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Bromouridine Triphosphate Inhibits Transcription Termination and mRNA Release by Vaccinia Virions*

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Termination of transcription *in vitro* by purified vaccinia virus RNA polymerase occurs downstream of a *cis*-acting signal UUUUUNU in the nascent RNA strand and requires a *trans*-acting termination factor, VTF, that is associated with the viral mRNA capping enzyme. Factor-dependent termination can be inhibited specifically by incorporation of BrUMP (from BrUTP) into nascent RNA in place of UMP. The relevance of VTF action to early vaccinia mRNA biogenesis was demonstrated in the present study of the effects of BrUTP on mRNA synthesis and release by permeabilized vaccinia virions. BrUMP incorporation inhibited the release of newly made transcripts from the virus particle, resulting in the accumulation of transcripts within virus cores. This effect was observed also with IUMP, but not with BrCMP or IMP incorporation. Transcripts synthesized in the presence of BrUTP were heterogeneous in size and severalfold larger than transcripts made in the presence of UTP. The progressive increase in the size of the core-associated, BrUMP-containing transcripts indicated that they were still engaged by elongating RNA polymerase. These results are consistent with a predominant pathway of mRNA 3′-end formation by virions that involves VTF-dependent transcription termination. These data do not support an alternative model of 3′-end formation by endonucleolytic cleavage of larger RNA precursors.

Insights into vaccinia virus early transcription have come from recent studies using soluble *in vitro* systems derived from extracts of virus cores (1–3). The soluble extract synthesizes accurately initiated and terminated RNAs when programmed by linear duplex DNA templates containing early viral genes and displays the requirement for ATP hydrolysis that is characteristic of the virion-associated transcriptase (3, 4). Fractionation of the soluble system has established that purified vaccinia RNA polymerase does not initiate transcription at early promoters but requires an accessory early transcription factor (5). Early transcription factor binds specifically to early promoter sequences and has an associated DNA-dependent ATPase (5, 7). Transcription termination requires a third factor (VTF),[1] purified by Shuman *et al.* (6), that is identical with the vaccinia mRNA capping enzyme.

Does the three-component reconstituted system accurately mimic the physiologic pathway of primary transcript formation, as inferred from studies of intact virus cores? This appears to be the case with respect to transcription initiation and elongation, as discussed by Broyles *et al.* (5, 7). The answer is less clear, however, with respect to 3′-end formation. In the reconstituted system, VTF induces RNA polymerase to terminate at heterogeneous sites downstream of a *cis*-acting sequence, TTTTTTNT, in the non-template DNA strand (6, 8). The conclusion that VTF induces termination rather than processing is based on the absence of endonuclease activity in VTF preparations (6). The early transcripts made under optimal conditions in the virion system tend to be of discrete size (the poly(A) tail notwithstanding), implying that the formation of a 3′-OH primer for polyadenylation is a specific process. Under nonoptimal conditions, however, notable exceptions arise. Certain reaction conditions or treatments (*e.g.* high virus concentration, limiting ATP concentration, ATP analog substitution, and thermal inactivation) lead to the synthesis of RNAs that are considerably longer than normal early transcripts (9–13). In most instances, these RNAs lack poly(A) and fail to be extruded from the virus particle. These findings have been interpreted variously as a failure to terminate transcription or a failure to process a larger RNA precursor. Studies of Paoletti (9, 10) support a precursor cleavage model by demonstrating that putative precursors can be chased into mRNA-sized molecules in a reaction requiring nucleoside triphosphates, but not requiring further RNA synthesis. Cleavage is accompanied by poly(A) addition and extrusion of cleaved products from virions. A soluble endoribonuclease that cleaves purified precursor RNA into mRNA-sized fragments has been extracted from virions (14), but this activity does not require NTPs (12, 14).

What then is the physiologic mechanism of 3′-end formation? If, as implied from the purified system, site-specific termination is the normal state of affairs, then termination is clearly not occurring under the conditions used to generate longer precursor molecules. Is the cleavage mechanism an alternative or backup pathway to generate 3′ ends? Do both pathways operate in parallel, with some early transcription units using termination and others using processing? Finally, can the retention of transcripts in virus cores under certain conditions shed light on the normal pathway of mRNA transport out of the particle?

The present report readdresses the mechanism of 3′-end formation and mRNA release by virus cores under various conditions, including those known to inhibit VTF-dependent termination in the purified transcription system *in vitro*. The results show that VTF-dependent termination is the predom-

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[1] The abbreviations used are: VTF, vaccinia termination factor; AdoMet, *S*-adenosyl-L-methionine; kb, kilobase(s); AdoHcy, *S*-adenosyl-L-homocysteine.

inant pathway of 3'-end formation in virion mRNA synthesis, that inhibition of termination prevents mRNA release, and that cleavage is unlikely to represent a major alternative pathway in vaccinia mRNA biogenesis.

EXPERIMENTAL PROCEDURES

Transcription by Permeabilized Virions—Standard reaction mixtures (0.2 ml) containing 60 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM dithiothreitol, 5 mM ATP, 1 mM CTP, 1 mM GTP, 0.2 mM [α-^{32}P]UTP, 10 μM AdoMet, 0.05% Nonidet P-40, and purified vaccinia virions (6.8 A_{260}/ml) were incubated at 37 °C. At various times, aliquots (40 μl) were removed, diluted with 0.16 ml of 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mM dithiothreitol, 0.05% Nonidet P-40, and virions were recovered by centrifugation for 3 min in an Eppendorf microcentrifuge. The supernatant was removed and assayed by filtration for incorporation of labeled nucleotide into trichloroacetic acid-insoluble material (released RNA). The pellets were resuspended in 50 mM Tris-HCl, pH 8.0, 0.1% sodium dodecyl sulfate, and assayed for acid-insoluble radioactive material (core RNA). In nucleotide substitution experiments, the unlabeled NTP analog (BrUTP, IUTP, BrCTP, or ITP) and its normal NTP counterpart were included at 0.2 mM concentration. In experiments involving uridine nucleotide substitution, RNA labeling was achieved by including 0.2 mM [α-^{32}P]CTP in the reaction mixtures in place of labeled UTP. Labeling of cap methyl groups in some experiments was achieved by inclusion of 0.6 μM [*methyl*-^{3}H]AdoMet in the reaction mixtures.

Assay of ATP Hydrolysis—Standard transcription reactions containing 5 mM [γ-^{32}P]ATP (4100 cpm/nmol) were incubated at 37 °C. At various times, aliquots (20 μl) were removed and assayed for release of $^{32}P_i$ (20).

Electrophoretic Analysis of RNA—Transcription reactions containing either UTP or BrUTP were carried out as described above, except that the acid precipitation step was omitted. Samples of released RNA were made 0.1% in sodium dodecyl sulfate, extracted sequentially with phenol:$CHCl_3$:isoamyl alcohol (50:48:2) and $CHCl_3$:isoamyl alcohol (24:1), and precipitated with ethanol. Samples of core RNA were digested with 20 μg of proteinase K for 2 h at 37 °C, then were extracted and precipitated as described for released RNA. RNAs were analyzed by electrophoresis through 1% agarose gels containing formaldehyde, essentially as described (23). Gels were dried under vacuum, and labeled RNAs were visualized by autoradiography. The size distribution of labeled species was determined by densitometric scanning of the autoradiograms. Lanes containing coelectrophoresed unlabeled RNA size standards were cut out, and RNA bands were located by staining with ethidium bromide.

Materials—Nucleotide analogs were synthesized chemically as described (18). [*methyl*-^{3}H]AdoMet was obtained from Du Pont-New England Nuclear. All other radiochemicals were purchased from Amersham. RNA standards were a product of Bethesda Research Laboratories.

RESULTS

RNA Synthesis and Release by Permeabilized Virions—Early studies by Kates and Beeson (15) showed, using a nitrocellulose filter binding assay, that transcripts synthesized *in vitro* by vaccinia cores accumulated transiently in the core particle before being actively extruded from the virus. Release of RNA from the core required high concentrations of ATP containing a hydrolyzable β-γ bond (15, 16). Subsequent studies of mRNA extrusion, including the present one, have used Nonidet P-40-permeabilized virions in lieu of cores, and have separated released RNA from core-associated RNA products by simple centrifugation. Kinetic analysis of RNA appearance in core-associated and released forms (Fig. 1) confirmed that the absolute level of transcription (as measured by UMP incorporation) and the extent of transcript release were both strongly dependent on ATP concentration. Under optimal conditions (5 mM ATP), core RNA appeared at early times and reached a steady state by 10 min. Released RNA appeared after a time lag and continued to accumulate for at least 30 min of incubation, at which time 83% of the RNA was released. Extrusion appeared more sensitive to

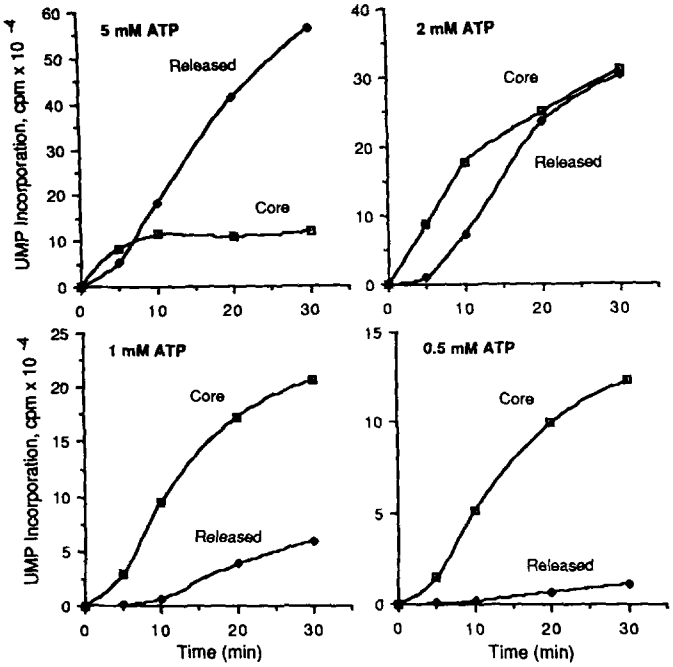

FIG. 1. **Kinetics of RNA synthesis and release by permeabilized vaccinia virions: effect of ATP.** Transcription reactions were performed as described under "Experimental Procedures." Radionucleotide incorporation into released and core-associated RNA is plotted as a function of time. The ATP concentration used in each experiment is indicated in the figure.

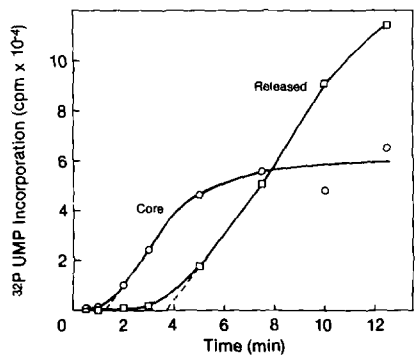

FIG. 2. **Early time course of RNA synthesis and release.** UMP incorporation into released and core-associated RNA is plotted as a function of time. The transcription reaction contained 5 mM ATP.

changes in ATP concentration than did RNA polymerization, *i.e.* total UMP incorporation was only decreased slightly at 2 mM ATP *versus* 5 mM ATP, while the distribution of RNA was shifted dramatically toward retention in cores. This retention was even more pronounced at lower ATP concentrations (Fig. 1). All further experiments were therefore performed at 5 mM ATP.

A finer analysis focusing on earlier time points is shown in Fig. 2. A brief time lag (1.4 min) preceded the onset of linear accumulation of RNA in the core, compared to a lag of 3.6 min for free RNA. The two RNA pools increase in parallel prior to reaching a steady state level of core RNA. Extrapolation of the slopes of the curves suggests that an interval of 2.2 min was required to synthesize, process, and release mRNAs. This estimate is consistent with the average size of early mRNAs (1200 nucleotides) and the estimated chain elongation rates for RNA polymerase (17 nucleotides/s (15)) and poly(A) polymerase (25 nucleotides/s (17)). That the core-

associated RNA is indeed destined for transport was supported by the pulse-chase experiment shown in Fig. 3. Transcripts were labeled in the cap methyl group during the first 10 min of synthesis (by which time a steady state level of methyl incorporation into core RNA had occurred, Fig. 3, *left panel*), followed by the addition of excess unlabeled AdoMet. In this way, RNA synthesis continued unabated during the chase period while avoiding changes in NTP concentrations or resorting to centrifugation of virions to remove labeled NTP. A time-dependent decline in core-associated RNA from the steady state level was observed, concomitant with an increase in released RNA (Fig. 3, *right panel*).

Inhibition of Factor-dependent Termination Prevents RNA Release—VTF-dependent termination in a reconstituted *in vitro* transcription system is abolished by substitution of BrUTP or IUTP for UTP in the transcription reaction (18). This effect requires incorporation of the base-substituted nucleotide into nascent RNA and is specific for uridine analogs. It has been proposed therefore that the *cis*-acting signal for factor-dependent termination is actually the sequence UUUUUNU in nascent RNA. Previous studies of the base substitution effect were carried out with linear DNAs containing defined early transcription units. It was of interest therefore to test the effect of these nucleotides on the more complex virion transcription reaction.

The kinetics of RNA synthesis and release were studied in reactions containing UTP or BrUTP. Substitution of BrUTP for UTP resulted in a marked retention of newly synthesized RNAs in the virus core (compare Fig. 4, *top left* and *top right panels*). This could not be attributed to a global inhibition of RNA synthesis, since the initial rates of CMP incorporation were similar in UTP- and BrUTP-containing reactions. Indeed, about twice as much RNA accumulates at steady state in the cores of BrUTP reactions compared to the steady state levels of control samples. The analog effect of RNA transport was specific for uridine base substitution, since substitution of BrCTP for CTP in virion transcription reactions had no effect on the distribution of transcripts (compare Fig. 4, *lower left* and *right panels*). Additional experiments revealed that substitution of IUTP for UTP affected RNA release in the same was as BrUTP substitution, while substitution of ITP for GTP had no effect on RNA release (data not shown).

In light of the strict dependence of RNA release on ATP concentration (Fig. 1), the possibility was considered that BrUTP might somehow accelerate the turnover of ATP (*e.g.*

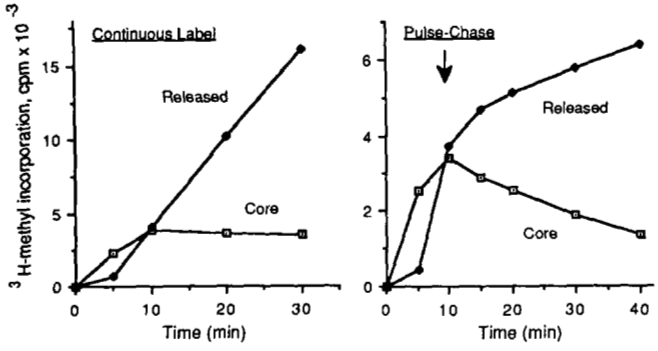

FIG. 3. **Pulse-chase analysis of RNA release.** *Left panel*, continuous incorporation of label from [*methyl*-^{3}H]AdoMet into core-associated and released RNA is plotted as a function of time. *Right panel*, after 10 min of pulse-labeling in the presence of 0.6 μM [*methyl*-^{3}H]AdoMet, the transcription reaction mixture was adjusted by addition of unlabeled AdoMet to 0.2 mM final concentration. Radiolabeling of core-associated and released RNA is plotted as a function of time. The initiation of the chase period is indicated by the *vertical arrow*.

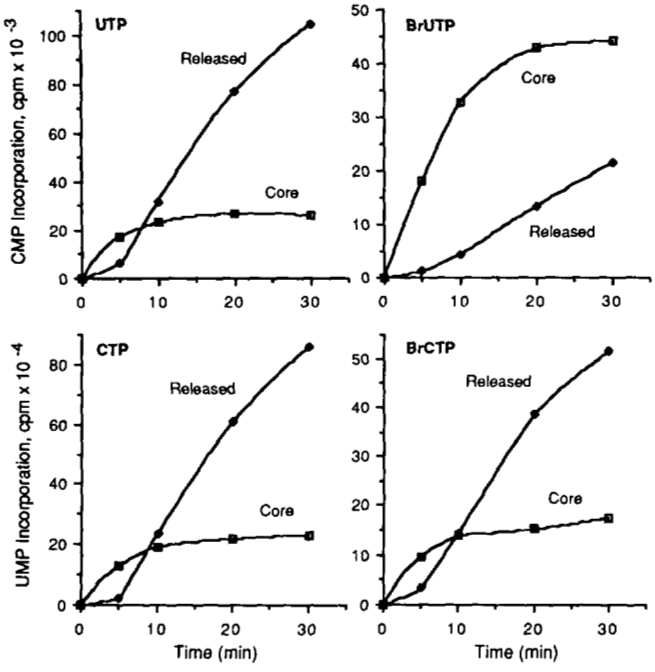

FIG. 4. **BrUTP substitution inhibits mRNA release.** Nucleotide incorporation into released and core-associated RNA is plotted as a function of time for control reactions (*top left* and *bottom left* panels) and for reactions containing BrUTP in place of UTP (*top right*) and BrCTP in lieu of CTP (*bottom right*).

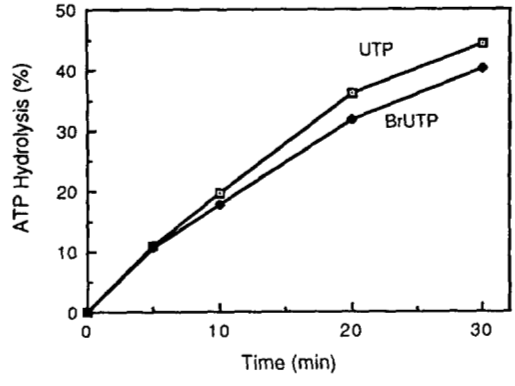

FIG. 5. **ATP hydrolysis by permeabilized virions is unaffected by BrUTP.** Release of ^{32}Pi from [γ-^{32}P]ATP (expressed as percent of input ATP hydrolyzed) is plotted as a function of time for transcription reactions containing UTP or BrUTP.

due to an effect on the virion-associated nucleic acid-dependent ATPases) and thereby indirectly influence RNA release. Accordingly, the rate of ATP hydrolysis by permeabilized virions was measured in the presence of UTP and BrUTP and was found to be essentially the same in both cases (Fig. 5).

Radiolabeled core-associated and released RNAs synthesized in the presence of UTP (control) or BrUTP were analyzed by electrophoresis in agarose gels containing formaldehyde (Fig. 6). In control reactions, RNAs of discrete sizes were apparent after 5 min of incubation, again reaching a steady state level by 10 min, while maintaining the same size distribution throughout the reaction. Released RNAs of discrete size and identical distribution to core RNA appeared after a time lag and accumulated with time. Densitometric scanning of the autoradiographic data (Fig. 7) revealed a peak at 1.2 kb, a results consistent with prior estimates of early mRNA size. Thus, under optimal conditions, there appeared

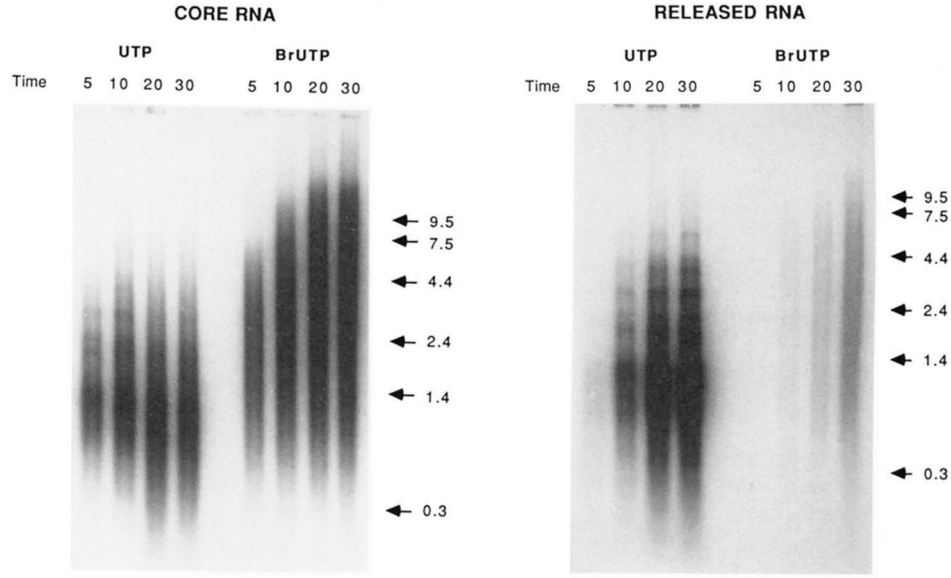

FIG. 6. **Effect of BrUTP substitution on RNA size.** Core-associated (*left panel*) and released (*right panel*) RNAs synthesized in transcription reactions containing UTP or BrUTP were isolated and analyzed by agarose gel electrophoresis as described under "Experimental Procedures." Autoradiograms of the gels are shown in the figure. The times at which samples were taken are indicated *above the lanes.* The positions and sizes (in kilobases) of marker RNAs are indicated by the *arrows.*

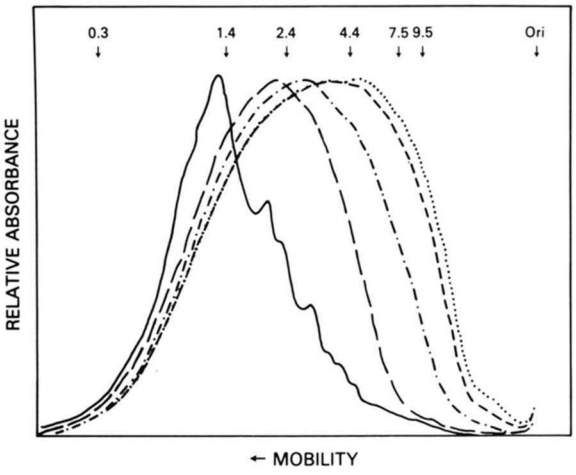

FIG. 7. **Size distribution of virion-associated transcripts.** The figure shows normalized densitometry tracings of autoradiograms depicted in Fig. 6 of radiolabeled core RNAs present in control (UTP-containing) reactions at 10 min (——), and in BrUTP-containing reactions at 5 (— —)-, 10 (—·—)-, 20 (– – – –)-, and 30-min (· · · · ·) time points. The positions and sizes (in kilobases) of marker RNAs and of the electrophoretic origin are indicated by the *vertical arrows.*

to be no evidence for high molecular weight virion-associated precursors to mature released transcripts.

The effects of BrUTP substitution were dramatic. No RNAs of discrete size were detected in cores; instead, transcripts were broadly heterogeneous in size and, even at 5 min, obviously longer than control RNAs (Fig. 6). Significantly, the average sizes of the labeled core transcripts increased throughout the reaction, suggesting that these were nascent RNAs still engaged by elongating RNA polymerase. Densitometry showed peak RNA sizes of 2.1, 3.0, 4.0, and 5.0 kb at 5, 10, 20, and 30 min, respectively. More telling is the presence of significant amounts of RNA greater than 10 kb in length in BrUTP-containing reactions; such large RNAs are not detected in control reactions. The released RNA from BrUTP reactions, although not abundant, reflected the size distribution of core RNA. The absence in the released fraction of discrete transcripts such as those seen in control reactions implies that an endonucleolytic processing mechanism, if it exists, was not operative under the reaction conditions used in this experiment. The nature of the BrUTP effect suggests that VTF-dependent termination of transcription is the predominant mode of 3′-end formation in early transcription units and that this step is required for RNA release from the virion.

Inhibition of Cap Formation Does Not Affect RNA Release—Studies using the reconstituted transcription system have shown that VTF/capping enzyme is required for termination, independent of its ability to modify the 5′-end of nascent transcripts. To test whether the same properties applied to RNA release, we analyzed the kinetics of release in reactions containing 10 μM AdoHcy in place of AdoMet; these conditions prevent cap methylation, and RNA 5′-ends contain either diphosphate termini or unmethylated capped ends (19). AdoHcy had no effect on either the level of RNA synthesis or the kinetics of release (data not shown).

DISCUSSION

Studies of the accumulation of high molecular weight RNA in vaccinia cores under certain transcription conditions *in vitro* have not distinguished clearly whether these transcripts arise due to failure to terminate transcription, to a failure of endonucleolytic processing, or both. Conditions employed to generate such large transcripts have often involved altering the concentration of ATP or the use of adenosine nucleotide analogs (9–12). Since ATP hydrolysis is clearly required at multiple stages in the formation of early mRNAs (11, 12, 16, 20), it seemed appropriate to readdress the problem of 3′-end formation and RNA release by virions using conditions that did not perturb the adenosine nucleotide pool. BrUMP incorporation specifically prevents VTF-dependent transcription termination by purified vaccinia RNA polymerase in a fractionated transcription system *in vitro*, without a significant effect on transcriptional initiation or elongation (18). Therefore, this nucleotide analog was used in the present study to assess the consequences of inhibition of VTF action during virion RNA synthesis. It was shown that, under conditions of efficient transcription and RNA extrusion, BrUTP specifically blocked RNA release from cores, leading to the accumulation of virion-associated transcripts. This effect on release, like the BrUTP effect on VTF action, was specific for halogenated uridine nucleotides. As might be anticipated with selective inhibition of termination, the RNAs made in the presence of BrUTP were many times longer than normal mRNAs and remained core-associated (presumably because

the transcripts remained in an elongation complex with RNA polymerase). The minority of RNAs that were released in the presence of BrUTP were the same size as core RNAs. Conceivably, some random termination by RNA polymerase occurs during extensive elongation, thereby accounting for transcripts that were released in the presence of BrUTP. Unless one invokes an RNA endonuclease that is specifically unable to cleave BrUMP (but not BrCMP)-containing RNA, the present data suggest that most or all early transcription units terminated via a VTF-dependent mechanism and that no specific alternative pathway exists (at least in permeabilized virions) for release of RNA in the absence of transcription termination.

The proposal that 3′-end formation in virion transcription is mediated by VTF/capping enzyme is sustained by the earlier work of Harper *et al.* (13) showing that heat treatment of virions resulted in synthesis of high molecular weight RNA as well as a defect in RNA transport (13). Arguing against a precursor cleavage model and in favor of selective thermal inactivation of transcription termination, the authors provided data correlating the transcription defect with selective heat inactivation (90%) of the virion-associated RNA methyltransferase. This is noteworthy since it is now appreciated that RNA methyltransferase is but one enzymatic component of the multifunctional VTF/capping enzyme protein, and that RNA methyltransferase and termination factor activities have identical thermal inactivation profiles (6).

If termination is a prerequisite for RNA transport, then does VTF have any role in RNA extrusion beyond generating a 3′-end? Preliminary results suggest not, insofar as abnormal transcription conditions (*i.e.* single nucleotide depletion) shown to favor premature termination and production of short, capped, and polyadenylated RNAs (21, 22) do not appear to prevent release of such RNAs from virus particles.[2]

The consequences of failure to terminate *in vitro* are a lower overall extent of mRNA production (due perhaps to prevention of reinitiation by RNA polymerase) and retention of RNA in a core-associated state. *In vivo*, RNA associated with cores may not be as accessible to ribosomes as released RNA, thereby providing some rationale for the existence of a mechanism to ensure site-specific termination of early transcription. Alternatively, termination may serve to avoid promoter occlusion and/or undue topological strain; these are problems that might well arise during transcription of the approximately 100 divergently and convergently oriented early genes with the vaccinia virus genome. Ultimately, an understanding of the physiologic role of transcription termination (and of mRNA transport) in the vaccinia virus growth cycle will hinge on the isolation of mutant viruses defective in these transcriptional events.

[2] S. Shuman and B. Moss, unpublished data.

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