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A Freeze-Frame View of Eukaryotic Transcription During Elongation and Capping of Nascent mRNA

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Ribonuclease footprinting of nascent messenger RNA within ternary complexes of vaccinia RNA polymerase revealed an RNA binding site that encompasses an 18-nucleotide RNA segment. The dimensions of the binding site did not change as the polymerase moved along the template. Capping of the 5′ end of the RNA was cotranscriptional and was confined to nascent chains 31 nucleotides or greater in length. Purified capping enzyme formed a binary complex with RNA polymerase in solution in the absence of nucleic acid. These findings suggest a mechanism for cotranscriptional establishment of messenger RNA identity in eukaryotes.

THE PRODUCTION OF MRNA IN EUkaryotic cells is regulated at multiple steps (*1*). A key regulatory target during transcription elongation and termination is the nascent mRNA chain, whose structure and sequence can be "sensed" by the ternary complex at distal sites on the template. Transduction of nascent RNA signals to the elongating polymerase may require the participation of accessory proteins that interact with the RNA, the polymerase, or both (*2*). Nascent chains are also the substrates for processing enzymes that cap, splice, cleave, and polyadenylate the mRNA precursor. How these proteins identify pre-mRNAs among other classes of transcripts is unknown. The mRNA identity may be established by recognition of the RNA polymerase II elongation apparatus or may be conferred upon the nascent RNA, perhaps through an RNA polymerase II–specific modification.

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The question of how nascent mRNA interacts with the elongating transcription apparatus and with the RNA modifying enzymes can be most effectively approached in an in vitro system that mimics the process in vivo and is sufficiently pure to permit manipulation of the individual components. A model system that meets these criteria is provided by vaccinia virus. Vaccinia, which replicates in the cytoplasm of mammalian cells, encapsidates within the virion all the enzymes required for the synthesis of early mRNAs, including a virus-encoded multisubunit RNA polymerase with structural and functional similarity to cellular RNA polymerase II (*3*). Faithful synthesis of early mRNAs can be recapitulated in vitro on exogenous DNA templates with enzymes purified from virus particles (*4, 5*). Initiation and elongation of RNA chains require the vaccinia RNA polymerase and a vaccinia early transcription factor (VETF) that binds to the promoter. VETF is a heterodimer of 80-kD and 70-kD subunits, has intrinsic DNA-dependent adenosine triphosphatase (ATPase) activity (*5*), and is the functional equivalent of the several RNA polymerase II general transcription factors (*1*). Termination of early transcription requires a cis-acting sequence UUUUUNU in the nascent RNA strand (*6*) and a vaccinia-encoded termination factor (VTF) that is identical to the vaccinia mRNA capping enzyme (*4, 7*). Capping enzyme, a heterodimer of 95-kD and 31-kD subunits, is a multifunctional protein that catalyzes three separate reactions leading to the synthesis of a ^{m7}GpppN RNA terminus (*8*).

The finding that capping enzyme is involved both proximally (in capping) and distally (in 3′ end formation) raises questions about the timing of its interaction with the ternary complex. We addressed this issue by examining the structure of nascent RNA contained within ternary transcription complexes assembled in vitro. We took advantage of a series of DNA templates that allowed us to pause the elongating polymerase at discrete positions downstream of the major transcription initiation site (Table 1). These templates are named according to the position of the first G residue in the transcript (Gn). Pausing could be restricted to a single template position (Gn) by inclusion of 3′-*O*-methyl guanosine triphosphate (GTP) in the reactions (Fig. 1, pulse lanes P). The G18, G21, and G27 templates yielded a single major [^{32}P]cytidine phosphate (CMP)–labeled transcript *n* bases long; minor species of apparent length *n*+2 and *n*+3 are 3′ coterminal with the major RNA but arose via initiation at the −2U and −3U positions of the template (*9*). The major doublet RNAs transcribed from G31, G34, and G51 templates are 3′ coterminal but differed in the state of 5′ terminal modification.

The integrity of the ternary complexes containing pulse-labeled, 3′-*O*-methyl guanosine phosphate (3′OMeGMP)–paused transcripts was confirmed by the ability of these RNAs to be elongated during a "chase" in the presence of GTP (*10*). Addition of excess GTP to the 3′OMeGMP-paused ternary complexes allowed nearly quantitative elongation to the end of each linear template (Fig. 1, chase lanes C). Reversal of the elongation block was a result of pyrophosphorolytic removal of 3′OMeGMP and incorporation of GTP during the chase (*9, 11*). Some minor short species that were not elongated represented abortive transcripts. The analysis of active ternary complexes as they move away from the promoter provides a "freeze-frame" view of transcription elongation.

To study the interaction of nascent RNA with the transcriptional apparatus, we treated discretely paused ternary complexes containing [^{32}P]CMP-transcripts with increas-

ing concentrations of ribonuclease A (RNase A) (Fig. 2). At higher RNase levels (10 μg/ml), the G27, G31, G34, and G51 transcripts yielded a predominant protected species 18 nucleotides (nt) in length. The protected species derived from the G21 nascent RNA was 19 nt long. Unlike the longer chains, the G18 nascent transcript was almost fully protected from RNase digestion. Thus, an 18- or 19-nt segment of the nascent RNA within the ternary complex was protected from nuclease digestion regardless of the length of the transcript. The longer digestion products generated by lower RNase concentrations (1 μg/ml) were in the range of 24 to 27 nt (Fig. 2), indicating that an additional 6 to 9 nt were partially protected by the transcription complex, again independent of overall chain length.

Resistance to RNase can, in principle, be attributable either to exclusion of nuclease by bound protein or to formation of a stable RNA:DNA hybrid. To clarify the basis for nuclease protection, we analyzed the RNase sensitivity of G34 ternary complexes that had been stripped of polymerase by addition of SDS (Fig. 3B). Detergent treatment should dissociate the polymerase from the ternary complex but not denature a stable nucleic acid secondary structure. Although RNase activity was reduced in the presence of SDS, no protected 18-bp species was detected at 10 μg of RNase per milliliter. We conclude that the footprint of 18 or more bases results from the interaction of nascent RNA with the protein components of the elongation complex and not from the existence of RNA:DNA hybrid structures of equivalent length.

We next determined the location of the 18-nt protected segment within the G34 nascent transcript. The region of protection included the 3′ growing point of the transcript, as determined by the ability to elongate the partial RNase digestion products to the end of the linear template (Fig. 3C, lane 3). No elongation was observed if GTP was omitted from the chase (Fig. 3C, lane 2). When the chase was performed with GTP but without RNase inhibitor, the labeled RNA was degraded (Fig. 3C, lane 4), sug-

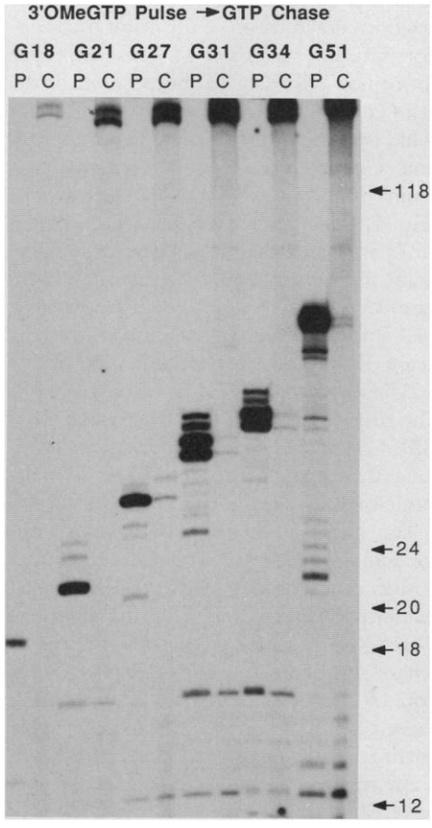

Fig. 1. Ternary transcription complexes paused stably at discrete sites on template DNA. Standard transcription reactions (*19*) containing the indicated G-less template DNA were incubated for 10 min and either processed directly (pulse reactions, lanes P) or else "chased" by adjustment to 1 mM ATP, 1 mM CTP, 1 mM UTP, and 1 mM GTP followed by an additional 10 min incubation (lanes C). CMP-labeled transcription products were resolved by denaturing gel electrophoresis. An autoradiogram of the gel is shown. The positions and chain lengths of coelectrophoresed-labeled DNA size markers are indicated by arrows at the right.

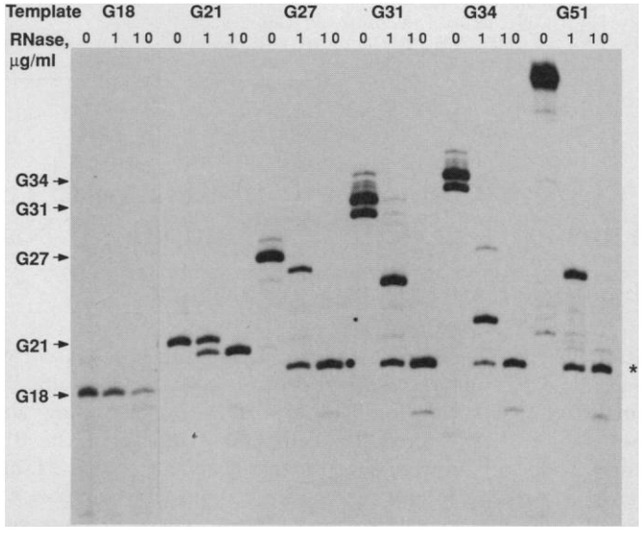

Fig. 2. Ribonuclease footprinting of nascent RNA in paused ternary transcription complexes. Standard transcription reactions containing the indicated G-less template DNAs were incubated for 10 min and then treated for 2 min at 30°C with RNase A at the concentrations (in micrograms per milliliter) indicated above each lane. CMP-labeled RNA was isolated by phenol extraction and ethanol precipitation. The precipitation step permitted efficient recovery of RNA chains 7 nt or greater in length (*9*). An autoradiogram of the gel is shown. Primary transcripts are identified at the left by arrows. The 18-nt protected species common to several nascent RNAs is indicated by the asterisk. The 18-nt protected species migrated more slowly than the G18 primary transcript because of the difference in their 5′ termini. The RNase cleavage product is predicted to contain a 5′ OH-terminus, whereas the G18 primary transcript is expected to contain a 5′ triphosphate terminus.

Table 1. The nucleotide sequences of the paused nascent transcripts encoded by the G-less series of DNA templates (Gn) for vaccinia early transcription are shown. The predominant initiation site is indicated by +1A. Minor upstream initiation sites occur at the −2U and −3U positions (*9*). The internucleotide bonds susceptible to RNase in the context of the ternary complex were assigned on the basis of the electrophoretic mobilities of the RNase digestion products and are denoted by double underlines (major RNase resistant fragments) or single underlines (partial digestion products at intermediate RNase concentrations).

Stably paused transcripts in ternary elongation complexes
+1 CUUUCAUACUUCUAUCACUAUAG (G18)
+1 CUUUCAUAACCCACUUCUAUCACUAG (G21)
+1 CUUUCAUAACUUCUAUCACUACUAUCACUCAG (G27)
+1 CUUUCAUAACCCACUUCUAUCACUACUAUCACUCAG (G31)
+1 CUUUCAUAACCCACUUCUAUCAUCUAACUUCUAUCACUAG (G34)
+1 CUUUCAUAAACUUCUAUCACUACUAUCACUCAACUUCUAUCACUACUAUCACUCAG (G51)

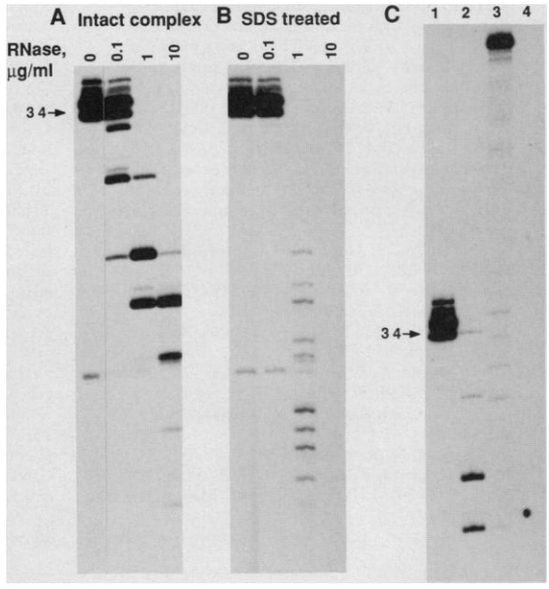

Fig. 3. Protection of nascent RNA from RNase requires an intact transcription complex. (**A**) Standard transcription reactions containing G34 template DNA were incubated for 10 min and then treated for 2 min with RNase A at the concentrations (in micrograms per milliliter) indicated above each lane. (**B**) Transcription reactions were adjusted to 0.1% SDS before RNase digestion. (**C**) Standard transcription reactions containing the G34 template were constituted as follows: lane 1, control reaction pulse-labeled with CTP for 14 min; lane 2, pulse-labeled for 10 min, digest with 1 μg of RNase per milliliter for 2 min, add RNase inhibitor (RNasin, 10 units) and 1 mM CTP, 1 mM ATP, 1 mM UTP, and then incubated for an additional 2 min; lane 3, constituted as in lane 2, except that 1 mM GTP was included during the chase; lane 4, constituted as in lane 3, except that RNase inhibitor was omitted.

gesting that the nuclease accesses the transcript only after it is extruded from the transcription complex during synthesis.

The freeze-frame analysis of elongation was used to determine when the 5′ cap is added to the nascent mRNA. Vaccinia capping enzyme cotranscriptionally incorporates [α^{32}P]dGTP into the cap structure in the presence of *S*-adenosyl methionine (AdoMet) to yield a stable ^{m7}dGpppN terminus, but dGTP is not appreciably incorporated into RNA by vaccinia RNA polymerase (*7*). We measured incorporation of dGTP into variously sized nascent RNAs (Fig. 4). In this experiment CMP-labeling reactions were performed in parallel to normalize for the extent of transcription. Capping was restricted to the G34 and G31 RNAs (Fig. 4). We conclude that vaccinia capping enzyme cannot access the 5′ triphosphate terminus of the growing transcript until it is greater than 27 nucleotides in length but can readily modify the 5′ terminus upon further extension. This is not attributable to an inherent RNA size preference on the part of the capping enzyme per se (*9*). That the dGMP labeling (Fig. 4) was cap-specific was confirmed directly by thin-layer chromatographic analysis of the labeled RNA product of the G34 template after digestion with nuclease P1, which revealed incorporation into a phosphatase-resistant species identical to ^{m7}dGpppA (*9*). The [^{32}P]dGMP transcripts programmed by the G34 template were elongated to the end of the template upon chase with unlabeled GTP, confirming that the transcription complexes were active (*9*).

It has been suggested that capping enzyme might interact with RNA polymerase at or shortly after the time of transcription initiation and remain associated with the transcription complex during elongation (*7*). Such an interaction might be advantageous in two respects. First, capping enzyme would be poised to cap the 5′ end as soon as it is extruded from the RNA polymerase and, subsequently, to recognize the UUUUUNU termination signal. We examined the ability of capping enzyme to form a complex with RNA polymerase in solution. A transcriptionally active holoenzyme RNA polymerase preparation (*12*) was mixed with purified radiolabeled capping enzyme and the mixture was subjected to sedimentation analysis. Capping enzyme can be labeled specifically at its active site by formation of the covalent enzyme–[^{32}P]GMP intermediate and recovery of native enzyme by gel filtration (*13*). Glycerol gradient sedimentation of capping enzyme alone revealed the expected single peak of radioactivity sedimenting at 6.5 *S* (Fig. 5). On incubation with RNA polymerase and subsequent centrifugation, the labeled capping enzyme sedimented more rapidly and overlapped with the leading portion of the RNA polymerase activity profile (Fig. 5). The shift in capping enzyme sedimentation was not observed when vaccinia RNA polymerase was pretreated by heating at 55°C (*9*). No comparable shift could be detected when capping enzyme was incubated with purified *Esche-*

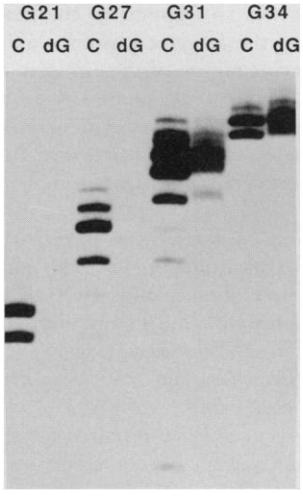

Fig. 4. Timing of mRNA capping during early elongation. To achieve dGMP labeling of the 5′ RNA cap, we modified standard transcription mixtures containing Gn template to include 50 μM AdoMet, 0.4 μM [α^{32}P]dGTP (3000 Ci/mmol), 1 μM unlabeled CTP, 1 mM UTP, and 100 fmol of capping enzyme. Incubation was for 10 min at 30°C. 3′OMeGTP was omitted from the reactions during the labeling phase because it acted as a cap donor for the RNA guanylyltransferase component of the capping enzyme (*9*). CMP labeling reactions contained unlabeled dGTP and 1 μM [α^{32}P]CTP (1000 Ci/mmol). Reactions were chased for 10 min in the presence of 0.1 mM 3′OMeGTP and then processed for analysis. Use of either CTP (C) or dGTP (dG) as labeled precursor is noted above each lane. G-less template DNAs were included as indicated.

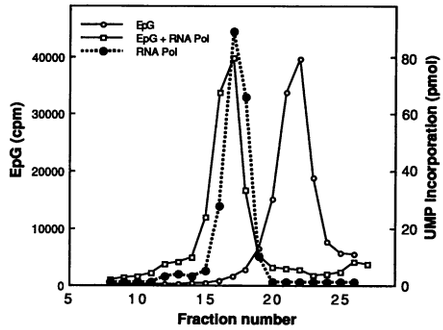

Fig. 5. RNA polymerase and capping enzyme form a binary complex in solution. Transcriptionally active vaccinia RNA polymerase depleted of capping enzyme was isolated by glycerol gradient sedimentation as described (*4*). Capping enzyme-[^{32}P]GMP complex (EpG) was prepared and isolated free of unbound radionucleotide as described (*13*). RNA polymerase (2.8 units) was mixed with EpG in 0.2 ml of buffer containing 10 mM tris-HCl, pH 8.0, 30 mM NaCl, 0.5 mM dithiothreitol (DTT), 0.2 mM EDTA, 0.002% NP40, 5% glycerol and incubated initially for 15 min at 4°C, then for 2 min at 30°C. A control reaction contained EpG without RNA polymerase. The samples were each applied to 4.8 ml 10- to 30% glycerol gradient in buffer A (*4*) containing 20 mM NaCl. Centrifugation was at 55,000 rpm for 3.5 hours at 4°C in a Beckman SW55 rotor. Fractions (0.2 ml) were collected from the bottom and each fraction was counted for Cerenkov radioactivity (counts per minute, solid lines). An aliquot (15 μl) of each fraction was assayed for RNA polymerase activity [as described (*20*)] with M13 ssDNA as template and [α^{32}P]UTP as labeled precursor.

richia coli RNA polymerase (*9*).

Cotranscriptional events such as 5′ ^{m7}G capping, 3′ cleavage–polyadenylation, and pre-mRNA splicing are specific for RNAs synthesized by RNA polymerase II (*14*). In the case of capping, the presence of a 5′ triphosphate or diphosphate RNA terminus is all that is needed in vitro to permit cap formation by cellular RNA guanylyltransferase (*15*). Such termini are not restricted to pre-mRNAs, yet only polymerase II transcripts are capped with the standard ^{m7}GpppN structure. To account for this specificity, it is conceivable that cellular capping enzyme interacts specifically with RNA polymerase II or some other component of the mRNA transcription apparatus. The results in the vaccinia system provide evidence for such a binary interaction.

The timing of the various nuclear cotranscriptional mRNA processing events is not clear. It has been suggested that RNA polymerase II products synthesized in vitro in nuclear extracts are uncapped when shorter than 20 nt, but they are capped when they are 79 nt long (*16*). The refinement of the timing of capping in the vaccinia system and the interpretation that the failure to cap shorter transcripts is caused by sequestration within the RNA polymerase may be applicable to cellular transcription, as these observations are consistent with recent studies of the RNA polymerase II–elongation complex. RNase footprinting of calf thymus RNA polymerase II ternary complex paused at a unique site on a synthetic DNA template suggests that the polymerase has an RNA binding domain sufficient to protect 24 nt extending from the 3′ terminus of the paused transcript (*12*).

We conclude that acquisition of the cap structure is the earliest modification event in mRNA biogenesis. There is evidence that the presence of the cap may target the nascent transcript for splicing and polyadenylation and even for mRNA transport (*14, 18*), in which case the timely interaction of capping enzyme with the transcript would be crucial (if not actually sufficient) to establish mRNA identity.

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19. Standard transcription reaction mixtures (20 μl) contained 20 mM tris-HCl, pH 8.0, 6 mM $MgCl_2$, 0.1 mM adenosine 5′-triphosphate (ATP), 0.1 mM uridine 5′-triphosphate (UTP), 1 μM [α^{32}P]CTP (1000 Ci/mmol), 0.1 mM 3′OMeGTP, 1.8 ng of DNA template (gel-purified Pvu II restriction fragment containing a vaccinia early promoter fused to a G-less cassette), vaccinia RNA polymerase [0.13 unit; phosphocellulose fraction purified from vaccinia cores as described by S. Shuman, M. I. Surks, H. M. Furneaux, J. Hurwitz, *J. Biol. Chem.* **255**, 11588 (1980)] and vaccinia capping enzyme (10 fmol). After incubation at 30°C, reactions were halted by addition of SDS and urea. Labeled RNA products were recovered by phenol extraction and ethanol precipitation [as described (*4*)] and analyzed by electrophoresis through a 17% polyacrylamide gel containing 7 M urea in TBE (90 mM tris, 90 mM borate, 2.5 mM EDTA). Reaction products were visualized by autoradiographic exposure of the gel.
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Spatial Control of the Gap Gene *knirps* in the *Drosophila* Embryo by Posterior Morphogen System

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The gap genes of *Drosophila* are the first zygotic genes to respond to the maternal positional signals and establish the body pattern along the anterior-posterior axis. The gap gene *knirps*, required for patterning in the posterior region of the embryo, can be activated throughout the wild-type embryo and is normally repressed from the anterior and posterior sides. These results provide direct molecular evidence that the posterior morphogen system interacts in a fundamentally different manner than do *hunchback* and *bicoid*, which are responsible for anterior pattern formation.

IN *DROSOPHILA MELANOGASTER* DEVELOPMENT, determination of polarity along the longitudinal axis is carried out by the maternal genes (*1*). The anterior determinant is *bicoid* (*bcd*), whose gene product is localized at the anterior pole of the egg (*2*). The posterior determinant is *nanos* (*nos*), and its gene product is localized at the posterior pole (*3*). The polarized distribution of these components ultimately leads to the regional expression of the zygotic gap genes (*4*). Genetic studies indicate that the anterior gap gene *hunchback* (*hb*) and the posterior gap gene *knirps* (*kni*) are key zygotic targets of *bcd* and *nos*, respectively (*4, 5*). Despite these formal similarities, the mode of action of the two maternal systems is fundamentally different. In the anterior, *bcd* directly activates and sets the expression border of *hb* (*6*). In the posterior, by contrast, *nos* does not seem to interact directly with *kni* but functions by eliminating a factor whose absence apparently allows *kni* to be expressed (*7*). This unexpected result then raised the question of how *kni* becomes initially expressed and how its spatial borders are established.

The gap gene *kni* is required for abdominal segmentation of the embryo (*5, 8*). Consistent with this function, *kni* is expressed in a posterior region of the blastoderm embryo that will give rise to the abdomen (*9*). A second expression domain is found in the anterior tip, but the function of this domain is not known and its regulation will not be discussed in this paper. We have shown that a 4.4-kb upstream sequence of *kni* directs the expression of a reporter gene in a region corresponding to the endogenous *kni* transcripts (*10*). To investigate how *kni* was spatially regulated, we made various deletion constructs of this fragment (Fig. 1), transformed flies, and assayed for reporter gene expression in early embryos (Fig. 2). The results show one

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