

A Kinetic Model for the Early Steps of RNA Synthesis by Human RNA Polymerase II*

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Eukaryotic mRNA synthesis is a highly regulated process involving numerous proteins acting in concert with RNA polymerase II to set levels of transcription from individual promoters. The transcription reaction consists of multiple steps beginning with preinitiation complex formation and ending in the production of a full-length primary transcript. We used pre-steady-state approaches to study the steps of human mRNA transcription at the adenovirus major late promoter in a minimal *in vitro* transcription system. These kinetic studies revealed an early transition in RNA polymerase II transcription, termed escape commitment, that occurs after initiation and prior to promoter escape. Escape commitment is rapid and is characterized by sensitivity to competitor DNA. Upon completion of escape commitment, ternary complexes are resistant to challenge by competitor DNA and slowly proceed forward through promoter escape. Escape commitment is stimulated by transcription factors TFIIE and TFIIH. We measured forward and reverse rate constants for discrete steps in transcription and present a kinetic model for the mechanism of RNA polymerase II transcription that describes five distinct steps (preinitiation complex formation, initiation, escape commitment, promoter escape, and transcript elongation) and clearly shows promoter escape is rate-limiting in this system.

Eukaryotic transcription is a multistep process subject to regulation by promoter-specific transcriptional activators and repressors. The general RNA polymerase II transcription machinery consists of greater than 30 protein subunits (1). The RNA polymerase II core enzyme can synthesize RNA from a DNA template, but requires additional general transcription factors for promoter-specific initiation of transcription. The general transcription factors can be classified into two subgroups: the basal transcription factors (TFIIA,¹ TFIIB, TFIID,

TFIIE, TFIIF, and TFIIH) that are thought to function in transcription at all promoters, and the cofactors that mediate transcriptional activation and appear to be more promoter- and/or regulatory protein-specific (as reviewed in Refs. 2–4). In current models, the transcription reaction consists of multiple steps, including preinitiation complex formation, open complex formation, promoter escape, promoter clearance, transcript elongation, termination, and reinitiation, all of which have the potential to be regulated by promoter-specific transcriptional activators and repressors (1). Biochemical studies have established that the first step in basal (or unregulated) transcription is the binding of TFIID to core promoter sequences. After TFIID binding, RNA polymerase II and the other general transcription factors assemble on the promoter DNA to form stable preinitiation complexes that become open complexes upon melting of the DNA in the region around the transcription start site (5–9).

When open complexes are provided with nucleoside triphosphates, transcription is initiated and RNA polymerase II begins a transformation that results in the dissolution of open complexes and the formation of elongation complexes. This period of the transcription reaction minimally includes the steps of initiation and promoter escape. Initiation begins with synthesis of the first phosphodiester bond, and promoter escape is complete by synthesis of a 15-nt RNA at the adenovirus major late promoter (AdMLP) (10). During these steps numerous protein-protein and protein-DNA contacts established in the open complex must be broken so that RNA polymerase II can begin to move forward. In addition, new protein-protein and protein-DNA contacts must be established to form an elongation complex. It is thought that during early transcription, TFIID remains bound to the promoter while TFIIB, TFIIE, and TFIIH release from initiation complexes as they proceed forward (11). TFIIF remains associated with RNA polymerase II in the ternary elongation complex (11). Promoter clearance, a step distinct from promoter escape, is complete when RNA polymerase II has moved far enough to allow another round of initiation at the promoter. RNA transcripts are then elongated by RNA polymerase II in a process that can be interrupted by pausing and influenced by multiple accessory factors (12). Finally, mRNA synthesis terminates and RNA polymerase II releases from the template and is free to participate in another round of preinitiation complex formation.

Studies of RNA polymerase II transcription in reconstituted transcription systems have revealed transitions, which occur during early transcription, that are facilitated by general transcription factors, including TFIIH (as reviewed in Ref. 13). TFIIH contains (d)ATP-dependent helicase activity that has been implicated in promoter melting and promoter escape, as

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¹ The abbreviations used are: TFII-, transcription factor of RNA polymerase II; AdMLP, adenovirus major late promoter; (d)ATP, adenosine triphosphate or deoxyadenosine triphosphate; nt, nucleotides; TBP, TATA binding protein; mt, mutant; ctDNA, calf thymus DNA; RP_{EC}, escape-committed complex; R_E, elongation complex; wt, wild type; PCF, preinitiation complex formation; R, general transcription

factors in the minimal system and RNA polymerase II; P, promoter DNA; PIC, preinitiation complex; RP_I, initiated complex; RP_A, aborted complex.

well as the suppression of promoter proximal pausing under conditions of low nucleotide concentrations (9, 14–25). Two transitions were identified using permanganate footprinting to characterize the melted regions of early transcription complexes on the AdMLP on linear DNA templates in a highly purified transcription system (19). The first transition, which required TFIIE, TFIIH, and (d)ATP, resulted in an extension of the downstream end of the transcription bubble and was complete when a 4-nt RNA was made. The second transition occurred at +11 and resulted in closing of the upstream end of the transcription bubble and opening at the downstream end. Similar conclusions concerning the role of (d)ATP in propagation of the melted region during early transcription were reached in studies of the adenovirus E4 promoter using a nuclear extract (8, 18, 26), however, the point of the first transition occurred upon synthesis of a 3-nt RNA product. In these studies it was concluded that the TFIIH helicase functions during the early stages of RNA polymerase II transcription. Deciphering the roles that RNA polymerase II itself plays in transitions that occur during early RNA synthesis would be greatly aided by a kinetic model of the molecular mechanisms that govern the RNA polymerase II transcription reaction under conditions where the TFIIH helicase is not required.

A reductionist strategy for developing a quantitative kinetic model for human RNA polymerase II transcription is to measure rate constants for discrete steps in the transcription reaction using a minimal transcription system. Additional factors and transcriptional activators can then be added to the minimal system to study their effects on individual rate constants. This is possible because not all of the general transcription factors are required for basal (non-regulated) transcription *in vitro*. The TFIID complex, consisting of the TATA binding protein (TBP) and multiple associated factors, can be replaced by the single subunit TBP in basal transcription at TATA-containing promoters (27, 28). In addition, TFIIE and TFIIH can be omitted during basal transcription if the promoter is contained on a negatively supercoiled DNA template (14, 17, 29, 30). It is thought that negative superhelicity facilitates promoter melting and escape in the absence of the TFIIH helicase (14, 17, 31).

Using a minimal transcription system, we previously characterized the kinetics of three stages of human RNA polymerase II transcription at the AdMLP (10): preinitiation complex formation, promoter escape (synthesis of a 15-nt RNA), and transcript elongation (elongation of a 15-nt RNA into a 390-nt RNA). These kinetic studies revealed that synthesis of a 15-nt RNA from preinitiation complexes limited both the rate and extent of basal transcription in a minimal system. TFIIE and TFIIH, in a (d)ATP-dependent manner, significantly increased the fraction of complexes that produced a 15-nt RNA product. We proposed a model in which early transcription branches into at least two pathways: one that results in functional promoter escape and full-length RNA synthesis, and another in which transcription aborts prior to the completion of promoter escape. TFIIH, in a (d)ATP-dependent reaction, stimulates the fraction of functional complexes that successfully escape the promoter, leading to an increase in the amount of RNA produced (10). These initial studies did not distinguish between initiation and promoter escape nor did they consider other possible steps that may exist during early RNA synthesis. Our observations, however, suggested that the early steps of RNA synthesis are likely to be a key point for regulating levels of transcription in cells and hence warranted further study.

Here we have used a highly purified *in vitro* transcription system to further study the mechanism and kinetics of the early events of RNA synthesis by human RNA polymerase II at

the AdMLP. To aid our studies we used competitor DNA that both inhibits an early step in RNA synthesis and limits transcription to a single round. This enabled us to characterize a specific transition that occurs after initiation and commits RNA polymerase II to the subsequent step of promoter escape. Using pre-steady-state approaches, we measured forward and reverse rate constants for distinct steps in basal transcription at the AdMLP. Because the studies described here were performed in a transcription system containing the minimal number of components necessary to obtain site-specific transcription from the AdMLP (TBP, TFIIB, TFIIF, and core RNA polymerase II), the kinetic model for basal transcription that we established represents the most basic pathway for the RNA polymerase II reaction at this promoter.

EXPERIMENTAL PROCEDURES

Preparation of Transcription Factors and Other Reagents—Recombinant (TBP, TFIIB, TFIIE, and TFIIF) and native (TFIIH and RNA polymerase II) human transcription factors were prepared as described previously (Ref. 10, and references therein). The +5mt AdMLP was constructed by site-directed mutagenesis with a primer that anneals to the non-template strand of the promoter (5'-AGGGGAAGTGAGTGAGGACGAACG-3'). Calf thymus DNA (ctDNA, Sigma) was subjected to extensive sonication, phenol-chloroform extraction, and ethanol precipitation, and was used at 275 μ g/ml.

In Vitro Transcription Assays—Transcription reactions were performed in buffer A containing 10 mM Tris-HCl (pH 7.9), 10 mM Hepes (pH 8.0), 10% glycerol, 1 mM dithiothreitol, 4 mM MgCl₂, 50 mM KCl, 50 μ g/ml bovine serum albumin, and 15 units of RNA Guard (Amersham Pharmacia Biotech). Reactions contained the following amounts of transcription factors: 5 ng of TBP, 10 ng of TFIIB, 6 ng of TFIIF, 50 ng of RNA polymerase II, 15 ng of TFIIE-34, 6 ng of TFIIE-56, and ~14 ng of TFIIH (where indicated). The DNA template (0.8–1.2 nm) was negatively supercoiled plasmid DNA containing the AdMLP core promoter (–53 to +10) fused to a 380-base pair G-less cassette (14). Nucleotides were added at final concentrations of 625 μ M ATP, 625 μ M CTP, 25 μ M [α -³²P]UTP (5 μ Ci per reaction), and, when used, 1 mM ApC. A general scheme for the transcription reactions follows, with details and exceptions included in the figures and figure legends. All transcription factor proteins were preincubated in buffer A for 2 min at 30 °C (10 μ l per reaction), after which time promoter DNA in buffer A at 30 °C (10 μ l per reaction) was added. The incubation continued for 10 min to allow preinitiation complexes to form, after which either limited nucleotides, a complete set of nucleotides, and/or ctDNA was added as indicated in the figures. Transcription was allowed to proceed for 20 min at 30 °C unless otherwise indicated. Reactions were stopped with 100 μ l of a stop solution containing 3.1 M ammonium acetate, 10 μ g of carrier yeast RNA, and 15 μ g of proteinase K. The samples were ethanol-precipitated and resolved by 6% denaturing polyacrylamide gel electrophoresis.

Rate Constant Calculations—The amount of RNA produced per *in vitro* transcription reaction was quantitated using a Molecular Dynamics PhosphorImager. After subtracting background, PhosphorImager units from full-length RNA produced at each time point were divided by the average PhosphorImager units produced at the longest time points (in the plateau region) to obtain the fractional completion at each time point. For forward rates, these values were plotted and fit to the equation $F_c = 1 - e^{-kt}$, where F_c is fractional completion at each time point, t is time in seconds, and k is the rate constant. Rate constants for decay rates were determined using the equation $F_c = e^{-kt}$. Observed rate constants for those steps too fast for obtaining data prior to the plateau region were estimated to be greater than the inverse of the fastest time point taken.

RESULTS

In previous studies we found that synthesis of a 15-nt RNA from preinitiation complexes limited both the rate and the extent of a single round of transcription by RNA polymerase II at the AdMLP (10). At this point in the transcription reaction, promoter escape is complete. Given the importance of the early steps of RNA synthesis in limiting the rate and level of transcription, we decided to study the mechanism of early transcription in greater detail using a minimal RNA polymerase II transcription system consisting of TBP, TFIIB, TFIIF, RNA polymerase II, and the AdMLP contained on a negatively su-

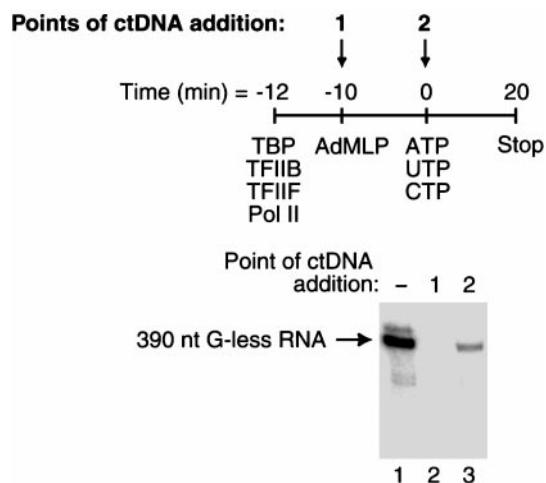


FIG. 1. Calf-thymus DNA (ctDNA) is a useful competitor for kinetic studies of a single round of RNA polymerase II transcription. An overview of the method is shown in the schematic at the top. ctDNA was added with the promoter DNA (lane 2) or with the nucleotides (lane 3). The position of the 390-nt G-less RNA transcript is indicated.

percoiled DNA template. Under these conditions other transcription factors, including TFIIE and TFIIH, are not required for transcription (14, 17, 29, 30). In addition, transcription at the AdMLP on a negatively supercoiled DNA template is not dependent on a source of (d)ATP that is hydrolyzable at the β - γ bond (14, 31). All this is consistent with the lack of requirement for the TFIIH helicase in transcription when DNA templates are negatively supercoiled. Therefore, studying transcription under these conditions allows us to assess the intrinsic properties of the RNA polymerase II enzyme in the mechanism of early transcription under conditions where an ancillary helicase activity is not required.

An Early Step in RNA Synthesis Is Inhibited by ctDNA—To facilitate mechanistic studies and limit transcription to a single round we added sonicated calf thymus DNA (ctDNA) to transcription reactions as a competitor. ctDNA sequesters free TBP and RNA polymerase II thereby preventing them from binding to the promoter and potentially initiating additional rounds of transcription. Furthermore, adding ctDNA at different time points allows us to isolate individual steps in the transcription reaction by considering only those events that occur either before or after the addition of ctDNA. The effect of ctDNA on transcription is demonstrated in Fig. 1. Lane 1 shows the level of transcription in the absence of ctDNA. Lane 2 demonstrates that ctDNA prevents preinitiation complex formation when added with the AdMLP, prior to the addition of proteins. Lane 3 shows the level of transcription when ctDNA was added with the nucleotides, after preinitiation complexes were allowed to form. Under this latter condition, the amount of transcript decreased approximately 10-fold from that produced in reactions lacking ctDNA (compare lane 1 to lane 3). This 10-fold decrease in transcription could result entirely from ctDNA inhibiting second and later rounds of initiation. Alternatively, all or part of the 10-fold decrease could be caused by ctDNA inhibiting a specific step in the transcription reaction during a single round of RNA synthesis.

To determine whether ctDNA inhibits an early step of a single round of RNA synthesis, we developed the method shown in Fig. 2A. Preinitiation complexes are formed in the absence of nucleotides. After preinitiation complexes form, transcription is initiated by adding a limited set of nucleotides (ApC, UTP, and CTP). Under these conditions RNA polymerase II progresses to +15 and stably pauses at this position due to

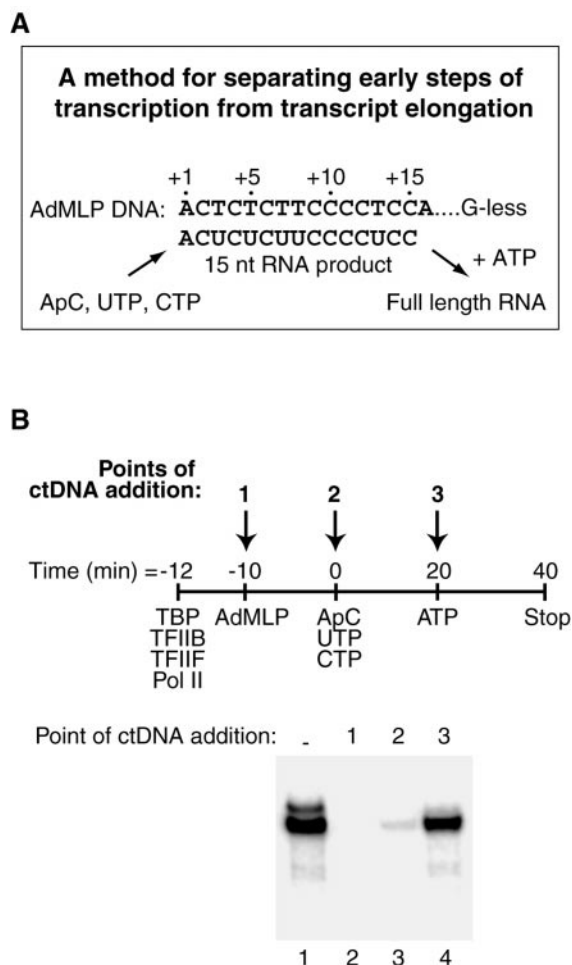


FIG. 2. Competitor DNA inhibits an early event in transcription. A, overview of the method used to study stages of transcription. The sequence of the nontemplate strand of the adenovirus major late promoter (AdMLP) early transcribed region is shown. A 15-nt RNA is made when preinitiation complexes are given the limited nucleotides ApC, UTP, and CTP. Upon addition of ATP, the 15-nt RNA is elongated to a 390-nt G-less RNA. B, competitor DNA can inhibit a transition during early transcription. Reactions were performed as diagrammed in the schematic, and ctDNA was added at points 1, 2, and 3. 390-nt G-less product was monitored.

the lack of ATP in the system. At this point promoter escape is complete, but the promoter is not yet accessible for a second round of initiation. When ATP is added, the pause releases and 15-nt RNAs are elongated into full-length 390-nt transcripts. ctDNA can be added at different points during the staged reaction to ask whether it inhibits any of the aforementioned stages.

As diagrammed in Fig. 2B, we either omitted ctDNA, added ctDNA with the AdMLP as a control (point 1), added ctDNA with the limited nucleotides so it was present during initiation and promoter escape (point 2), or added ctDNA with the ATP after promoter escape had occurred (point 3). Interestingly, there was a 10-fold decrease in the amount of RNA produced when ctDNA was added with the limited nucleotides (lane 3) compared with its addition with ATP (lane 4). Therefore, prior to the completion of promoter escape, 90% of transcription complexes were inhibited by ctDNA and did not produce full-length RNA in the presence of the competitor (compare lane 3 to lane 1). In contrast, ctDNA did not significantly reduce the level of transcription when added with ATP (compare lane 4 to lane 1). This indicates that even in the absence of ctDNA approximately one round of transcription occurred under these

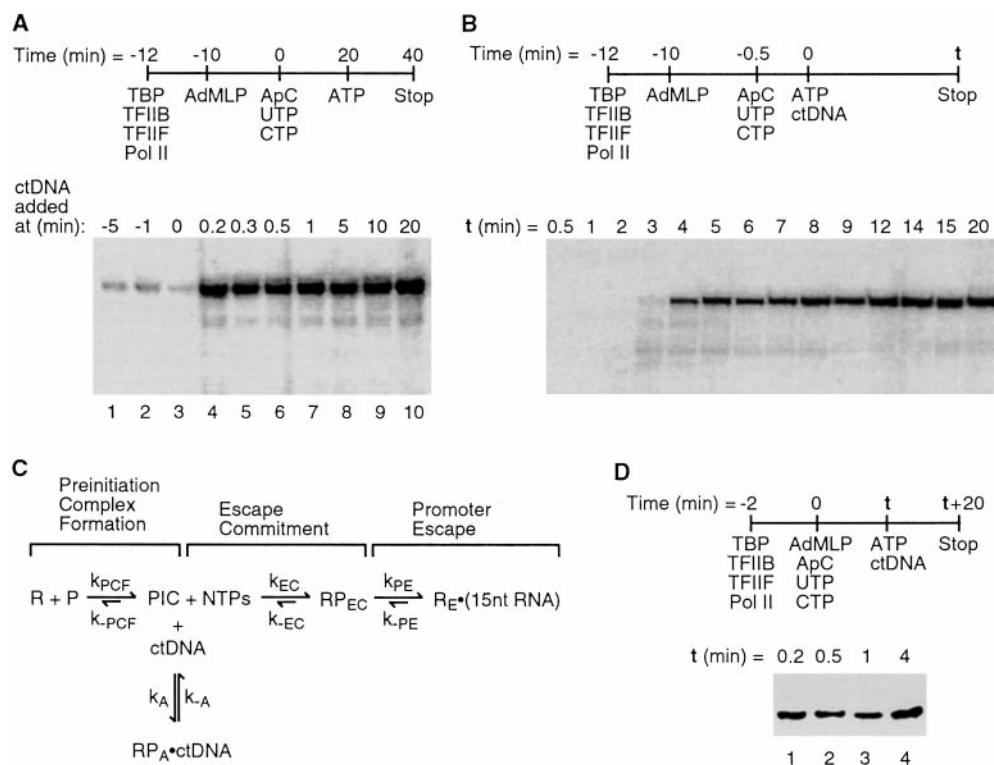


FIG. 3. A rapid transition, termed escape commitment, occurs prior to the rate-limiting step of promoter escape. *A*, the transition to ctDNA-resistant complexes occurs within seconds after adding ApC, UTP, and CTP. Reactions were assembled and initiated as diagrammed, and ctDNA was added at the time points indicated above the lanes. 390-nt G-less product was monitored. *B*, the rate-limiting step occurs after the transition to ctDNA-resistant complexes. Reactions were assembled and initiated in a large volume as shown in the schematic. Transcription was stopped at varying time points after the addition of ATP and ctDNA by removing 20- μ l aliquots and adding them to stop mix. 390-nt G-less product was monitored. *C*, model showing escape commitment. During escape commitment, complexes commit to proceeding forward and escaping the promoter. Escape-committed complexes are characterized by stability to ctDNA. Before the completion of escape commitment the transcription pathway branches and aborted complexes can form in the presence of ctDNA. See text for a complete discussion. Abbreviations are as follows: *R*, general transcription factors in the minimal transcription system (TBP, TFIIIB, TFIIIF, and RNA polymerase II); *P*, adenovirus major late core promoter; *PIC*, preinitiation complex; *RP_{EC}*, escape-committed complex; *R_E**(15nt RNA), elongation complex containing a 15-nt RNA; *RP_A*, aborted complex; *ctDNA*, sonicated calf thymus DNA. *D*, all events prior to promoter escape are rapid. Reactions were performed as shown in the schematic. ATP and ctDNA were added at the time points indicated above the lanes. 390-nt G-less product was monitored.

conditions. These results suggest that prior to completion of promoter escape a transition occurs in which complexes become resistant to competitor DNA and produce full-length RNA product.

A Rapid Transition Caused by NTPs Occurs Prior to the Rate-limiting Step of Promoter Escape—We showed above that by the completion of promoter escape a transition occurs in which complexes become resistant to ctDNA. In addition, we previously determined that early steps of RNA synthesis are rate-limiting for a single round of transcription (10). Therefore, there are three possibilities as to when complexes become resistant to ctDNA: before, during, or after the rate-limiting step. We used the kinetic experiment shown in Fig. 3*A* to distinguish between these possibilities. This experiment allowed us to determine how long it took for complexes to become resistant to ctDNA. As shown in Fig. 3*A*, ctDNA was added at multiple time points both before and after the addition of limited nucleotides. When added either with or prior to limited nucleotides, a low amount of transcript was produced (lanes 1–3). Within 10 s after the addition of limited nucleotides, however, complexes became resistant to ctDNA, and the amount of transcript produced plateaued at the maximal level (lanes 4–10). These results indicate that a transition occurs within seconds after the addition of nucleotides, during which time a large fraction of complexes become resistant to challenge with ctDNA and produce an RNA product.

The finding that complexes rapidly become resistant to ctDNA upon the addition of nucleotides implies that the rate-

limiting step occurs subsequent to this transition. To test this, we performed the experiment shown in Fig. 3*B*. Preinitiation complexes were given limited nucleotides for 30 s to allow the transition to ctDNA-resistant complexes to occur, after which time ATP and ctDNA were added and the production of full-length product was monitored over time. The results show that the rate of RNA synthesis from these complexes was slow with a rate constant of $2.0 \pm 0.4 \times 10^{-3} \text{ s}^{-1}$. This demonstrates that the rate-limiting step of promoter escape occurs after the transition in which complexes became stable to ctDNA. Experiments carried out with ATP, CTP, and UTP gave similar results, thereby confirming that initiating transcription with the dinucleotide as opposed to ATP does not alter promoter escape or the rate at which complexes become resistant to ctDNA (data not shown).

These experiments revealed a kinetically distinct step in transcription that occurs within seconds after providing preinitiation complexes with nucleotides and prior to the rate-limiting step of promoter escape, as shown in the model in Fig. 3*C*. During this transition, which we term escape commitment, complexes commit to proceeding forward through promoter escape and are characterized by resistance to ctDNA. Escape-committed complexes (*RP_{EC}*) transform into elongation complexes (*R_E*) during the rate-limiting step of promoter escape, which is complete prior to or at the point of synthesis of a 15-nt RNA. Prior to the completion of escape commitment, in the presence of ctDNA, approximately 90% of preinitiation complexes abort and are likely to be bound by ctDNA (*RP_A*·ctDNA).

Only 10% of complexes successfully proceed through promoter escape in the presence of ctDNA. As a result we observed a 10-fold decrease in the level or extent of transcription when ctDNA was included in transcription reactions with the nucleotides. We will refer to the non-functional complexes that are inhibited by ctDNA as “aborted complexes” (RP_A), not to be confused with the term “abortive initiation,” which refers to the synthesis and release of short RNA products by RNA polymerases.

If promoter escape is rate-limiting for RNA polymerase II transcription at the AdMLP, then all steps before and after promoter escape should occur faster than promoter escape. We have previously found that stable preinitiation complex formation and transcript elongation through the G-less cassette are rapid ($k_{PCF} > 0.1 \text{ s}^{-1}$ and $k_E > 0.3 \text{ s}^{-1}$, respectively (10)). Here we have shown that escape commitment is also rapid ($k_{EC} > 0.1 \text{ s}^{-1}$) and occurs within seconds of adding nucleotides to preinitiation complexes (Fig. 3A). These observations led to the prediction that the formation of escape-committed complexes should occur within seconds of combining proteins with promoter DNA and nucleotides. It was formally possible, however, that a slow step existed after preinitiation complex formation and prior to escape commitment that was not detected by previous experiments. To ensure this was not the case and to confirm that all steps prior to promoter escape occur rapidly, we measured the rate at which escape-committed complexes form after combining proteins with promoter DNA and nucleotides. As shown in Fig. 3D, the significant difference between this experiment and previous experiments is that preinitiation complexes were not allowed to form prior to the addition of limited nucleotides. Therefore each time point encompassed preinitiation complex formation, escape commitment, and all steps in between. Within 15 s after adding AdMLP and limited nucleotides to the proteins, the level of transcription plateaued at the maximum level. Furthermore, the level of transcript observed in this experiment was equal to that observed in reactions lacking ctDNA, thereby confirming that escape commitment had occurred. This demonstrates that all steps prior to and including escape commitment occur rapidly.

Escape Commitment Is Complete upon Synthesis of a 4-nt RNA—We hypothesized that escape commitment occurs with the synthesis of a distinct phosphodiester bond during early transcript synthesis. To test whether escape commitment is complete upon formation of 3-nt RNA product, we added ApC and UTP to preinitiation complexes. RNA products 3 nt in length are made abortively when preinitiation complexes at the AdMLP are provided with ApC and UTP (14, 32). After variable amounts of time in the presence of ApC and UTP, we added ctDNA, ATP, and CTP and measured the amount of 390-nt RNA that was made. The data are plotted in *curve 1* of Fig. 4A and show that the number of complexes capable of producing full-length RNA remained low throughout the time course. This demonstrates that ApC and UTP are not sufficient to allow escape commitment, even after 20 min. For comparison, *curve 2* shows the rate at which escape commitment occurs after preinitiation complexes are provided with the limited nucleotides ApC, UTP, and CTP (data from Fig. 3A is plotted). Taken together, these results demonstrate that an RNA at least 4 nt in length must be synthesized to form escape-committed complexes.

To test whether synthesis of a 4-nt RNA is sufficient to allow escape commitment to occur, we constructed a mutant of the AdMLP (+5mt) in which the +5 position was changed to an adenosine (nontemplate strand, see sequence in Fig. 2A). With the +5mt promoter, a 4-nt RNA is the longest product that can be made in the presence of the limited nucleotides ApC, UTP,

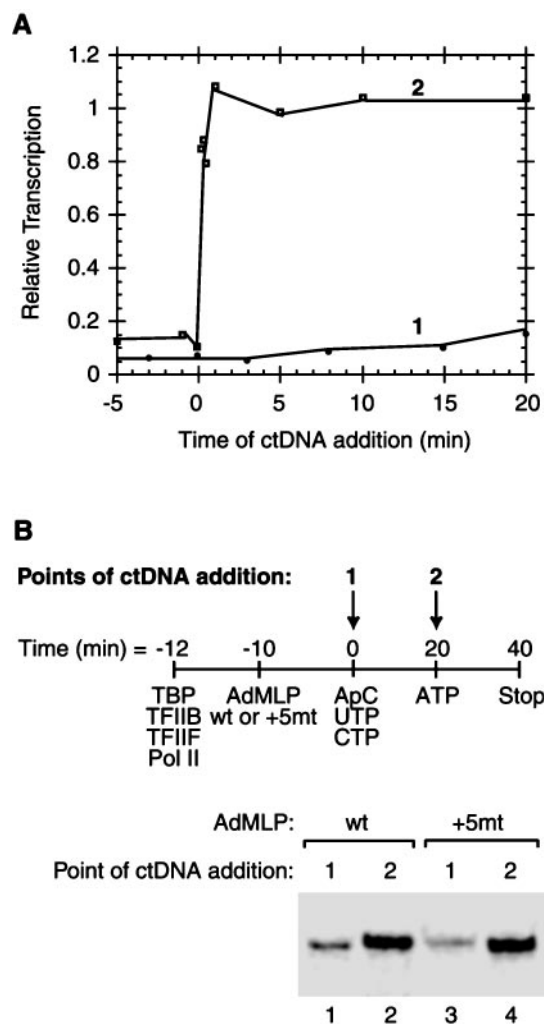


FIG. 4. Escape commitment occurs with synthesis of a 4-nt RNA. A, synthesis of a 3-nt RNA is not sufficient for escape commitment. In *curve 1* limited nucleotides that produce a 3-nt RNA (ApC and UTP) were added to reactions at time zero after preinitiation complexes formed. ctDNA, CTP, and ATP were added to reactions at varying time points as indicated in the plot, and RNA synthesis proceeded for 15 min. Full-length RNA product was quantitated and plotted with respect to the time of ctDNA addition. For comparison, *curve 2* shows the time course for formation of escape-committed complexes (data from Fig. 3A were plotted in the manner just described). B, synthesis of a 4-nt RNA is sufficient for escape commitment. A mutant AdMLP (+5mt) was constructed with an A at +5 (non-template strand) such that a 4-nt RNA would be produced upon the addition of ApC, UTP, and CTP. Reactions were assembled and initiated as diagrammed, and ctDNA was added at *point 1* or *point 2*. 390-nt G-less product was monitored.

and CTP. The +5mt behaved just as wt AdMLP with respect to the rate and extent of transcription (data not shown). We added ctDNA both during and after the formation of a 4-nt product, as diagrammed in Fig. 4B. The results using the +5mt template show that adding ctDNA with the limited nucleotides (*lane 3*) decreased the amount RNA produced by 10-fold when compared with adding ctDNA with ATP (*lane 4*). For comparison, similar results were obtained using the wt template (*lanes 1* and *2*). These results show that escape commitment is complete by formation of a 4-nt RNA. This distinguishes escape commitment from initiation in which 2- and 3-nt RNAs are made.

A potential caveat to the above experiment was that if read-through of the mutation at +5 occurred in the presence of ApC, UTP, and CTP, then we could not conclude with certainty that escape commitment was complete with synthesis of a 4-nt RNA. Various experimental evidence indicates, however, that RNAs longer than 4 nt are not made from the +5mt template

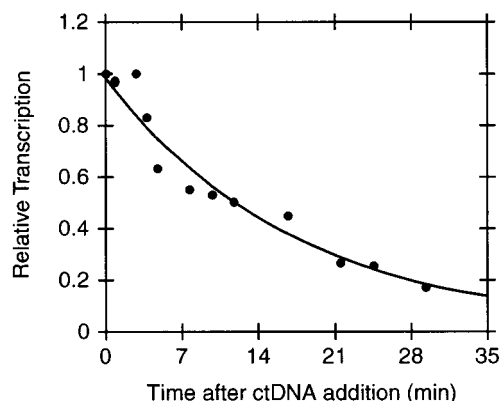


FIG. 5. **Escape-committed complexes decay slowly.** Escape-committed complexes were formed in a large reaction volume by providing limited nucleotides (ApC, CTP, UTP) to preinitiation complexes on the +5mt template for 1 min. ctDNA was then added, and 20- μ l aliquots were removed over time and added to ATP. Transcription was stopped after 15 min. The data were quantitated and fit to a single exponential.

using the limited nucleotides. First, the only RNA observed in ternary complexes on the +5mt template after 20 min of transcription with ApC, UTP, and CTP was 4 nt in length (data not shown). It is possible, however, that some of the 4-nt product arose from sites on the plasmid other than the AdMLP start site. Second, escape commitment was complete within 10 s after adding ApC, UTP, and CTP on the +5mt template, thus any read-through would have had to occur in less than 10 s, which seems unlikely (data not shown). Third, other nucleotide combinations did not result in escape commitment. For example, in Fig. 4A, read-through past +3, which could have resulted in escape commitment, did not occur over the course of 20 min despite high concentrations of ApC and UTP. Furthermore, escape commitment did not occur on the +5mt template when only UTP and CTP were added to reactions for 20 min and ApC was omitted (data not shown). Under these conditions transcription initiated at the +2 position and a 3-nt RNA was the longest product that could be produced. These data, along with that discussed above, confirm both that a 4-nt RNA is required for the completion of escape commitment and that read-through does not occur on the +5mt template under our reaction conditions.

We propose that escape commitment is a transition in the transcription reaction that results in ternary complexes that proceed forward through promoter escape even in the presence of ctDNA. One prediction of this proposal is that escape-committed complexes will be stable and will not rapidly decay back to aborted complexes in the presence of ctDNA. To test this prediction, we formed escape-committed complexes on the +5mt AdMLP template. On this template, escape-committed complexes were not able to proceed forward through promoter escape in the absence of ATP. ctDNA was then added and portions of the reaction were removed and added to ATP over time. The amount of full-length RNA at each time point was quantitated, and the data were fit to a single exponential equation (Fig. 5). The rate constant measured, $9.5 \pm 0.6 \times 10^{-4} \text{ s}^{-1}$, is slow relative to the rate at which escape-committed complexes form ($k_{\text{EC}} > 0.1 \text{ s}^{-1}$, from Fig. 2A). This demonstrates that once formed, escape-committed complexes are kinetically stable and do not rapidly revert to aborted complexes.

Escape Commitment Is Facilitated by TFIIE and TFIIH—In previous studies we found that TFIIE and TFIIH, in an (d)ATP-dependent reaction, increased the fraction of complexes that were functional rather than abortive when preinitiation complexes were provided with ATP, UTP, CTP, and ctDNA (10). In these studies TFIIE and TFIIH acted after preinitiation com-

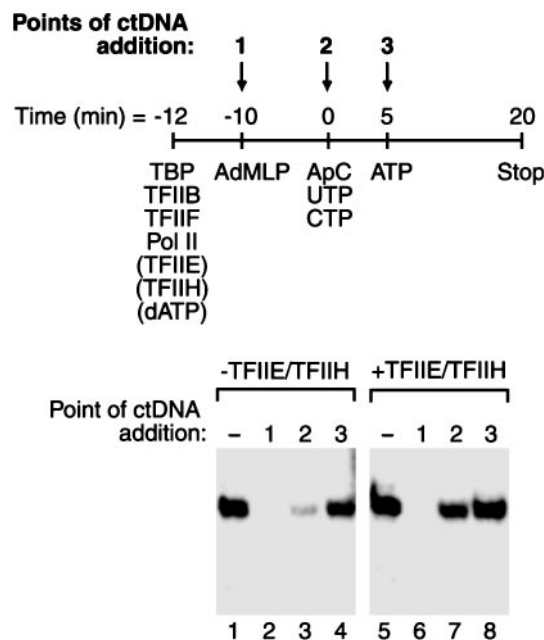


FIG. 6. **TFIIE and TFIIH facilitate escape commitment.** Reactions were performed as diagrammed in the schematic, and ctDNA was added at points 1, 2, and 3. dATP was included where shown at a final concentration of $5 \mu\text{M}$. 390-nt G-less product was monitored.

plex formation and prior to the completion of promoter escape. Upon finding that ctDNA inhibits the formation of escape-committed complexes, we hypothesized that TFIIE and TFIIH would facilitate escape commitment. To test this we studied the effects of TFIIE, TFIIH, and dATP on transcription levels when ctDNA was added at different points in the staged reaction. For reference, lanes 1–4 of Fig. 6 show the effect of ctDNA on transcription in the absence of TFIIE and TFIIH. In the presence of TFIIE and TFIIH, ctDNA only decreased transcription 2-fold when added at point 2, with ApC, UTP, and CTP (compare lane 7 to lane 5). Moreover, when ctDNA was added at this point, the amount of RNA produced was 10-fold greater in the presence of TFIIE and TFIIH than in their absence (lane 7 versus lane 3). The effect of TFIIE and TFIIH was dependent on dATP (data not shown). These results indicate first that TFIIE and TFIIH increase the fraction of complexes that are functional and produce RNA in the presence of ctDNA, and second that they act at the step of escape commitment.

Kinetics of the Steps of Early RNA Synthesis by RNA Polymerase II—The experiments shown above and published previously (10) allowed us to derive a detailed reaction pathway for a single round of transcription at the AdMLP on negatively supercoiled DNA as shown in Fig. 7. We include five distinct stages, each of which we experimentally isolated: preinitiation complex formation, initiation, escape commitment, promoter escape, and transcript elongation. As shown in Table I, we measured forward and reverse rate constants for each of the five stages of transcription depicted in Fig. 7. The forward rates for all of these steps are fast, except promoter escape, which is rate-limiting. Reverse rates were determined by measuring the rate at which complexes decayed after challenge with ctDNA. Preinitiation complexes, escape-committed complexes, and elongation complexes were all relatively stable. These experiments did not allow us to determine the pathway by which complexes decayed, therefore, the reverse rate constants are presented as upper limits. Together these rate constants provide a kinetic profile for a single round of transcription at the AdMLP that experimentally distinguishes multiple steps and

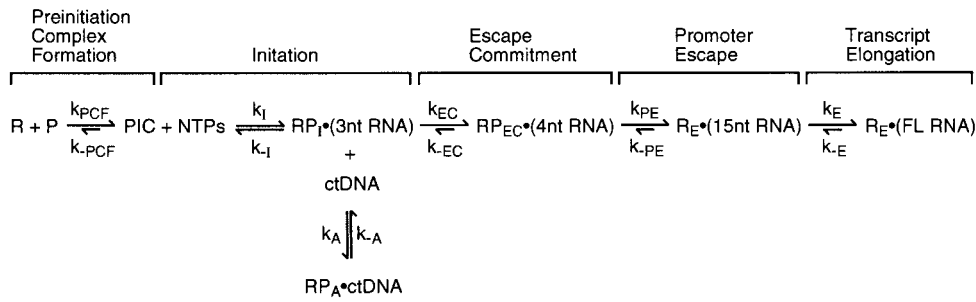


FIG. 7. **A kinetic model for the RNA polymerase II transcription reaction.** Escape commitment occurs after initiation and prior to the rate-limiting step of promoter escape. It is complete after the synthesis of a 4-nt RNA. See the text for a complete discussion of the model. For rate constants associated with each step see Table I. Most abbreviations are described in the legend to Fig. 3, except: $RP_I(3nt\ RNA)$, initiated complex containing 3-nt RNA; $RP_{EC}(4nt\ RNA)$, escape-committed complex containing 4-nt RNA; $R_E(FL\ RNA)$, elongation complex containing full-length RNA.

TABLE I
Rate constants for the steps of transcription at the adenovirus MLP

Constant	Value	Method
	s^{-1}	
$k_{PCF(obs)}$	>0.1	<i>a</i>
k_{-PCF}	$\leq 1.3 \pm 0.4 \times 10^{-3}$	<i>b</i>
k_I	>0.1	<i>c</i>
k_{-I}	Not determined	
k_{EC}	>0.1	Fig. 3A
k_{-EC}	$\leq 9.5 \pm 0.6 \times 10^{-4}$	Fig. 5
k_{PE}	$2.0 \pm 0.4 \times 10^{-3}$	<i>d</i>
k_{-PE}	$< 5 \times 10^{-4}$	<i>e</i>
k_E	>0.03	<i>a</i>
k_{-E}	Not determined	
k_A	(>0.1)	<i>f</i>
k_{-A}	Not determined	

^a Kugel and Goodrich (10).

^b Preinitiation complexes were challenged with ctDNA for variable times prior to adding ATP, UTP, and CTP. 390-nt RNA was quantitated. The value is the average of three experiments, and the error is one standard deviation.

^c The rate constant for initiation is inferred to be rapid because the formation of escape committed complexes from free protein and DNA was complete within 10 s (Fig. 3D).

^d The method used is shown in Fig. 3D. The value is the average of three experiments, and the error is one standard deviation.

^e Preinitiation complexes were provided with ApC, UTP, and CTP to allow the formation of 15-nt RNA. Ternary complexes were challenged with ctDNA for variable amounts of time prior to adding ATP to allow elongation of 15-nt RNA to 390 nt. There was no detectable decay over 30 min.

^f We have not measured this rate constant directly; however, the observations that preinitiation complexes transform into escape-committed complexes within 10 s and that aborted complexes form rapidly in the presence of ctDNA imply that this rate constant (shown in parentheses) is greater than $0.1\ s^{-1}$ at the concentration of ctDNA used.

clearly shows promoter escape is rate-limiting in this system. The model shown in Fig. 7 is discussed in more detail below.

DISCUSSION

We investigated the kinetic mechanism of transcription at the AdMLP using a highly purified human transcription system. In doing so we identified and characterized a transition, escape commitment, that precedes the rate-limiting step of promoter escape. Escape commitment is complete after formation of a 4-nt RNA, which distinguishes it from initiation in which 2- and 3-nt RNA transcripts are made. Interestingly, escape commitment can be inhibited by competitor ctDNA. We also developed a detailed model for a single round of transcription that kinetically isolates several steps and clearly shows promoter escape is rate-limiting in a minimal transcription system.

A Kinetic Model for RNA Polymerase II Transcription at the AdMLP—The model shown in Fig. 7 consists of five steps in the transcription reaction at the AdMLP that we have studied.

Below we discuss each of these steps and relate our findings to previous studies on the early steps of RNA polymerase II transcription, most of which were performed in transcription systems containing additional factors than those used here. It is important to note that in our earlier work (10) and in the work we describe here we have utilized a minimal RNA polymerase II transcription system consisting of TBP, TFIIB, TFIIF, and core RNA polymerase II under conditions where the TFIIF helicase and (d)ATP hydrolysis are not required. Therefore, the model shown in Fig. 7 represents the intrinsic mechanism of promoter specific transcription by RNA polymerase II in the absence of an ancillary enzymatic activity that facilitates DNA melting.

During preinitiation complex formation (PCF), general transcription factors (R) assemble on promoter DNA (P) to form preinitiation complexes (PIC) that are stable to challenge with calf thymus DNA (ctDNA) used as a competitor. Surprisingly, we found that preinitiation complexes form within 10 s of adding promoter DNA to RNA polymerase II and the general transcription factors (10). Hence, under our experimental conditions, the observed rate constant for preinitiation complex formation ($k_{PCF(obs)}$) is greater than $0.1\ s^{-1}$. Clearly the rate of preinitiation complex formation will depend on the concentration of transcription factors. However, because preinitiation complexes form faster than we can sample manually under our experimental conditions, we have not determined the dependence of this rate constant on the concentration of transcription factors; therefore, we present $k_{PCF(obs)}$ in units of s^{-1} . Once formed, preinitiation complexes are kinetically stable ($k_{-PCF} \leq 1.3 \pm 0.4 \times 10^{-3}\ s^{-1}$). In the model shown in Fig. 7 we have not divided preinitiation complex formation into the steps of closed complex formation and open complex formation, because it has not yet been determined if preinitiation complexes formed in a purified transcription system at the AdMLP on negatively supercoiled DNA contain melted DNA around the transcription start site. These preinitiation complexes, however, are likely to be in an open conformation because they are capable of producing full-length RNA.

Transcription is initiated when preinitiation complexes are provided with nucleoside triphosphates. At the AdMLP, the stage of initiation occurs with the synthesis of 2- and 3-nt RNAs to form ternary initiated complexes (for example, $RP_I(3nt\ RNA)$). Initiation occurs rapidly with a rate constant (k_I) that is greater than $0.1\ s^{-1}$. We have not directly measured the rate of decay of initiated complexes (k_{-I}), however, the fact that 2- and 3-nt RNAs are synthesized and abortively released by RNA polymerase II under conditions of limited nucleotides indicates that ternary initiated complexes are not kinetically stable (14, 19, 32, 33).

The step of initiation is followed by a transition, termed escape commitment, that precedes the rate-limiting step of

promoter escape. Specifically, escape commitment results in stable ternary complexes (RP_{EC}) containing 4-nt RNAs that are committed to subsequently escape the promoter. The rate constant for formation of escape-committed complexes (k_{EC}) is greater than 0.1 s^{-1} . Once formed, escape-committed complexes are kinetically stable ($k_{-EC} \leq 9.5 \pm 0.6 \times 10^{-4} \text{ s}^{-1}$). In our reactions, performed with negatively supercoiled DNA templates, (d)ATP is not required for escape commitment in the absence of ctDNA; however, (d)ATP, TFIIE, and TFIIH are required to obtain maximal escape commitment in the presence of ctDNA. Previous studies have observed transitions at this same position that were dependent on (d)ATP. First, Luse and colleagues (33, 34) found that stable ternary complexes formed when an RNA 4 nt in length was produced and that the formation of these stable complexes was dependent on (d)ATP. Second, in studying the status of the transcription bubble at the AdMLP on a linear DNA template during early transcription, Timmers and colleagues (19) identified a transition that was complete with the synthesis of a 4-nt RNA. On linear DNA templates this transition was dependent on TFIIE, TFIIH, and (d)ATP and resulted in an extension of the downstream end of the transcription bubble. We suggest that escape commitment observed here in the absence of TFIIE, TFIIH, and (d)ATP is the same as the transitions observed in these two earlier studies using assays different from ours, with the exception that TFIIE, TFIIH, and (d)ATP were essential for escape commitment in the earlier studies even in the absence of competitor DNA.

Other transitions have been characterized during early transcription at the AdMLP. First, mechanistic studies on transcription at the AdMLP in a cruder system defined a distinct transition during early transcription that resulted in resistance to specific concentrations of the competitor sarkosyl (35). Formation of the sarkosyl-resistant “initiated complexes” only required nucleotides sufficient to form a 2-nt RNA. Because it is more difficult to control accessibility to small amounts of contaminating nucleotides in cruder transcription systems, it is possible that these initiated complexes are the same as the escape-committed complexes observed here. Second, an “escape competent intermediate” was previously characterized (36). Formation of this intermediate required 40–50 base pairs of DNA downstream of the transcription start site and appeared to occur at register 13 in RNA synthesis. Given these characteristics, we believe that this transition occurs after escape commitment and during promoter escape. Third, studies of RNA polymerase II transcription at the adenovirus E4 promoter in human nuclear extracts identified a transition in the region of melted DNA that occurred upon synthesis of a 3-nt RNA product (8, 18, 26). Hydrolyzable (d)ATP was required to observe this transition even though negatively supercoiled DNA templates were added to reactions. Determining how this transition at the adenovirus E4 promoter relates to the step of escape commitment studied here will require future studies aimed at characterizing the sensitivity of early transcription at the E4 promoter to ctDNA.

In the presence of ctDNA, 90% of initiated complexes at the AdMLP do not successfully undergo escape commitment, but instead form aborted complexes that are probably sequestered by ctDNA ($RP_A \cdot \text{ctDNA}$). Only 10% of initiated complexes successfully proceed through escape commitment in the presence of saturating amounts of ctDNA. We have not directly measured forward and reverse rate constants for aborted complex formation and decay (k_A and k_{-A}). The observation, however, that preinitiation complexes proceed through initiation and escape commitment within 10 s implies that the formation of aborted complexes is at least as rapid; therefore, we estimate k_A at the

concentration of ctDNA used to be greater than 0.1 s^{-1} . We have drawn the branch to aborted complexes as occurring after a 3-nt RNA is synthesized and prior to the completion of escape commitment, because this is the last point in the reaction at which it can occur. Once escape commitment is complete, ctDNA cannot inhibit subsequent steps of transcription. Establishing the exact point in the reaction at which the branch occurs will require future studies. Models for the molecular changes that may occur during escape commitment and aborted complex formation are discussed in the following section.

Our kinetic studies revealed that the rate-limiting step of transcription occurs between synthesis of the fourth and fourteenth phosphodiester bonds. We now refer to this stage of transcription as promoter escape, which is complete by the synthesis of a 15-nt RNA. This is a refinement of our previous use of the term promoter escape, which included synthesis of the first 14 phosphodiester bonds (10) due to the identification of escape commitment. The rate constant for promoter escape (k_{PE}) is $2.0 \pm 0.4 \times 10^{-3} \text{ s}^{-1}$. Once complexes escape the promoter, they are considered elongation complexes (R_E). These ternary complexes are kinetically stable ($k_{-PE} < 5 \times 10^{-4} \text{ s}^{-1}$), and the 15-nt RNA can be rapidly elongated to full-length 390-nt RNA in our reactions (10).

Together the model shown in Fig. 7 and the rate constants contained in Table I provide a kinetic profile for a single round of transcription at the AdMLP that experimentally distinguishes multiple steps and clearly shows promoter escape is rate-limiting in this system. That two steps in early transcript synthesis, escape commitment and promoter escape, limit the extent and rate of transcription, respectively, suggests that this point in the transcription reaction will be targeted by regulatory factors such as transcriptional activators.

Possible Molecular Changes That Occur during Escape Commitment—We propose that escape commitment is an intrinsic transition that RNA polymerase II undergoes during early transcription from promoters and that this transition can be facilitated by either negative supercoiling or TFIIH. Studies that we have performed with TFIIH using linear and negatively supercoiled templates support this proposal. For example, we previously reported that a branch occurs during the early stages of transcription that results in the formation of aborted complexes (10). These experiments were performed in the presence of ctDNA and we observed that TFIIH stimulated functional promoter escape over aborted complex formation. Given the studies described here we revised this model and propose that TFIIH, in a (d)ATP-dependent reaction, stimulates functional escape commitment over aborted complex formation. As discussed previously, it is likely that escape commitment is the same as the (d)ATP- and TFIIH-dependent transition, which was previously observed on linear DNA templates and resulted in extension of the downstream end of the transcription bubble (19). That escape commitment occurs in the absence of TFIIH and (d)ATP on negatively supercoiled DNA, however, suggests that the transition observed at this same register in permanganate footprinting studies (19) is an intrinsic property of the RNA polymerase II reaction that can be facilitated by either negative supercoiling or TFIIH. In other words, TFIIH does not cause the transition that we refer to as escape commitment, it simply allows initiated complexes to undergo this transition under conditions where DNA templates are not inherently underwound.

Escape commitment was identified because escape-committed complexes were resistant to challenge by ctDNA, whereas prior to the completion of escape commitment the majority of complexes were inhibited by ctDNA. Studies to understand the molecular changes that occur during escape commitment are

ongoing, however, there are at least two models to explain the observation that ctDNA inhibits the formation of escape-committed complexes. First, one of the general transcription factors might release from the promoter during aborted complex formation and would then have to rebind to participate in a second attempt at escape commitment. ctDNA could sequester the released general transcription factor and prevent it from rebinding. Second, ctDNA might directly attack complexes that contain all of the general transcription factors and decrease the number of complexes that undergo escape commitment. For example, if one of the DNA or RNA binding sites on RNA polymerase II is exposed during escape commitment, the ctDNA could enter this site on the polymerase and block the transition to escape-committed complexes. In the absence of ctDNA the polymerase would "lock" onto the promoter DNA and the short RNA transcript rendering the binding sites resistant to competitor. Studies of *Escherichia coli* RNA polymerase transcription from the rrnB P1 promoter indicate that when a 4-nt RNA, initiated at the -3 position, enters the RNA binding site on the polymerase, the enzyme then stably locks onto the DNA (37).

One appealing hypothesis for the molecular events that occur during escape commitment by RNA polymerase II was raised when we considered a recent x-ray structure of a T7 RNA polymerase initiation complex published by Cheetham and Steitz (38). The crystallized complex consisted of phage T7 RNA polymerase, template DNA, and a trinucleotide RNA transcript. The trinucleotide RNA forms a duplex with the template DNA strand. With respect to the model proposed here, the most interesting part of the structure relates to the orientation of the trinucleotide transcript in the catalytic pocket. Cheetham and Steitz predict that after synthesis of the third phosphodiester bond by transcribing T7 RNA polymerase, the 5'-end of the transcript peels away from the template DNA and enters the RNA channel from which the elongating transcript will exit the polymerase. In our transcription system with human RNA polymerase II, it is after the synthesis of the third phosphodiester bond that transcribing RNA polymerase becomes resistant to competition by ctDNA. It is possible that at this point the growing 4-nt transcript blocks the RNA channel from attack by ctDNA. Perhaps the mechanisms of early transcription by T7 RNA polymerase and human RNA polymerase II are similar and conserved throughout evolution. Future experiments aimed at determining if either the RNA exit channel or the downstream DNA binding channel are targeted by ctDNA will be greatly aided by the recently published crystal structure of yeast RNA polymerase II (39).

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