Discrete Functional Stages of Vaccinia Virus Early Transcription during a Single Round of RNA Synthesis in Vitro*

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The dynamics of protein-protein and protein-nucleic acid interactions during the synthesis of early mRNAs are unknown. In seeking to address this point we were confronted by two constraints of the in vitro system as presently constituted, these being (i) difficulty in restricting synthesis to a single round of transcription, and (ii) lack of methodology to analyze discrete steps in the transcription cycle, i.e., initiation, elongation, and termination. These impediments have been surmounted through the use of specialized DNA templates and pulse-chase RNA labeling strategies, as described in this report. We focus on the formation and properties of a stable ternary transcription complex, the nature of the termination signal in the nascent transcript, and the access of VTF to the ternary complex. Furthermore, we show that vaccinia-capping enzyme expressed in (and purified from) Escherichia coli (32) promotes transcription termination in vitro.

Experimental Procedures

DNA Templates—Plasmid pSB24, containing a G-less cassette (33) downstream of a synthetic vaccinia early promoter element, was constructed by Dr. Steven Broyles (Purdue University). The synthetic promoter (shown in Fig. 1B) is considerably more active in directing vaccinia transcription in vitro than are various "natural" early promoters; the choice of this particular sequence was inspired by the comprehensive study of early promoter strength reported by Davison and Moss (9). Plasmid pYL1, containing a vaccinia early termination

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1 The abbreviations used are: VETF, vaccinia early transcription factor; VTF, vaccinia termination factor; nt, nucleotide(s); AdoMet, S-adenosylmethionine.
signal downstream of the G-less cassette, was constructed by replacing the sequences from the BamHI site to the HindIII site of the pSB24 polymerase with a duplex oligonucleotide (shown in Fig. 1C) using standard molecular cloning techniques. The inserted sequence is identical to the well-studied terminator at the 3'-end of the vaccinia growth factor gene (34) and includes three tandem copies of the TTTTTTNT termination signal. Plasmids pC2AT and pML are described by Sawadogo and Roeder (33), and were the gift of Dr. Danny Reinberg (Rutgers/University of Medicine and Dentistry of New Jersey).

**Enzyme Purification**—Vaccinia DNA-dependent RNA polymerase was purified as described (11). The phosphocellulose elution fraction (150 units/ml; 1 unit catalyzed incorporation of 1 nmol of UMP into acid-insoluble material under standard conditions (11)) was used routinely unless specified otherwise. The glycerol gradient polymerase fraction (6) was used in certain experiments where indicated. All RNA polymerase preparations contained core polymerase and the vaccinia early transcription factor, VETF. VTF/capping enzyme was purified from vaccinia virions as described (6). The phosphocellulose preparation of the virion-capping enzyme was used in the present study. Capping enzyme was expressed in E. coli and purified from bacterial lysates as described (32). The molar concentration of active capping enzyme was determined by enzyme-GMP complex formation as described (6).

**Transcription in Vitro**—Reaction mixtures containing 20 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 6 mM MgCl₂, NTPs, linear duplex DNA template, RNA polymerase, and other proteins as indicated were incubated at 30 °C. Samples were processed as described (6). Transcription reaction products were analyzed by electrophoresis through 4% polyacrylamide gels under denaturing conditions. Radiolabeled RNAs were visualized by autoradiographic exposure of the gels.

**Materials**—Radiolabeled nucleotides were purchased from Amer sham Corp. BrUTP was synthesized chemically from UTP (30). Restriction endonucleases were obtained from Bethesda Research Laboratories and New England Biolabs.

**RESULTS**

**A Synthetic Early Promoter Directs Transcription of a G-less Cassette by Vaccinia RNA Polymerase**—Partially purified vaccinia RNA polymerase (that contained VETF and core polymerase) was used to transcribe the linear duplex template pSB24. This DNA contained a 382 nt G-less cassette situated immediately downstream of a synthetic vaccinia early promoter (Fig. 1). Plasmid DNA was linearized at the end of the G-less cassette by treatment with endonuclease SmaI and tested for template activity as a function of DNA concentration. In the absence of added DNA, no radiolabeled RNA products were detected (Fig. 2B, lane 0). Addition of template lead to the appearance of a prominent labeled species of 390 nt, consistent with transcription of the entire G-less cassette from the promoter-driven initiation site to the end of the linear molecule. A less abundant transcript of approximately 350-360 nt was evident at lower DNA concentrations; the level of this RNA diminished steadily as template concentration was increased. In other experiments in which low template concentrations were employed, longer autoradiographic exposures revealed additional smaller RNA products with regular electrophoretic spacing (data not shown). The smaller RNA could arise by synthesis of more than one transcript/template with inefficient release of the leading polymerase from the linear end of the DNA. This would result in the backup of lagging polymerase molecules such that the entire G-less cassette cannot be transcribed by those enzymes. Increasing template concentration would be expected to limit multiple initiations and reduce the abundance of the smaller RNA without affecting overall synthesis, just as was observed in the present case (Fig. 2B). Parenthetically, the size interval between the leading and lagging transcripts (30–40 nt) should reflect the amount of template DNA occupied by a single polymerase molecule. The presence of regularly spaced smaller RNAs has also been detected in studies of transcription of the G-less cassette by RNA polymerase II (33, 35, 36).

**Promoter Specificity**—Two other plasmids were employed as control templates for in vitro transcription: (i) pC2AT, containing the G-less cassette alone with no viral promoter, and (ii) pML, containing a G-less cassette downstream of the adenovirus 2 major late promoter element. pML has been shown to be an effective template for transcription in vitro by RNA polymerase II (33). Each DNA was linearized with SmaI and tested at high and low DNA concentration for template activity in parallel with pSB24. No radiolabeled RNA was detected in the absence of DNA (Fig. 2A, lane 1) or in the presence of either pC2AT (Fig. 2A, lanes 4 and 5) or pML (Fig. 2A, lanes 6 and 7) at either DNA concentration. Thus, the vaccinia RNA polymerase was highly specific for its own early promoter and did not recognize a "strong" RNA polymerase II promoter even when the segment of DNA to be transcribed was invariant. The absence in transcription reactions containing the control templates of any RNA corresponding to the putative "lagging" nascent transcript seen at low concentration of pSB24 template (Fig. 2A, lane 2) excluded the possibility that such transcripts arise via initiation within the G-less cassette in a promoter-independent reaction.

**Analysis of a Single Round of Transcription**—We have exploited the early promoter-driven G-less template to restrict our analysis to a single complete cycle of transcription in vitro. This has been accomplished as follows: (i) by developing a novel method to cotranscriptionally label RNA specifically in the 5' cap, and (ii) by defining a stable ternary complex of template, polymerase, and nascent RNA paused at the 3'-end of the G-less cassette that could be induced to resume elongation by the addition of GTP to the reaction.

In order to label the cap, advantage was taken of the ability of the vaccinia virus RNA guanylyltransferase (a component of the multifunctional capping enzyme) to utilize dGTP in lieu of GTP in the formation of a blocked 5' terminus (37). Because dGTP is not a substrate for RNA polymerase (11),

![Fig. 1. cis-Acting transcription signals in pYL1 template DNA. The pUC-based pYL1 plasmid was constructed as described under "Experimental Procedures." The arrangement of salient transcription signals in the template DNA is shown in A. The location of restriction sites used to linearize the template are indicated by the arrows. The sequence of the synthetic vaccinia early promoter (non-template strand) is shown in B. The site of transcription initiation is indicated by +1; the direction of transcription is shown by the arrow. The sequence of the duplex termination signal is indicated in C. The pSB24 DNA template (shown in D) lacks the terminator present in pYL1.](image-url)
FIG. 2. Transcription of pSB24 DNA by partially purified vaccinia RNA polymerase. A, promoter specificity. Transcription reaction mixtures (50 µl) containing 1 mM ATP, 1 mM CTP, 0.1 mM \([\alpha-\text{P}]\)UTP, RNA polymerase (phosphocellulose fraction, 0.6 unit), and template DNA were incubated at 30 °C for 60 min and then processed for electrophoretic analysis as described under "Experimental Procedures." The DNA templates (each of which had been linearized by digestion with SmaI) were as follows: lane 1, no added DNA; lane 2, 30 ng of pSB24; lane 3, 900 ng of pSB24; lane 4, 30 ng of pC2AT; lane 5, 900 ng of pC2AT; lane 6, 30 ng of pML; lane 7, 900 ng of pML. An autoradiogram of the gel is shown. The positions and sizes (in nucleotides) of radiolabeled denatured DNA markers are indicated on the left. B, effect of DNA template concentration. Transcription reaction mixtures (50 µl) containing 1 mM ATP, 1 mM CTP, 0.1 mM \([\alpha-\text{P}]\)UTP, RNA polymerase (0.6 unit), and the indicated amounts (ng) of SmaI-cut pSB24 DNA were incubated at 30 °C for 60 min and then processed for electrophoretic analysis. An autoradiogram of the gel is shown. The positions and sizes (in nucleotides) of radiolabeled denatured DNA markers are indicated on the left. C, effect of NaCl concentration. Transcription reaction mixtures (50 µl) containing 1 mM ATP, 1 mM CTP, 0.1 mM \([\alpha-\text{P}]\)UTP, RNA polymerase (0.6 unit), and the indicated amounts (ng) of SmaI-cut pSB24 DNA were incubated at 30 °C for 60 min and then processed for electrophoretic analysis. An autoradiogram of the gel is shown. The positions and sizes (in nucleotides) of radiolabeled denatured DNA markers are indicated on the left. The concentration of added NaCl (mM) is indicated above each lane.

\([\alpha-\text{P}]\)dGTP added to the transcription reaction should be excluded from internal positions in the RNA chain. Also, each RNA molecule should be labeled only once with dGMP, obviating the need to normalize the extent of labeling to nucleotide composition and transcript length. Stable incorporation of dGMP into the cap should be enhanced by inclusion of AdoMet in the reaction. AdoMet serves as a methyl donor for N-7 methylation of the blocking guanylate (or deoxyguanylate) moiety (37, 38). Methylation can be expected to render the dGMP-labeled cap structure resistant to pyrophosphoryl reversal of the capping reaction that would otherwise be promoted by PP, generated during RNA synthesis (37). These predictions were borne out experimentally (Fig. 3, top panel). Inclusion of purified capping enzyme and \([\alpha-\text{P}]\)dGTP in transcription reactions containing vaccinia RNA polymerase and SmaI-cut pSB24 template resulted in trace labeling of a 390-nt product, the size expected for a run-off transcript of the G-less cassette. dGMP labeling of this RNA was stimulated dramatically by the inclusion of AdoMet and was completely dependent on the presence of template DNA (Fig. 3, top panel). Incorporation of dGMP into cap dinucleotide was not verified directly by chromatographic analysis of nuclease digests of the labeled transcript; however, it is extremely unlikely that the radionucleotide could have been assimilated at any other position in the RNA chain given that AdoMet has no stimulatory effect on the rate or extent of NTP incorporation into RNA by the fractionated in vitro system (6) or by permeabilized virions (31, 39).

Whether RNA polymerase could pause at the 3'-end of the G-less cassette was studied using a pSB24 template linearized with nuclease NdeI. This enzyme cleaves the plasmid 251 nt downstream of the SmaI site (where the first G residue is downstream of the SmaI site) or by permeabilized virions (31, 39).

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of the G-less cassette. The total amount of cap-labeled RNA remained relatively constant up to 15 min of chase, as predicted.

Single-round transcription conditions could also be achieved using a conventional pulse-chase protocol in which the nascent transcript was labeled by inclusion of \(^{32}\text{P}\)UTP in the reaction mixture (Fig. 4). Analysis of UMP-labeled products after a 5-min pulse phase of synthesis in the presence of HindIII-cut pSB24 template revealed a major transcript of 390 nt consistent with pausing at the end of the G-less cassette (Fig. 4, lane 1, indicated by \(P\)). The chase was performed by addition of excess unlabeled UTP and 1 mM GTP. This resulted in the near-quantitative conversion of the 390-nt transcript to a species of approximately 430 nt, indicative of read-through to the site of linearization by HindIII, 37 nt downstream of the 3'-end of the G-less cassette (Fig. 4, lane 3, indicated by \(H\)). The size of the read-through transcript was strictly a function of the site of linearization of the plasmid DNA; when similar pulse-chase experiments were performed using as template pSB24 cut with NdeI, elongation of the pulse-labeled transcript yielded a 630-nt product (Fig. 4, lane 2, indicated by \(N\)). When NdeI-cut and HindIII-cut pSB24 templates were present together at the same concentration during the pulse-phase of the reaction, the transcripts were chased into approximately equal amounts of 630 and 430 nt run-off RNAs (Fig. 4, lane 4). However, when NdeI-cut template was included in the pulse-phase and a second HindIII-cut template was added upon initiation of the chase, only the 630-nt transcript of the NdeI-cut DNA was radiolabeled (Fig. 4, lane 5). Thus, no new transcripts were labeled during the chase.

These experiments (Figs. 3 and 4) established the feasibility of analyzing a single round of RNA synthesis via a pulse-chase protocol and provided a method for temporal separation of the initiation/elongation and termination phases of the transcription reaction (see below).

Properties of the Ternary Transcription Complex: Stability to Salt and Sarkosyl—Transcription of pSB24 template by vaccinia RNA polymerase was extremely sensitive to increasing NaCl concentration. Addition of salt to the reaction mixture prior to the addition of RNA polymerase resulted in a concentration-dependent inhibition of synthesis (Fig. 2C). Reactions supplemented with \(\geq100\) mM NaCl (over the approximately 25 mM NaCl contributed to the standard reaction by the polymerase) were completely inactive. In contrast, the ternary transcription complex of RNA polymerase, template, and nascent RNA was remarkably insensitive to salt (Fig. 5). Ternary complex containing cap-labeled nascent RNA was generated by pulse-labeling for 5 min as described above. Analysis of the RNA at the end of the pulse revealed a product of the size expected for a paused transcript (Fig. 5, lane \(\Delta\)). Reaction mixtures were supplemented with increasing concentrations of NaCl (indicated above the lanes in Fig. 5) and then chased by the addition of 1 mM GTP. NaCl concentrations up to 300 mM had little effect on the ability of polymerase to elongate to the end of the template. NaCl concentrations \(\geq400\) mM reduced incrementally the amount of full-length read-through RNA and resulted in the appearance of new transcripts of discrete size between that of the paused RNA and the read-through RNA. At 500 mM NaCl, a significant fraction of the pulse-labeled RNAs were not extended during the chase (Fig. 5). This was due to inhibition of nucleotide incorporation by polymerase or to salt-induced destabilization of the ternary complex leading to transcript release (termination)? Additional experiments suggested that the nonelongated RNAs observed at high salt had been released from the transcription complex, insofar as paused ternary complexes that were adjusted to 450 mM in NaCl and then diluted to 150 mM NaCl in transcription buffer did not catalyze further elongation to full-length transcripts when chased with GTP (data not shown).

Inclusion of the detergent Sarkosyl in the reaction mixture prior to the addition of RNA polymerase resulted in a concentration-dependent inhibition of transcription of a SmaI-cut pSB24 template (Fig. 6A). UMP incorporation into the runoff transcript (assayed by cutting out the appropriate region of the dried gel and counting the gel slice in a liquid scintillation fluid) was inhibited by 80% at 0.0125% Sarkosyl and by 96% at 0.02% Sarkosyl; no labeled RNA product was discernable at detergent concentrations in excess of 0.02% (Fig. 6A).

Similar experiments were performed using NdeI-cut pSB24 template in reactions lacking GTP. Under these reaction conditions, only a single cycle of initiation events was permitted. It was observed that the concentration-dependent inhibition of synthesis of paused nascent RNA by Sarkosyl was essentially identical to that depicted in Fig. 6A (data not shown). In contrast, elongation of preformed ternary complex was refractory to Sarkosyl up to 0.03% (Fig. 6B). Partial inhibition of elongation was observed at 0.05% and elongation was completely abrogated at 0.1% detergent.
Initiation, Elongation, and Termination by Vaccinia RNA Polymerase

Fig. 6. Differential effect of Sarkosyl on transcription initiation and elongation. Panel A, transcription reaction mixtures (50 µl) contained 1 mM ATP, 1 mM CTP, 0.1 mM α-32PUTP, and 600 ng of Smal-cut pSB24 DNA template. Reactions were supplemented with Sarkosyl prior to the addition of RNA polymerase (0.3 unit). Incubation was at 30 °C for 60 min. An autoradiogram of the gel is shown. The concentration of added Sarkosyl (%) is indicated above each lane. Panel B, transcription reactions were performed in two stages. In the pulse-phase, reactions (25 µl) contained 1 mM ATP, 1 mM CTP, 0.1 mM α-32PUTP, 300 ng of NdeI-cut pSB24 DNA, and RNA polymerase (0.1 unit). Samples were incubated for 5 min at 30 °C and then supplemented with Sarkosyl. The chase-phase was initiated by addition of 1 mM unlabeled UTP and 1 mM GTP, followed by incubation at 30 °C for another 5 min. An autoradiogram of the gel is shown. The concentration of Sarkosyl (%) added to each reaction mixture after the pulse-phase is indicated above the lanes. Transcription reaction products corresponding to paused nascent RNA (P) and read-through RNA (RT) species are indicated on the right.

Studies of Transcription Termination Nature of the Termination Signal—In order to study transcription termination in a single round of RNA synthesis, we constructed the template pYL1 by inserting downstream of the G-less cassette of pSB24 a synthetic oligonucleotide containing three copies (in tandem or overlapping) of the TTTTTNT early termination signal (Fig. 1). NdeI-cut pYL1 was used to program transcription in vitro in the absence of GTP, leading to the accumulation of pulse-labeled ternary complexes that were paused at the 3′ end of the G-less cassette (Fig. 3, bottom panel). Addition of GTP chased the nascent RNA into two classes of product (i) an RNA of 455 nt, arising via termination approximately 35 nt downstream of the first encountered TTTTTNT signal. The synthesis of this later class of transcript depended on the presence in the template of the cis-acting termination motif (Fig. 3, bottom panel, compare SB24 and YLI templates).

Prior studies had indicated that the cis-acting signal for factor-dependent termination was contained within the nascent RNA; this was based on the selective abrogation of factor-dependent termination when BrUTP or IUTP was substituted for UTP in the in vitro transcription reaction (30). We have now applied this strategy to the pulse-chase transcription reaction in order to probe the influence of specific regions of the nascent transcript on the termination signal transduction. As shown in Fig. 7, inhibition of termination by BrUTP was observed only when analog substitution occurred during the chase period, i.e. specifically when the TTTTTNT signal was being transcribed into UUUUUNU. BrUMP substitution for UMP throughout the 390-nt portion of the nascent RNA immediately preceding the termination signal had no appreciable inhibitory effect, provided that UTP was present in 20-

Fig. 7. BrUTP prevents transcription termination by incorporation of BrUMP into the distal segment of nascent RNA containing the UUUUUNU termination signal. Transcription reactions were performed in two stages. In the pulse-phase, reactions contained 1 mM ATP, 1 mM CTP, 50 µM UTP, or BrUTP (as indicated), 0.33 µM [α-32P]dGTP (3000 Ci/mmol), 600 ng of NdeI-cut pYL1 DNA template, 50 µM AdoMet, RNA polymerase (0.3 unit), and 100 fmol of purified vaccinia-capping enzyme. Samples were incubated for 5 min at 30 °C and then either processed immediately for electrophoresis (indicated in the figure by a Δ symbol for the chase phase) or chased by adding 1 mM UTP or BrUTP (as indicated) followed by addition of 1 mM GTP. Incubation was continued for 5 min at 30 °C. An autoradiogram of the gel is shown. Transcription reaction products corresponding to paused nascent RNA (P), read-through RNA (RT), and terminated RNA (T) species are indicated on the left.

Fig. 8. VTF activity of capping enzyme expressed in E. coli. Transcription reaction mixtures contained 20 mM Tris-HCl, pH 8.0, 2 mM dithiothreitol, 6 mM MgCl2, 1 mM ATP, 1 mM GTP, 1 mM UTP, 50 µM [α-32P]CTP, 600 ng of SspI-cut pYL1 DNA (left-most three lanes) or 600 ng of SspI-cut pSB24 DNA (rightmost two lanes), and RNA polymerase (glycerol gradient fraction; 0.25 unit). Also included were capping enzyme purified from vaccinia virions (phosphocellulose fraction; 100 fmol, indicated by V above the lanes) or from E. coli (glycerol gradient fraction; 8 fmol, indicated by E above the lanes). Incubation was for 60 min at 30 °C. Transcription reaction products corresponding to read-through RNA (RT) and terminated RNA (T) species are indicated on the left.

fold excess over BrUTP during the chase. Only about 45 nt separate the pause site at the end of the G-less cassette and the approximate site of termination; this region of RNA includes 19 U residues of which 14 are within the tandemly arranged termination signals. The data suggested that BrUMP specifically prevented interaction of VTF and/or RNA polymerase with the segment of the RNA containing the termination signal and that no other region of the transcript contributed to the analog effect.

Recombinant Capping Enzyme Promotes Termination in Vitro—Vaccinia capping enzyme is a multifunctional protein that catalyzes three mechanistically distinct reactions leading to the synthesis of the cap zero structure (38, 40). Studies to date indicate that capping enzyme and VTF are intimately associated (6). Active capping enzyme was recently produced in E. coli by coexpressing the viral genes encoding the M, 95,000 and 31,000 subunit polypeptides (32, 41). In the heterologous system, the two subunits associated with the proper stoichiometry to form a heterodimeric protein with RNA triphosphatase, RNA guanylyltransferase, and RNA (guanine-7) methyltransferase activities (32).

Does the capping enzyme expressed in E. coli also have VTF activity? This was addressed by assaying the ability of "native" and "recombinant" capping enzyme preparations to restore termination competence to termination-defective RNA polymerase (6). As shown in Fig. 8, the RNA polymerase synthesized a 1000-nt read-through transcript when programmed with SspI-cut pYL1 template (lane −). Addition of capping enzyme purified from E. coli resulted in the appear-
ance of a 435-nt terminated transcript (Fig. 8, lane E on left) identical in size to that of the terminated RNA made in the presence of capping enzyme purified from vaccinia virions (Fig. 8, lane V on left). The amount of recombinant capping enzyme used in this experiment was less than the amount of native enzyme. This was because the addition of higher amounts of the E. coli protein preparation resulted in complete inhibition of overall transcription. We attributed this inhibitory effect to bacterial proteins present in the enzyme fraction (which was shown to be 32% pure with respect to the vaccinia capping enzyme (32)). Nonetheless, the termination-promoting effect of the bacterial protein was dependent on the amount added within a narrow range of concentration (data not shown). Significantly, the appearance of the 435-nt transcript depended upon the presence in the template of the cis-acting termination signal, i.e., no RNA species the size of the terminated transcript was induced by addition of E. coli capping enzyme to reactions programed by SspI-cut pSB24 (Fig. 8, lane E on right). We infer from this experiment that capping enzyme and VTF are indeed identical.

**VTF Promotes Termination by Preformed Ternary Complex**—Capping of vaccinia mRNA (in virions) is believed to occur on short nascent chains, i.e., soon after initiation of transcription. The present study shows clearly that capping in the *in vitro* system also occurs on nascent transcripts (Fig. 3). Does the same enzyme molecule that caps the 5′-ends of an RNA mediate termination hundreds or even thousands of bases downstream and, if so, how? Or are capping and termination wholly unrelated, mediated by different enzyme molecules (e.g., from a pool of exchangeable molecules) that act at opposite ends of the transcription unit? The fact that the number of enzyme molecules/virus particle is estimated at 80–100 for both capping enzyme and RNA polymerase (12, 42, 43), and is roughly the same as the number of early transcription units, argues against a physiologic pool of excess capping enzyme.

One plausible model is that capping enzyme is engaged in the transcription process from the outset and remains associated with the transcription complex (template, polymerase, nascent RNA, etc.) during elongation. In this way, efficient capping of nascent chains might be ensured, and VTF would remain poised to “read” the UUUUNU termination signal during synthesis of the proximal portion of the complete RNA. The number of enzyme molecules/virus particle is estimated to be 80–100 for both capping enzyme and RNA polymerase (12, 42, 43) and is roughly the same as the number of early transcription units, arguing against a physiologic pool of excess capping enzyme.

**VTF-dependent Termination Is Sensitive to Salt and Sarkosyl**—We began to address the functional interaction of VTF with the transcription elongation complex by examining the sensitivity of the termination reaction *per se* to salt and Sarkosyl. In these experiments, VTF was added to preformed UMP-labeled ternary complex on an Ndel-cut pYL1 template and further elongation was permitted by provision of cold UTP and GTP (Fig. 10, A and B). The appearance of an appropriately terminated transcript during the chase (denoted by T in Fig. 10) depended entirely on VTF added after the pulse (compare *lanes* −VTF and 0 in Fig. 10, A and B). Our analysis showed that Sarkosyl abolished termination at 0.02% concentration, i.e., at a level of detergent well below that required to inhibit elongation (Fig. 10B). A parallel series of experiments was performed in which VTF was included in the reactions prior to addition of Sarkosyl and subsequent chase (e.g., VTF added at the same time as polymerase). It was observed that the Sarkosyl sensitivity of termination was unaltered (compared with the results of Fig. 10B) by varying the order of addition (data not shown). Further experiments were performed in which Sarkosyl was added to paused ternary complexes synthesized on Ndel-cut pYL1 template and elongation was then resumed by chasing in the absence of added VTF. Under these circumstances, only read-through RNA was produced during the chase (e.g., see Fig. 10B, lane −VTF). The effects of increasing Sarkosyl concentration on resumption of elongation was essentially identical to the results of Fig. 6B (data not shown). Thus, the addition of Sarkosyl during elongation and synthesis of RNA containing the UUUUUNU signal did not confer upon the elongating polymerase the ability to terminate in a factor-independent manner.
DISCUSSION

Single Round of RNA Synthesis in Vitro—Discrete steps in transcription by DNA-dependent RNA polymerase have been studied in depth in bacteria (reviewed in Ref. 44) and, more recently, in eukaryotic systems (e.g. 45–48). A common experimental strategy is the use of reagents that disrupt protein-nucleic acid and/or protein-protein interactions critical to a specific stage of the initiation or elongation reaction; such agents or treatments include Sarkosyl, heparin, high salt, thermal shift, and template challenge. It is a general property of RNA polymerases that the initiation and elongation phases of transcription display different sensitivities to such agents; typically the formation of a ternary complex is accompanied by enhanced stability to salt, Sarkosyl, etc. We have shown this to be the case for vaccinia virus RNA polymerase and have defined the sensitivities of the initiation, elongation and termination stages of vaccinia early transcription to both salt and Sarkosyl. In addition, we have employed pulse-chase labeling strategies (involving either cap labeling at the 5’-end with dGTP or internal labeling with rNTPs) to examine the fate of transcripts made within a single round of synthesis without having to specifically block the capacity for subsequent initiation events.

Factor-dependent Transcription Termination—We have examined transcription termination using a linear template in which the termination signal TTTTTTN is placed downstream of the G-less cassette. Polymerase paused at the end of the G-less cassette can, when allowed to resume elongation by addition of GTP, terminate transcription in response to the inserted element. The ability to temporally separate initiation and termination in a single transcriptional cycle has facilitated study of the termination signal. Specifically, by selectively incorporating the analog BrUMP into proximal and distal portions of the nascent transcript, we localize the termination signal within or near the sequence UUUUUNU in the nascent RNA. Selective incorporation of other labeled or derivatized precursors into specific segments of the transcript may prove useful as well in cross-linking experiments to address dynamics of protein-RNA interactions.

Our finding that VTF/capping enzyme can promote termination by preformed ternary complex containing a 390-nt nascent transcript rules out an obligate requirement for assembly of a “termination competent” apparatus either prior to initiation or during early phases of elongation. The data do not, however, exclude the possibility that VTF/capping enzyme does actually interact with polymerase and/or nascent RNA at those phases when mRNA synthesis occurs in a physiologic setting (i.e. within the virion core particle). The issue of how the capping enzyme gains access to the elongating
transcription complex in the reconstituted system, e.g., via RNA-protein interactions, protein-protein interaction, or both, remains open. Clearly, this putative interaction step for VTF is more sensitive to salt and Sarkosyl than is the maintenance of an intact elongation complex. The inability to override the salt inhibition of termination by prior addition of VTF can be interpreted in several ways. It is possible that (i) VTF does not interact at all with the ternary complex prior to the appearance of the UUUUUNU sequence in the nascent RNA (indeed the signal has not yet been transcribed by the paused complexes used in our experiments), (ii) VTF can bind to ternary complex but this interaction is either unstable or in rapid equilibrium with free VTF and therefore readily dissociated by salt, (iii) stable interaction of VTF with elongation complex occurs only after synthesis of the UUUUUNU signal and would therefore not be revealed by order of addition experiments using pYL1 as a template. Further studies addressing the timing and mode of ingress of VTF into the transcriptional apparatus will require the physical isolation of the ternary complex paused upstream of the termination signal (devoid of NTPs and free proteins) and the construction of new templates that allow pausing of the elongation complex downstream of the UUUUUNU signal.

Finally, the observation that heterologously produced capping enzyme promotes termination by vaccinia polymerase circumvents the caveats inherent in earlier studies demonstrating association of VTF and capping enzyme by copurification (6). It also opens up a molecular genetic approach to VTF function via the heterologous expression of mutated alleles of the genes encoding the enzyme subunits.

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