# Elongation Properties of Vaccinia Virus RNA Polymerase: Pausing, Slippage, 3′ End Addition, and Termination Site Choice†

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ABSTRACT: We have analyzed the elongation properties of vaccinia virus RNA polymerase during a single round of transcription in vitro. RNA-labeled ternary complexes were halted at a unique template position located upstream of a T-run (TTTTTTTTT) in the nontemplate strand; this element encodes an RNA signal for factor-dependent transcription termination at distal sites on the template. The halted ternary complexes were purified and allowed to resume elongation under a variety of conditions. We found that the T-run constituted a strong elongation block, even at high nucleotide concentrations. The principal sites of pausing were at a C position situated two nucleotides upstream of the first T in the T-run and at the first three to four T positions within the T-run. There was relatively little pausing at the five downstream Ts. Intrinsic pausing was exacerbated at suboptimal nucleotide concentrations. Ternary complexes arrested by the T-run at 10 $\mu$M NTPs rapidly traversed the T-run when the NTP pool was increased to 1 mM. Limiting GTP (1 $\mu$M) resulted in polymerase stuttering at the 3′ margin of the T-run, immediately prior to a templated G position; this generated a ladder of slippage synthesis products. We found that vaccinia ternary complexes remained intact after elongating to the very end of a linear DNA template and that such complexes catalyzed the addition of extra nucleotides to the 3′ end of the RNA chain. The 3′ end addition required much higher concentrations of NTPs than did templated chain elongation. Finally, we report that factor-dependent transcription termination by vaccinia RNA polymerase downstream of the T-run was affected by nucleotide concentration. Limiting UTP caused the polymerase to terminate at sites closer to the UUUUUNU termination signal. This is consistent with the kinetic coupling model for factor-dependent termination.

Vaccinia early mRNAs are synthesized by a virus-encoded multisubunit RNA polymerase in conjunction with virus-encoded accessory factors. Transcription initiation is specified by the vaccinia early transcription factor (ETF), a heterodimeric virus-encoded protein that binds to the early promoter element and recruits vaccinia RNA polymerase to the template (*1*−*5*). The 3′ ends of early mRNAs are formed by transcription termination, which is dictated by a *cis*-acting sequence UUUUUNU in the nascent RNA chain (*6*, *7*). Termination requires the participation of a *trans*-acting vaccinia termination factor (VTF/capping enzyme) and is coupled to hydrolysis of ATP (*8*, *9*). The termination event occurs at a heterogeneous array of sites downstream of the RNA signal. We have proposed that termination site choice is determined by a kinetic balance between the rate of signaling and the rate of polymerase movement (*9*). As a necessary step toward understanding the mechanism of factor-dependent termination, we are interested in the parameters that influence both aspects of kinetic coupling—signaling and elongation.

In the present study, we focused on the elongation reaction. Our strategy was to purify RNA-labeled ternary complexes paused at a unique template position located upstream (5′) of a TTTTTNT termination signal and then to allow these complexes to resume elongation under a variety of conditions. We report the following observations: (i) the T-run is an impediment to polymerase elongation, especially at suboptimal nucleotide concentrations; (ii) under certain circumstances, polymerase will stutter at the 3′ margin of the T-run and reiteratively incorporate UMP; (iii) ternary complexes remain intact after elongating to the end of a linear DNA template; (iv) such complexes catalyze the addition of extra nucleotides to the 3′ end of the RNA chain; (v) manipulating NTP concentration elicits effects on VTF-dependent termination site choice that support the kinetic coupling model.

## EXPERIMENTAL PROCEDURES

*Immobilized DNA Templates.* The pBS-based G21 and G21(TER) plasmids containing a vaccinia early promoter fused to a 20-nucleotide G-less cassette have been described (*9*). G21(TER) contains a TTTTTTTTT terminator element located downstream of the G-less cassette (refer to Figure 1). G21(TER) and G21 plasmids were linearized with *Acc*65-1, which cleaved the DNA template upstream of the vaccinia early promoter region. Biotinylated dAMP 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) as described (*9*).

*Transcription in Vitro.* Ternary transcription complexes were formed in standard reaction mixtures containing 20 mM Tris·HCl (pH 8.0), 6 mM $MgCl_2$, 2 mM DTT, 1 mM ATP, 0.1 mM UTP, 1 $\mu$M [$\alpha$-$^{32}$P]CTP (1000 Ci/mmol), 0.1 mM 3′-OMeGTP, vaccinia RNA polymerase (holoenzyme frac-

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**G21(TER)**

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+1
ATAACCCACTTCTATCACTAGGGGATCCGCCGTTTTTTTTTGACGTCTAG (50)

AGCGGCCGCCACCGCGGTGGAGCTCCAATTCGCCCTATAGTGAGTCGTAT (100)

TACGCGCGCTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCC (150)

TGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAG   (198)
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**G21**

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+1
ATAACCCACTTCTATCACTAGGGGATCCACTAGTTCTAGAGCGGCCGCCA (50)

CCGCGGTGGAGCTCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTC (100)

ACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCC (150)

AACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAG              (187)
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FIGURE 1: G21(TER) and G21 DNA templates. A vaccinia virus early promoter element specifies the initiation of transcription at position +1 of a 20-nucleotide G-less cassette, which is flanked by a run of four consecutive G residues. A termination signal (TTTTTTTTT) is situated downstream of the G-less cassette in G21(TER) but not G21. The complete sequences of the transcribed regions (nontemplate strands) are shown.

tion containing ETF), and bead-linked G21(TER) or G21 DNA (*9*). Reaction mixtures were incubated at 30 °C for 10 min and then concentrated by microcentrifugation for 15 s. The beads were held in place with a magnet while the supernatant was removed and replaced with 0.1 mL of 20 mM Tris·HCl (pH 8.0), 2 mM DTT. 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 mixtures containing 20 mM Tris·HCl (pH 8.0), 6 mM $MgCl_2$, 2 mM DTT, and NTPs as specified. Where indicated, recombinant VTF/capping enzyme (*10*) was added to the chase reaction mixtures prior to the NTPs. After incubation at 30 °C, the elongation reactions were quenched by addition of a stop solution containing 10 mM EDTA, 0.5% SDS, 125 $\mu$g/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 containing TBE (90 mM Tris, 90 mM borate, 2.5 mM EDTA), and radiolabeled transcripts were visualized by autoradiography.

## RESULTS

*Pausing by Vaccinia RNA Polymerase within a T-Run.* Transcription was programmed by linear DNA templates linked to streptavidin-coated paramagnetic beads. The G21 transcription unit consisted of an early promoter fused to a 20-nucleotide G-less cassette, which was flanked by a run of four G residues at positions +21 to +24 (Figure 1). The G21(TER) transcription unit was identical to G21, except that a 17-bp sequence, GCCGTTTTTTTTTGACG, was

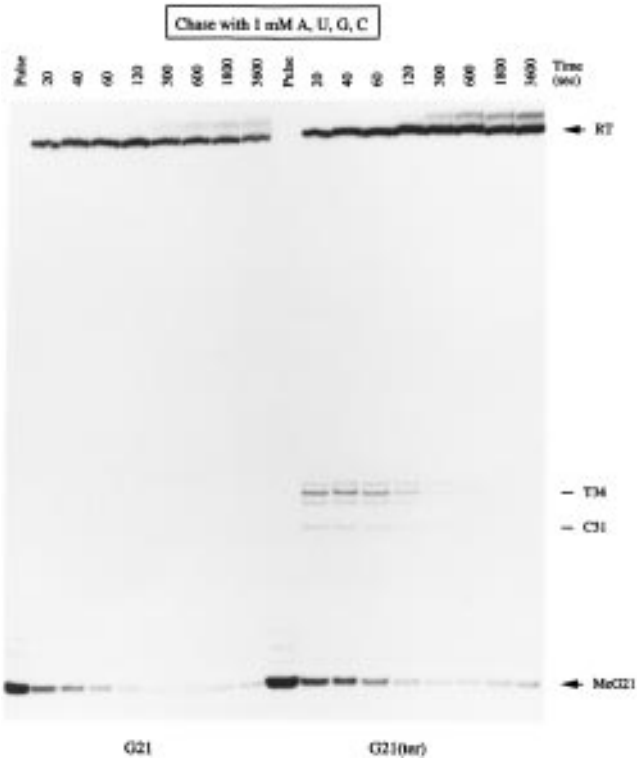


FIGURE 2: Intrinsic pausing by vaccinia RNA polymerase. Transcription reactions were programmed by the bead-bound G21 and G21(TER) templates that had been linearized with *Pvu*II. Pulse-labeled elongation complexes were then purified as described in Experimental Procedures. The pulse-labeled RNA associated with the isolated complexes is shown in lanes marked Pulse. Purified complexes were allowed to resume elongation during a 60 min chase in the presence of 1 mM ATP, 1 mM GTP, 1 mM CTP, and 1 mM UTP. Aliquots were withdrawn at the times indicated and quenched immediately in stop mix. Labeled transcription products were resolved by polyacrylamide gel electrophoresis. The positions of pulse-labeled 3′-OMeG21 RNA (MeG21) and the chased runoff transcript (RT) are indicated by arrows at the right of the autoradiogram. Several paused RNA species are denoted according to the location of their 3′ ends on the G21(TER) DNA template (refer to Figure 1).

inserted from positions +29 to +45 in G21(TER) in lieu of the 6-bp sequence ACTAGT present from +29 to +34 in G21 (Figure 1). This 17-mer insert included a run of nine T residues from positions +33 to +41 (Figure 1). Pulse-labeling transcription reactions contained ATP, UTP, [$\alpha$-$^{32}$P]-CTP and 3′-OMeGTP. The template-engaged ternary complexes containing radiolabeled 3′-OMeGMP-arrested 21-mer RNA were recovered by centrifugation and concentration of the beads with a magnet, followed by washing the beads with buffer lacking nucleotides and magnesium (Figure 2, Pulse). Minor 22-, 23-, and 24-mer RNA species were also recovered; these are 3′ coterminal transcripts that initiated from upstream template positions (*11*).

Prior studies showed that the purified transcription complexes resume elongation when provided with NTPs and magnesium (*9*, *12*). Traversing the arrest site at +21G depends upon removal of the blocking 3′-OMeGMP moiety by a hydrolytic activity intrinsic to the vaccinia RNA polymerase elongation complex (*13*, *14*). A kinetic analysis of elongation is shown in Figure 2. In this experiment, the OMeG21 RNAs were chased in the presence of high concentrations of nucleotides (1 mM ATP, CTP, GTP, and UTP), and aliquots were removed at various times for RNA size analysis. About half the nascent chains were extended

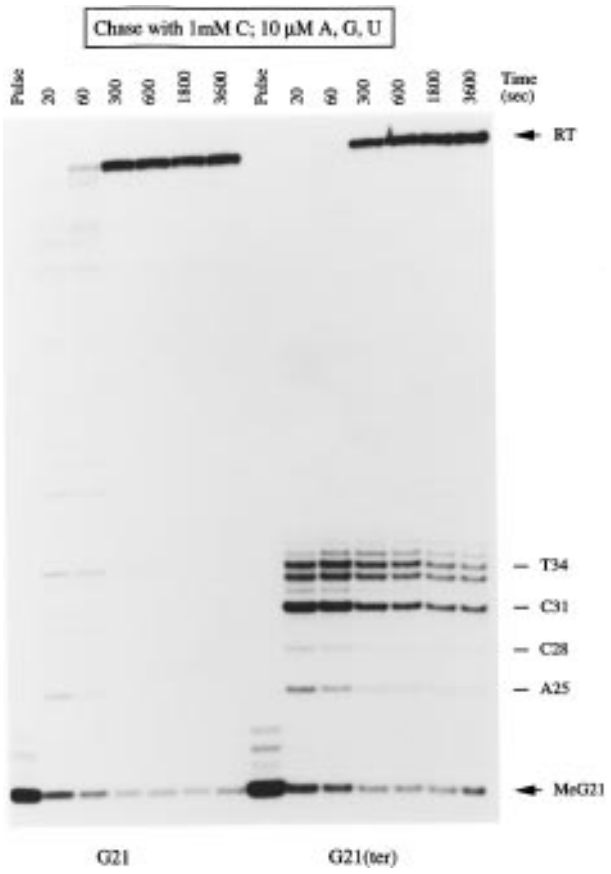


FIGURE 3: Pausing is enhanced by a reduction in NTP concentration. Bead-purified pulse-labeled G21 and G21(TER) complexes were allowed to resume elongation during a 60 min chase in the presence of 1 mM CTP, 10 $\mu$M GTP, 10 $\mu$M ATP, and 10 $\mu$M UTP. Aliquots were withdrawn at the times indicated and quenched immediately. Labeled transcription products were resolved by polyacrylamide gel electrophoresis. The positions of pulse-labeled 3′-OMeG21 RNA (MeG21) and the chased runoff transcript (RT) are indicated by arrows at the right of the autoradiogram. Paused RNA species are denoted according to the location of their 3′ ends on the G21(TER) template.

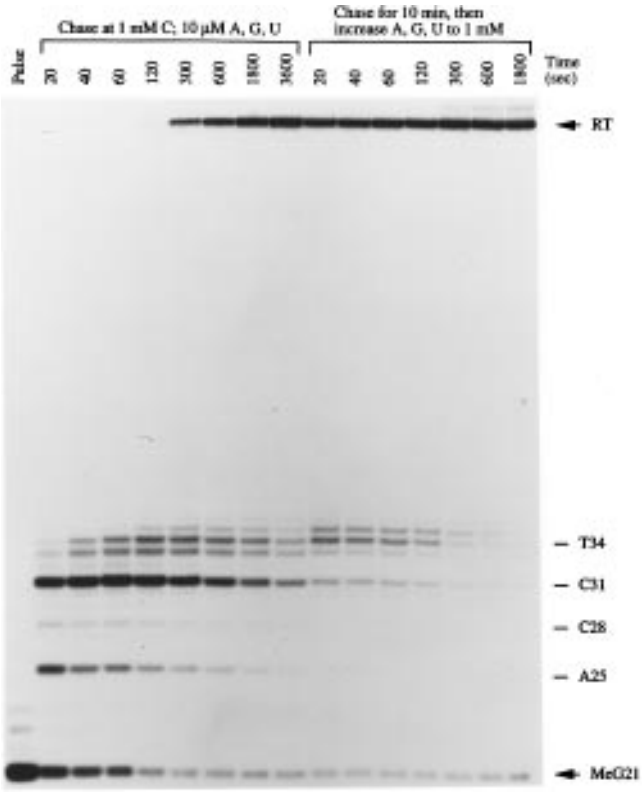


FIGURE 4: Complexes paused at limiting NTP concentration resume elongation upon NTP supplementation. Bead-purified pulse-labeled G21(TER) complexes were chased in the presence of 1 mM CTP, 10 $\mu$M GTP, 10 $\mu$M ATP, and 10 $\mu$M UTP. Aliquots were withdrawn at the times indicated. A portion of the reaction mixture was removed after 10 min of chase, and the concentration of all four NTPs was adjusted to 1 mM. Aliquots were then withdrawn at the indicated times after NTP supplementation.

to the end of the linear G21 template within 20 s. No RNAs of intermediate size were observed. The amount of runoff transcript increased up to 60 s, concomitant with a decrease in the level of OMeG21 RNA (Figure 2). Elongation by purified G21(TER) complexes differed from that of G21 complexes in one respect, i.e., we observed transient accumulation of a cluster of RNAs at early times (20−60 s). These species were paused at sites +33T, +34T, and +35T within the T-rich termination signal and at site +31C at the 5′ margin of the T-run (Figure 2).

The rate of elongation by vaccinia polymerase on the G21 template was slowed when the concentrations of ATP, GTP, and UTP were reduced to 10 $\mu$M (Figure 3). The concentration of CTP was maintained at 1 mM because CTP stimulates nascent chain cleavage by the vaccinia RNA polymerase (*13*). After 20 s of chase, a ladder of paused RNAs was apparent. The paused species persisted at 60 s, at which point some of the complexes had reached the end of the G21 template. The paused species were chased completely after 5 min. On the G21(TER) template, reducing the NTP concentration to 10 $\mu$M strongly enhanced pausing within and prior to the T-run (Figure 3). Most of the ternary complexes were arrested at +31C, +33T, and +34T after 20 s and remained paused at these sites up to 60 s. A finer time course (Figure 4) showed that the polymerase paused transiently at +25A before encountering a very strong elongation block at +31C. Although a fraction of the paused polymerases traversed the T-run at 5 min and reached the end of the template, most transcription complexes stayed paused (Figures 3 and 4). The paused RNAs declined slowly between 5 and 60 min, as full-length transcripts steadily increased (Figures 3 and 4). Complexes paused after 10 min of incubation in the presence of 10 $\mu$M NTPs were chased rapidly when the NTP concentration was increased to 1 mM (Figure 4). Most of the paused polymerases traversed the +31C and +33T sites within 20−40 s. After a transient delay at +34T and +35T (between 20 s and 2 min after adjustment of NTPs to 1 mM), the RNAs were extended to the end of the template. These results show clearly that the T-run constitutes a significant intrinsic pause site for the vaccinia RNA polymerase.

*Effects of Varying the Concentration of Individual NTPs.* Pulse-labeled OMeG21 complexes assembled on the G21-(TER) template were chased for 5 min in reaction mixtures in which the concentration of a single nucleotide (either UTP, GTP, or ATP) was varied in 10-fold increments from 1 to 1000 $\mu$M (Figure 5). Control reactions were performed in which a single nucleotide was omitted from the chase. Although one might expect that no elongation should occur under these circumstances, this was not the case in practice. For example, in reactions containing no added UTP, polymerase elongated past +21G and became arrested at +25A, immediately 5′ of the first templated T at +26 (Figure 5,

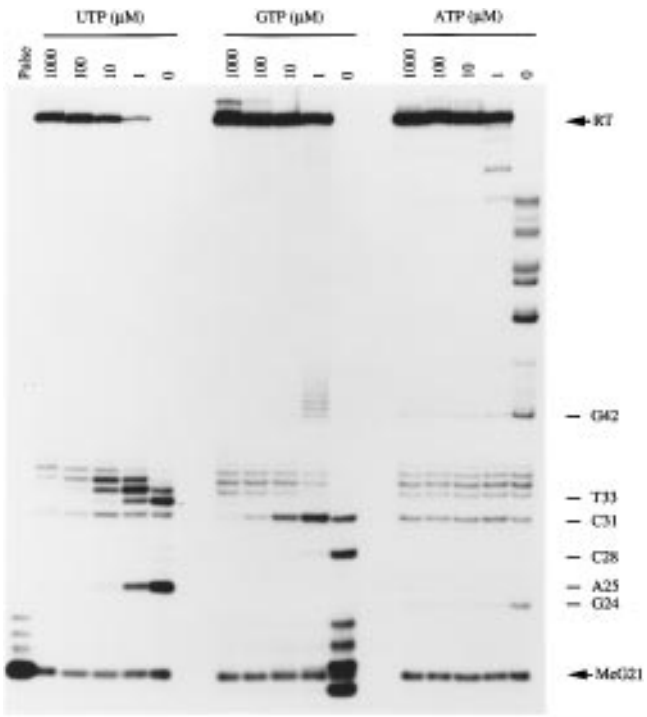


FIGURE 5: Effect of varying the concentrations of individual nucleotides on polymerase pausing. Bead-purified pulse-labeled G21(TER) complexes were chased for 5 min in the presence of 1 mM CTP, the indicated concentration of the variable NTP, and 0.1 mM of the remaining two NTPs. Labeled transcription products were resolved by polyacrylamide gel electrophoresis. The positions of pulse-labeled 3′-OMeG21 RNA (MeG21) and the chased runoff transcript (RT) are indicated by arrows at the right of the autoradiogram. Paused RNA species are denoted according to the location of their 3′ ends on the G21(TER) template.

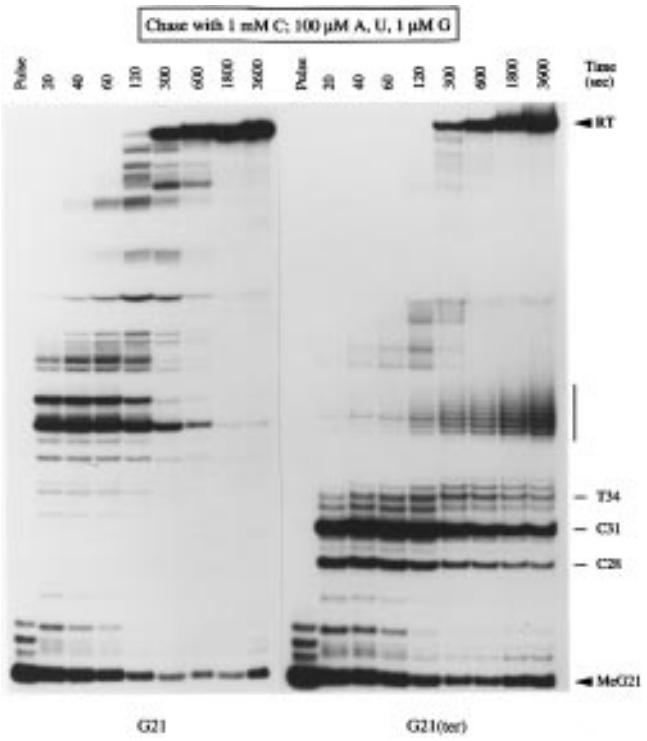


FIGURE 6: Slippage synthesis on the T-run. Bead-purified pulse-labeled G21 and G21(TER) complexes were chased in the presence of 1 mM CTP, 0.1 mM ATP, 0.1 mM UTP, and 1 μM GTP. Aliquots were withdrawn at the times indicated. The positions of pulse-labeled 3′-OMeG21 RNA (MeG21) and the chased runoff transcript (RT) are indicated by arrows at the right of the autoradiogram. Paused RNA species are denoted according to the location of their 3′ ends on the G21(TER) template. The ladder resulting from slippage synthesis in the T-run is indicated by the vertical bar.

left panel). A fraction of the complexes traversed this position only to arrest at +32G and +33T. (Trace UTP contamination was likely caused by spontaneous deamination of CTP.) As little as 1 μM UTP was sufficient to diminish the arrest at +25A and +32G. At 10 μM UTP, the polymerase bypassed these two arrest sites only to pause at +33T, +34T, and +35T. Full-length RNA was detected with as little as 1 μM UTP and increased progressively at 10 and 100 μM concentrations.

A ladder of incompletely elongated transcripts was seen in chase reactions lacking added ATP; these were arrested at sites immediately preceding templated A positions, e.g., at +24G and +42G, +60C, and +70G (Figure 5, right panel). With as little as 1 μM exogenous ATP, nearly all of the complexes that traversed the T-run were able to elongate to the end of the template. (We surmise from this that the concentration of contaminating ATP was <1 μM.) The relative paucity of consecutive A residues at proximal sites in the transcription unit may explain why polymerase elongated farther down the template in the absence of added ATP than it did in the absence of UTP.

In reactions lacking GTP, the OMeG21 RNA was shortened by a single nucleotide increment to A20 (Figure 5, middle panel). Cross contamination with sub-micromolar levels of GTP or ITP (arising by spontaneous deamination of ATP) permitted limited extension of the A20 cleavage product to form G21, G22, G23, C28, and C31 RNAs; each of these arrest sites precedes a templated G position (refer to Figure 1). [As noted previously (*11*), the OMeG21 RNA migrated during denaturing gel electrophoresis at a position in between A20 and G21.] The A20 cleavage product and the G21, G22, and G23 paused species were chased at 1 μM GTP. A significant fraction of the polymerases had reached the end of the template at 1 μM GTP; however residual pausing at +31C was noted along with a novel cluster of products at 42−50 nucleotides. The C31-paused RNA was reduced, but not eliminated, as GTP was increased to 10 and 100 μM. The etiology of the RNA cluster, which was eliminated at 10 μM GTP, is discussed below.

*Slippage in the T-Run.* The RNA cluster observed at 1 μM GTP consisted of at least 12 species separated by single nucleotide increments (Figure 5, middle panel). The shortest RNA in the cluster was halted at +41T, the last T base in the TTTTTTTTT sequence immediately 5′ of +42G. The ladder of RNAs extending upward from +41 was apparently generated by slippage of the RNA polymerase in the T-run, such that Us were reiteratively added to the 3′ end of the T41 nascent chain. A kinetic analysis of elongation by pulse-labeled G21 and G21(TER) complexes in the presence of 1 μM GTP verified that formation of this cluster depended on the presence of the TTTTTTTT sequence in the DNA (Figure 6). Polymerase elongation on the G21 template at 1 μM GTP was punctuated by a series of discrete pauses, none of which resembled the cluster of 1-nucleotide-spaced transcripts we observed on the G21(TER) template. The vanguard of polymerases reached the end of the G21 template after 2−5 min (Figure 6). All of the pause sites on G21 were traversed after 10−30 min.

Intrinsic pausing at the 5′ margin of the T-run was again noted during elongation on the G21(TER) template; the principal sites of elongation block at 20−120 s were at +28C and +31C, immediately preceding G positions, as expected. As polymerases traversed the T-run between 2 and 60 min, there was a steady accumulation of both runoff transcripts and the cluster of RNAs derived from slippage in the T-run (Figure 6). Because the slipped RNAs did not diminish in abundance over 60 min, we infer that the majority of the polymerases that engaged in slippage did not resume the continuous templated mode of elongation to the end of the template. This was borne out by the observation that the majority of the slipped RNA cluster was released from the bead-bound template after a 30−60 min chase at 1 $\mu$M GTP (data not shown). Because the chase reaction was performed in the absence of the vaccinia termination factor (VTF/capping enzyme), we infer that release of the slipped RNAs reflected an inherent instability of polymerase ternary complexes containing a 3′ poly(U) tract.

*End Addition to Full-Length RNAs.* The runoff RNAs synthesized on the G21 and G21(TER) templates during elongation in the presence of 1 mM NTPs were converted over time into a higher molecular weight species (Figure 2). Kinetic analysis suggested a precursor−product relationship between the runoff transcript, which was synthesized within 20 s, and the longer extension product, which appeared after 5 min and increased in abundance at 10 and 30 min (Figure 2). We observed no conversion of the runoff RNA into the longer extension product after 60 min when elongation reactions were performed at 10 $\mu$M ATP, GTP, and UTP (Figure 3). However, the longer RNA was formed after elongation reaction mixtures containing 10 $\mu$M ATP, CTP, and UTP were adjusted to 1 mM ATP, GTP, and UTP (Figure 4). Production of the longer RNA required high concentrations of all four NTPs. This was illustrated in the experiments shown in Figure 5, in which the concentrations of UTP, GTP, and ATP were individually titrated from 1 $\mu$M to 1 mM in the presence of 1 mM CTP and 0.1 mM of the other two nucleotides. These results show that synthesis of the 3′ extension product by RNA polymerase required much higher concentrations of NTPs than did templated synthesis of the 195-nucleotide runoff RNA. The size of the extension product could not be gauged accurately from its mobility in a 17% polyacrylamide gel; however analysis by electrophoresis through a 12% polyacrylamide gel indicated that approximately 15 extra nucleotides were added (data not shown).

In order to evaluate 3′ extension of runoff RNAs at single nucleotide resolution, we employed a truncated version of the G21 template that had been linearized with *Xba*I (see Figure 1). G21 ternary complexes formed on the bead-linked G21-*Xba*I template were chased within 20 s to form a 39-nucleotide transcript indicative of elongation to the very end of the template DNA strand (Figure 7). The 39-mer was subsequently elongated by a single nucleotide increment. The abundance of the 40-mer transcript increased steadily over 10−30 min, concomitant with a decrease in the level of the full-length G39 RNA (Figure 7). Longer extension products, 41 to ~70 nucleotides in length, accumulated between 2 and 30 min (Figure 7).

An additional experiment was conducted to determine whether 3′ extension of the 39-mer RNA occurred on the DNA template. The bead-bound and free reaction products were centrifugally separated after 1.5, 2, or 3 min of chase. (Note that these chase times include the ~60 s interval necessary to spin down the beads, remove the supernatant fraction, and denature each fraction by addition of stop mix.) The 39- and 40-mer RNAs were recovered quantitatively in the bead-bound fraction (data not shown). Low levels of 41−43 nucleotide transcripts were also associated exclusively with the template-bound fraction. This implies that initial incorporation of the extra 3′ nucleotides by RNA polymerase occurred in the context of a ternary complex.

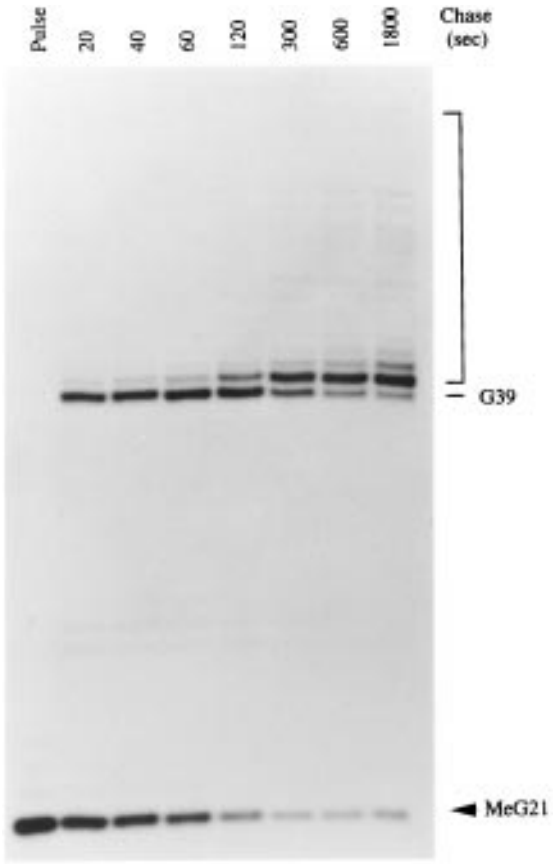


FIGURE 7: 3′ end addition to full-length RNAs. Transcription reactions were programmed by a bead-bound G21 template that had been linearized with *Xba*I at recognition site T↓CTAG↓A (nucleotides 35−40 in Figure 1). Bead-purified pulse-labeled G21 elongation complexes were chased in a reaction mixture containing 1 mM ATP, 1 mM GTP, 1 mM CTP, and 1 mM UTP. Aliquots were withdrawn at the times indicated. RNA species are denoted according to the location of their 3′ ends on the G21 template. The assignment of G39 was verified by electrophoretic analysis of the reaction products next to RNA ladders synthesized by vaccinia RNA polymerase on *Pvu*II-cut G21 DNA under conditions of single-nucleotide limitation.

*Effect of NTP Concentration on VTF-Dependent Transcription Termination.* Adding VTF/capping enzyme to the isolated ternary complexes immediately prior to the chase resulted in the appearance of a heterogeneous array of incompletely extended transcripts corresponding to terminated chains (Figure 8). Low UTP concentration favored the utilization of termination sites closer to the UUUUUNU signal (Figure 8). This finding underscores a considerable plasticity in termination site choice and provides additional evidence for the kinetic coupling model. According to this model, slowing elongation rate (e.g., by limiting UTP) without affecting signal transduction rate should result in utilization of more proximal termination sites, essentially as observed in Figure 8. Note that site choice is subject to the

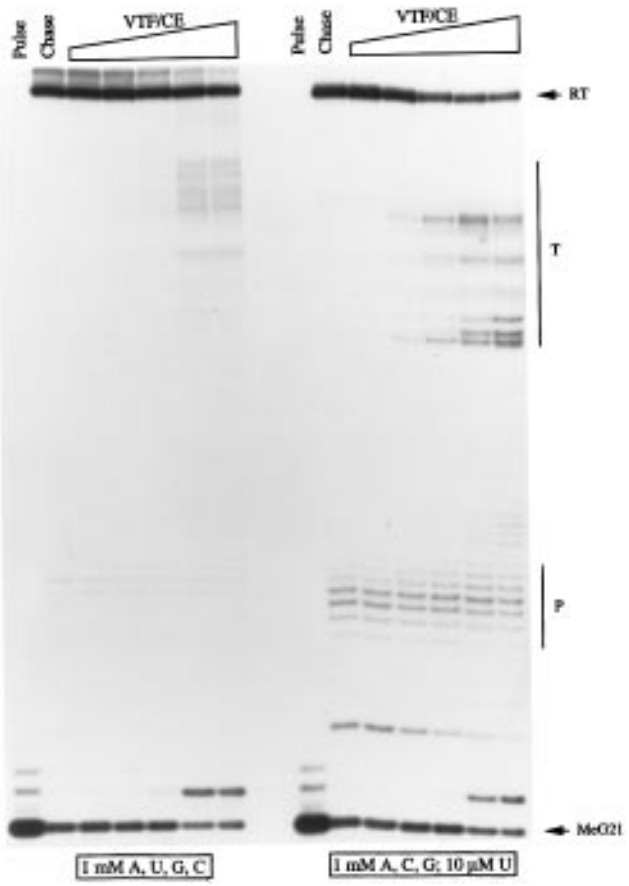


FIGURE 8: NTP limitation alters the distribution of VTF-induced termination sites. Purified G21(TER) transcription complexes containing pulse-labeled nascent RNA were chased for 10 min in elongation buffer containing either 1 mM each of CTP, ATP, GTP, and UTP (left panel) or 1 mM each of CTP, ATP, and GTP, plus 10 $\mu$M UTP (right panel). Extension products synthesized in the absence of added VTF/capping enzyme are shown in the lanes marked Chase. Other elongation reaction mixtures contained purified recombinant VTF/capping enzyme (12, 25, 50, 200, and 800 fmol, proceeding from left to right within the titration series) that was added prior to NTPs. Arrows indicate the positions of pulse-labeled 3′-OMeG21 (MeG21) and runoff transcripts (RT). Terminated transcripts (T) and paused RNAs (P) are indicated by vertical bars.

physical constraint that polymerase must elongate a critical distance (>20 nucleotides) beyond the UUUUUNU signal so that the signal can be extruded from the nascent RNA binding site on the polymerase (*9*, *14*, *15*).

## DISCUSSION

We have analyzed the elongation properties of vaccinia virus RNA polymerase during a single round of transcription in vitro. Ternary complexes containing a $^{32}$P-labeled 21-nucleotide RNA were purifed away from unincorporated nucleotides and then allowed to resume elongation to the end of a linear template. Purification of the elongation complexes was facilitated by immobilizing the DNA on a streptavidin bead. This allowed us to manipulate the concentration of NTPs during the chase phase. Our principal findings concern the effects of NTP concentration on pausing and slippage, and the fate of the ternary complex at the end of a linear DNA. We discuss the results as they pertain to vaccinia mRNA synthesis and relate them to findings reported for other RNA polymerases.

*Intrinsic Pausing.* Distinctive patterns of pausing were revealed as the concentration of individuals NTPs was lowered. For the most part, the polymerase paused at template positions preceding the limiting nucleotide, as would be expected. We found, however, that a run of Ts in the nontemplate strand constituted a strong elongation block, even at high nucleotide concentrations. Clusters of consecutive T residues have been shown to act as intrinsic pause sites for purified mammalian RNA polymerase II (pol II) (*16*−*18*). Yet, there are instructive differences between the behavior of pol II and vaccinia polymerase at such sites. Pol II arrests elongation within T tracts, yielding a cluster of transcripts with 3′ ends at many or even all of the T positions (*16*, *17*, *19*). At the adenovirus major late attenuation site, pol II arrests at a single site corresponding to the fifth T in the run (*20*). This contrasts with the behavior of the vaccinia polymerase, which arrests most prominently at position C31, situated two nucleotides upstream of the first T in the T-run. The vaccinia enzyme also paused at the first three or four T positions in the T-run. There was relatively little pausing at the five downstream Ts. It has been suggested that the propensity of T-runs to induce DNA bending is contributory to intrinsic pausing (*18*). It would appear that vaccinia RNA polymerase and pol II respond differently to such a distortion of the template, i.e., the vaccinia enzyme is inclined to pause prior to or at the 5′ margin of the T-run, whereas pol II arrests within or at the 3′ margin of the T-run. We speculate that the template distortion is sensed by the leading edge of the elongating polymerase. In the case of pol II the leading edge may become compressed relative to the active site when it encounters an arrest site (*21*). Compression of the leading edge footprint has not been observed with halted elongation complexes of vaccinia RNA polymerase (*3*). This may explain why pol II moves farther into a T-run before pausing than does the vaccinia enzyme. An alternative explanation is that vaccinia RNA polymerase does move farther into the T-run, but the nascent chains are immediately trimmed back to C31, T33, and T34 by 3′ hydrolysis.

Pol II intrinsic pausing can be exacerbated by nucleotide limitation; however, the elongation complexes, once paused, are not rescued by nucleotide supplementation (*20*). Arrested pol II is rescued by TFIIS, a factor that induces nascent RNA cleavage and allows resumption of elongation through the arrest site (*22*−*24*). In contrast, paused vaccinia polymerase does traverse the T-run-induced elongation block in response to increasing NTP concentration. The vaccinia elongation complex possesses an intrinsic nascent RNA hydrolase activity that is probably mediated by the rpo30 polymerase subunit, which is structurally homologous to TFIIS (*13*, *25*). Because rpo30 cannot be dissociated from the ternary complex, we cannot easily evaluate whether RNA cleavage plays a role in overcoming intrinsic pausing by vaccinia RNA polymerase.

Recent experiments suggest that a viral protein tightly associated with the vaccinia elongation complex may regulate the intrinsic pausing properties of the vaccinia RNA polymerase (*26*). Removal of that protein (designated factor X) by transient treatment of G21(TER) elongation complexes with high concentrations of heparin renders the polymerase much more susceptible to pausing at the T tract. (Heparin stripping has no apparent effect on nascent RNA cleavage.) Adding isolated factor X back to the heparin-stripped G21(TER) complexes restores the normal elongation behavior of the enzyme. The finding that factor X has an associated DNA-dependent ATPase activity suggests that it

functions by a novel mechanism (*26*). Factor X is also required in conjunction with VTF for transcription termination by vaccinia RNA polymerase (*26*).

*Slippage*. Slippage by vaccinia virus RNA polymerase is a normal occurrence during initiation of mRNA synthesis from vaccinia late promoters and from a subset of early promoters (*27*−*31*). Initiation on these genes occurs at an adenylate within a highly conserved sequence 5′-TAAAT (nontemplate strand), which constitutes an essential part of the vaccinia late promoter. Reiterative incorporation of adenylates via slippage on the sequence 3′ ATTTA in the template strand results in the production of a 5′ poly(A) "head" that adorns all known late mRNAs and selected early transcripts. The late mRNA poly(A) heads produced in vivo contain about 35 adenylates. Implicitly, the length of the head will be dictated by the probability of slipping and adding another A versus the probability of incorporating the next templated base (usually a U in late mRNAs). The latter must occur for productive mRNA synthesis. The factors that control this key decision point in vaccinia transcription are essentially unknown. Slippage during promoter-dependent initiation has also been described for T7 RNA polymerase (*32*), *E. coli* RNA polymerase (*33*−*38*), and yeast RNA polymerase III (*39*).

We now find that elongating vaccinia RNA polymerase can reiteratively incorporate U residues via slippage on a run of A residues in the template strand. We observed "elongation slippage" only when incorporation of the templated guanylate immediately following the T-run was disfavored by severely limiting the GTP concentration. After their synthesis, the slipped RNAs were released from the template. They differ from typical paused transcripts, which remained engaged in ternary complexes. We speculate that protein−RNA interactions (and perhaps RNA−DNA interactions) that normally stabilize the nascent chain are weakened when poly(U) occupies most of the nascent RNA binding pocket on the vaccinia polymerase. The dimensions of this pocket (extending ∼16−18 nucleotides back from the 3′ end of the nascent RNA (*14*, *15*)) are similar to the sizes of the 3′ poly(U) segments of the slipped chains. It will be interesting in future studies to determine the influence of tract length on internal slippage and to see whether other nucleotides besides UMP can be reiteratively incorporated in response to homomeric tracts in the template DNA.

A previous study suggested that *E. coli* RNA polymerase can slip in vivo during elongation on runs of 10 adenines or 10 thymines and then resume elongation; this has the effect of creating translational frame-shift mutations at the RNA level (*40*). Yeast RNA polymerase I (pol I) can reiteratively slip on a run of 10−12 T residues if the T tract is located upstream of a protein binding site on the template that serves as an elongation roadblock when the binding site is occupied (*41*). Slippage by pol I results in the addition of very long 3′ poly(A) tails (*41*). Slippage during elongation has also been described for RNA virus-encoded RNA-dependent RNA polymerases (reviewed in ref 42).

*End Addition.* It had been noted previously that a substantial fraction of the apparently full-length RNA synthesized by vaccinia RNA polymerase on *Pvu*II-cut G21(TER) remained associated with the bead-bound template (*9*). The same study also documented the presence of a minor transcript longer than the runoff RNA. We have now established a precursor−product relationship between the full-length transcript and the longer 3′ extension product. The ∼5 min lag between the appearance of full-length RNA and the 3′ extension product implies a rate-limiting step in this conversion that is much slower than the rate of chain elongation during templated RNA synthesis.

Using a shorter linear template, G21-*Xba*I, we found that vaccinia polymerase rapidly elongated to the very end of the template DNA strand and remained template-engaged. This differs from the behavior ascribed to RNA polymerase II by Izban et al. (*43*). Using linear DNAs containing analogous 5′ restriction endonuclease overhangs, they reported that pol II transcribed to within one to two nucleotides of the end of the template strand, at which point the ternary complexes were destabilized and the RNAs were released from the template (*43*). They found that during transcription on blunt-ended templates, pol II tended to arrest elongation 5−10 nucleotides prior to the end of the template strand and to remain template engaged. We found that vaccinia RNA polymerase elongated to the very end of the blunt-ended *Pvu*II-cut G21 DNA. The pol II results have been interpreted as indicative of the importance of contacts between pol II and the template DNA ahead of the ternary complex as determinants of elongation competence and ternary complex stability. Although the DNase footprint of the vaccinia polymerase elongation complex extends 22−24 nucleotides ahead of the site of nucleotide incorporation (*3*), it appears that leading-edge contacts are not as critical for progression of the ternary complex to the last template position. Terminally positioned vaccinia polymerase begins to incorporate several extra 3′ nucleotides while still engaged on the *Xba*I-cut template. Because end-addition also occurs on the blunt-ended *Pvu*II-cut DNA, we presume that the reaction is not simply being driven by slippage on a 5′ DNA overhang at the restriction site. Whether the DNA plays a direct role in vaccinia polymerase 3′ addition is not yet clear. Other RNA polymerases are capable of 3′ end addition reactions: most models invoke the action of newly made transcripts as primers for nontemplated 3′ addition or as template-primers for RNA-directed RNA synthesis (*44*−*46*).

*Elongation Rate and Termination Site Choice.* We previously elaborated a model whereby termination site choice is determined by a kinetic balance between the rate of signaling and the rate of chain elongation. Signaling is coupled to ATP hydrolysis whereas elongation is not. Signaling rate is dictated by the concentration of hydrolyzable ATP. The effect of limiting ATP concentration is to shift the distribution of VTF-induced termination sites farther away from the UUUUUNU signal (*9*). The hypothesis that elongation rate is an independent determinant of termination site choice is based on studies of the effects of substituting the nucleotide analog GMPPNP for GTP during the chase reaction on the G21(TER) template (*9*). Even at high concentrations (1 mM), GMPPNP alters the elongation properties of vaccinia RNA polymerase, e.g., slowing elongation rate, inducing novel pauses outside the T-run, and eliciting VTF-independent transcript release immediately 3′ of the T-run. The present study suggests an explanation for the cluster of "prematurely" released RNAs generated in the presence of GMPPNP (*9*), i.e., that the analog is poorly incorporated at template position +42G, which prompts the addition of extra UMP residues by slippage synthesis. We suspect that the effects of 1 mM GMPPNP substitution are qualitatively similar to those of severe GTP depletion with respect to

inducing polymerase slippage. The slipped RNAs are released from the template in both instances.

In this study, we adopted an alternative approach to test the contribution of elongation rate to termination site choice, i.e., by comparing termination site distribution at high versus low concentration of UTP. The finding that UTP limitation shifts the distribution of termination sites closer to the UUUUUNU signal lends further support to the kinetic coupling model.

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