrates. Chain elongation was assayed during a single round of synthesis on a bead-linked G21(TER) template containing a vaccinia promoter fused to a 20-nucleotide G-less cassette (Fig. 1). Elongation complexes containing 32P-labeled nascent RNA were assembled and purified as described (2). The complexes were arrested uniquely at the first G position on the template (G21) by incorporation of 3'-OMeGMP. The bead-purified G21 transcription complexes hydrolyzed the blocking 3’OMeGMP moiety (10) and resumed elongation during a chase in the presence of magnesium and 1 mM of each of the standard NTPs. A 195-nucleotide run-off transcript was produced within 20 s (Fig. 1). The amount of run-off transcript increased over time, concomitant with a decrease in the level of OMeG21 RNA. Note that because transcript shortening was rate-limiting in the chase reaction, the population of halted ternary complexes did not elongate synchronously. A fraction of the polymerases paused transiently at template positions C31 and T34 at the proximal margins of the transcription termination signal (Fig. 1). The full-length RNAs were converted at late times (5–30 min) into a higher molecular weight species (Fig. 1, left panel). These longer transcripts were formed via DNA template-independent 3’ extension of the run-off RNA by the vaccinia RNA polymerase.

GTPoS, ATPoS, UTPoS, and CTPoS were accepted as sub-

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1 The abbreviation used is: VTF, vaccinia termination factor.

2 L. Deng and S. Shuman, unpublished observations.
strates for chain elongation when substituted singly for the standard NTPs (Figs. 1 and 2). CTP\textsubscript{aS} had no detectable impact on the kinetics of elongation (Fig. 2). The other \textalpha-thiophosphate derivatives slowed chain elongation slightly by increasing polymerase dwell time at specific template positions. GTP\textsubscript{aS} substitution enhanced intrinsic pausing at C31 (immediately 5’ of a G at position 32) and induced a cluster of pause sites near the 3’ end of the template (Fig. 1). However, full-length RNAs were still evident at 20 s, and nearly all pause sites were traversed by 40 s. ATP\textsubscript{aS} enhanced pausing at a distinct set of distal sites that were traversed by 2 min (Fig. 1). UTP\textsubscript{aS} increased pausing at T33 and T34 within the T-run and at discrete downstream positions (Fig. 2). Full-length transcripts did not appear until 40 s; nonetheless, virtually all polymerases traversed the distal pause sites by 2 min.

The effects of phosphorothioate substitution on transcription termination are shown in Fig. 3. Pulse-labeled G21 ternary complexes were chased for 10 min in the presence or the absence of VTF. In control reactions containing 1 mM each of the standard NTPs, VTF induced the appearance of a heterogeneous array of transcripts terminated between about 40 and 100 nucleotides downstream of the first U residue of the UUUUUNU signal (denoted by T in Fig. 3). GTP\textsubscript{aS}, ATP\textsubscript{aS}, and CTP\textsubscript{aS} altered the distribution of termination sites but did not.

FIG. 1. Utilization of ATP\textsubscript{aS} and GTP\textsubscript{aS} as substrates for chain elongation. The architecture of the G21(TER) DNA template is shown. 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. A vaccinia virus early promoter element (P) specifies the initiation of transcription at position +1 of a 20-nucleotide G-less cassette. A termination signal (T) is situated downstream of the G-less cassette. The sequence of the first 50 nucleotides of the transcribed region (nontemplate strand) is shown. The termination signal TTTTTTTTTT and the GGGG element used to arrest transcription are underlined. Ternary complexes containing pulse-labeled OMeG21 RNA were assembled on G21(TER) and bead-purified as described under “Experimental Procedures.” The complexes were chased in reaction mixtures containing 1 mM each of CTP, UTP, ATP or ATP\textsubscript{aS}, and GTP or GTP\textsubscript{aS}, as indicated. Aliquots were removed at the times indicated and quenched immediately in stop buffer. The RNA products were resolved by polyacrylamide gel electrophoresis. The positions of the pulse-labeled MeG21 RNA and the chased run-off transcript (RT) are indicated by arrows. Individual paused RNA species C31 and T34 are denoted according to the location of their 3’ ends on the DNA template.
not diminish the extent of VTF-induced termination. Indeed, termination was actually enhanced by these analogs. In contrast, UTP\textsubscript{a}S prevented factor-dependent termination by vaccinia RNA polymerase (Fig. 3, lane 8).

We showed previously that termination site choice reflects a kinetic balance between the rate of polymerase elongation and the rate at which the signal is transduced to the ternary complex (2). VTF preferentially induces termination at transcriptional pause sites, at least under reaction conditions where pausing can be discerned, \textit{e.g.} in the presence of nucleotide analogs (2). The observed shifts in termination site preferences in the presence of 1 mM GTP\textsubscript{a}S, ATP\textsubscript{a}S, and CTP\textsubscript{a}S were consistent with their position-specific effects on polymerase dwell time. A kinetic analysis of elongation in the presence and the absence of VTF showed that the sites of VTF-induced termination in reactions containing ATP\textsubscript{a}S corresponded to sites of analog-induced pausing (not shown).

Incorporation of the \textalpha-thio-nucleotides by RNA polymerase results in inversion of chirality at the \textalpha-phosphorus from the Sp configuration of the NTP precursor to an Rp diastereomer of the NMP in the RNA chain (5). The results of Fig. 3 make clear that the presence of Rp thiophosphates 5’ of A, G, or C residues has no effect on termination signal recognition, whereas an Rp thiophosphate 5’ of U residues abrogated VTF-dependent termination. We infer that Rp thiophosphate substitution within the UUUUUNU signal was the basis for this effect.

The experiment in Fig. 3 focuses on the effects of \textalpha-thio-UMP incorporation on the transcript release step of the termination reaction. Our strategy was to purify RNA-labeled ternary complexes paused at G21, then “walk” the RNA polymerase through an A-less cassette to a defined template position (A78) located 50 nucleotides downstream of the first U residue of a UUUUUUUU termination signal, and then test for the release of the RNA from the ternary complex in response to VTF/capping enzyme. This assay system eliminates ongoing elongation as a variable. We demonstrated previously that release of the A78 nascent RNA requires the UUUUUNU signal, VTF, and an adenosine nucleotide energy cofactor (6). We also showed that VTF-dependent release is blocked by bromo-UMP substitution within the termination signal (6).

Pulse-labeled G21 complexes were walked to A78 on the G21(TER29)/A78 template in chase reactions containing CTP, GTP, 3’ dATP, and either UTP or UTP\textsubscript{a}S (Fig. 4). The arrested complexes were then challenged with VTF, and the bead-bound and free RNAs were separated. \textasciitilde70\% of the A78 RNA was released in the control reaction, compared with only 9\% in reactions containing UTP\textsubscript{a}S (Fig. 4). Thus, thiophosphate substitution at U residues interfered with transcript release.

We surmise from these experiments that recognition of the phosphate moieties of the UUUUUNU signal is essential for termination signal transduction. The substitution of a non-bridging oxygen by sulfur can viewed as a minimal structural alteration of the RNA signal (7). The net charge on the phosphates is unaltered, and no bulky substituents are introduced. Rather, the negative charge in the phosphorothioate becomes preferentially localized on the sulfur (7). Phosphorothioate interference with termination implies that the phosphates within the UUUUUNU element interact directly with a protein. Alternatively, the phosphates may coordinate an essential metal ion.

We have now documented four physical constraints to RNA signal recognition during transcription termination by vaccinia RNA polymerase.
RNA polymerase. These are: (i) the phosphates must be unperturbed (this study), (ii) the uracil bases must be unmodified (1, 6), (iii) the UUUUNU signal must be single-stranded (8), and (iv) the UUUUNU element must be extruded from the RNA binding site on the polymerase (6). VTF is known to be in contact with the nascent RNA chain even prior to synthesis of the UUUUNU sequence (2). We suggest that the encounter of VTF with the unperturbed termination signal as it is extruded from RNA polymerase elicits a conformational change in VTF that is transmitted to the elongation complex, leading to transcript release by RNA polymerase. The nature of the conformational change and its connection to ATP hydrolysis remain to be defined.

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FIG. 3. α-thio-UMP incorporation inhibits VTF-dependent transcription termination. Ternary complexes containing pulse-labeled OMeG21 RNA were assembled on G21(TER) and bead-purified. The complexes were processed directly for RNA analysis (lane 1, chase −) or else chased for 10 min in elongation reaction mixtures containing 1 mM nucleoside triphosphates (lanes 2–11, chase +). Control reactions included ATP, GTP, CTP, and UTP (lanes 2 and 3). α-thio-NTPs were included in lieu of standard NTPs as indicated above the lanes. Where indicated (lanes 3, 5, 7, 9, and 11), VTF/capping enzyme (0.5 pmol of recombinant protein purified as described (9)) was added to the reaction mixtures just prior to adding the NTPs. The RNA products were resolved by polyacrylamide gel electrophoresis. The positions of the pulse-labeled MeG21 RNA and the chased run-off transcript (RT) are indicated by arrows. The terminated (T) and paused (P) RNAs are indicated by vertical bars.

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