

An 8 nt RNA triggers a rate-limiting shift of RNA polymerase II complexes into elongation

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To better understand the critical conversions that RNA polymerase II complexes undergo during promoter escape, we determined *in vitro* the precise positions of the rate-limiting step and the last step requiring negative superhelicity or TFIIE and TFIIH. We found that both steps occur after synthesis of an 8 nt RNA during the stage encompassing translocation of the polymerase active site to the ninth register. When added to reactions just before this step, TFIIE and TFIIH overcame the requirement for negative superhelicity. The positions at which both steps occur were strictly dependent on RNA length as opposed to the location of the polymerase relative to promoter elements, showing that the transcript itself controls transformations during promoter escape. We propose a model in which completion of promoter escape involves a rate-limiting conversion of early transcribing complexes into elongation complexes. This transformation is triggered by synthesis of an 8 nt RNA, occurs independent of the general transcription factors, and requires under-winding in the DNA template via negative superhelicity or the action of TFIIE and TFIIH.

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Introduction

Eukaryotic mRNA transcription is an intricate, multistep process that requires the regulated interplay of promoter DNA, numerous protein subunits, and the transcript RNA. The promoter DNA controls levels of transcription via the sequences of the core and regulatory promoter elements (Smale and Kadonaga, 2003) as well the conformation of the DNA. The RNA polymerase II (Pol II) core enzyme catalyzes the synthesis of mRNA, but requires additional accessory factors for promoter-specific transcription (Orphanides *et al*, 1996). The general transcription factors,

which are believed to function at all promoters, include TFIIA, TFIIB, TFIID, TFIIE, TFIIIF, and TFIIH. Additional factors such as activators, repressors, co-regulators, and chromatin-modifying factors are involved in promoter-specific transcription in eukaryotic cells (Naar *et al*, 2001; Malik and Roeder, 2000; Narlikar *et al*, 2002). The RNA transcript itself is intimately involved in the process of transcription; it stabilizes elongation complexes as part of the RNA:DNA hybrid (Kireeva *et al*, 2000; Westover *et al*, 2004), and it influences critical transformations during early transcription (Kugel and Goodrich, 2002).

The transcription reaction involves many steps in a linear pathway including preinitiation complex formation, open complex formation, initiation, escape commitment, promoter escape, elongation, termination, and reinitiation. Pol II and the general transcription factors assemble at the promoter to form preinitiation complexes that subsequently transform into open complexes upon melting of the DNA surrounding the transcription start site (Holstege *et al*, 1996, 1997). *In vitro*, functional preinitiation complexes can be assembled from a subset of the general transcription machinery including TBP, TFIIB, TFIIIF, and Pol II, which are sufficient to produce promoter-specific transcripts if the template DNA is negatively supercoiled (Parvin and Sharp, 1993; Tyree *et al*, 1993; Goodrich and Tjian, 1994; Kugel and Goodrich, 1998). Using this minimal *in vitro* transcription system and the adenovirus major late promoter (AdMLP), we previously characterized five steps in the transcription reaction and measured the rate at which each occurs, resulting in the kinetic model for Pol II transcription shown in Figure 1 (Kugel and Goodrich, 2000).

In the presence of nucleoside triphosphates, preinitiation complexes initiate transcription and synthesize 2–3 nt RNA transcripts, often abortively (Luse and Jacob, 1987; Goodrich and Tjian, 1994). Stable ternary complexes form upon addition of the fourth nucleotide during a step termed escape commitment (Cai and Luse, 1987; Luse *et al*, 1987; Kugel and Goodrich, 2000, 2002). Complexes then proceed through promoter escape, a transition in which numerous protein–protein and protein–nucleic acid interactions are broken and new ones are established en route to formation of an elongation complex (Dvir, 2002). Promoter escape has been kinetically defined as complete by synthesis of a 15 nt RNA, because the rate at which a 15 nt RNA is elongated to full-length transcript is rapid (Kugel and Goodrich, 1998). Promoter escape also involves changes in the transcription bubble, transcript slippage, pausing of ternary complexes, release of TFIIB, and formation of a stable RNA:DNA hybrid (Zawel *et al*, 1995; Holstege *et al*, 1997; Pal and Luse, 2002, 2003; Pal *et al*, 2005). Predictions about the transformations that occur during promoter escape can be made by comparing the structures of Pol II elongation complexes to structures of the free polymerase (Boeger *et al*, 2005); however, there are no structures of early transcribing complexes yet.

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Figure 1 A working kinetic model for the Pol II transcription reaction obtained using a minimal *in vitro* transcription system. The rate-limiting step occurs during promoter escape. R, Pol II and the general transcription factors; P, promoter DNA; PIC, preinitiation complex; RP_i, initiated complex; RP_{EC}, escape committed complex; R_E, elongation complex.

In vitro studies have shown that the conformation of the promoter DNA is intimately tied to the mechanism of promoter escape. Factors that influence DNA conformation such as superhelicity and TFIIH, which contains two subunits having ATP-dependent helicase activity, can profoundly affect the early steps of transcription (Kumar *et al*, 1998; Kugel and Goodrich, 1998, 2000; Moreland *et al*, 1999; Bradsher *et al*, 2000; Kim *et al*, 2000). For example, the melted region of the DNA undergoes dramatic changes in size and position during early transcription, which are influenced by the helicase activity of TFIIH and negative superhelicity in the DNA template (Goodrich and Tjian, 1994; Holstege *et al*, 1997; Moreland *et al*, 1999; Pal *et al*, 2005). The requirement for TFIIH is alleviated by synthesis of a 15 nt transcript (Goodrich and Tjian, 1994); thus, negative superhelicity and TFIIH regulate one or more steps during promoter escape.

To further understand the conversion of early transcribing complexes into elongation complexes at the completion of promoter escape, we performed experiments to determine the precise positions of the rate-limiting step in early transcription and the last step at which DNA conformation influences the reaction. Using a minimal transcription system, we found that both of these steps occur after synthesis of an 8 nt RNA, during the stage encompassing translocation of the polymerase active site to the ninth register. The positions at which both steps occur are strictly dependent on the length of the RNA, suggesting that the transcript itself is intimately involved in the mechanisms of these steps. Moreover, the position of the rate-limiting step is independent of the general transcription factors. When TFIIE and TFIIH are added to ternary complexes containing a 7 nt RNA, they can overcome the requirement for negative superhelicity. Our studies lead to a model in which the completion of promoter escape involves a rate-limiting conversion of early transcribing complexes into elongation complexes. This conversion is triggered by synthesis of an 8 nt RNA and requires under-winding of the DNA via negative superhelicity or TFIIE and TFIIH.

Results

The rate-limiting step occurs between synthesis of a 7 nt and a 9 nt RNA

We previously found that the rate of transcription *in vitro* is limited by a step that occurs between synthesis of a 4 nt and a 15 nt RNA on the AdMLP in a minimal transcription system (Figure 1). We hypothesized that the rate-limiting step would occur at a distinct position between registers +4 and +15, concomitant with an important transformation during early transcription. To initially test this, we created a series of mutant promoters in which single base pairs in the +5 to +14 region of the AdMLP were changed to T/A base pairs (template/non-template), thereby allowing us to pause early transcribing complexes at specific positions and measure the

rates at which the short paused RNAs were extended to full-length transcripts. This is illustrated in Figure 2A for the wild-type AdMLP (+16T). In the absence of ATP, transcription proceeded to the first T in the template strand where it paused. Extension of the short paused RNA to full-length RNA was initiated by addition of ATP. 3'-O-methylguanosine triphosphate (3'-Me-GTP) was included during extension to terminate transcription at the first C in the template strand (position +101). Reactions were then stopped at various time points to measure the rates at which short paused RNAs were extended to 101 nt transcripts. Supplementary Figure 1 provides a more complete description of the method used, as well as representative data for the rates of extension of 4 and 15 nt RNAs. Control experiments showed that the mutations did not affect the overall rate of transcription (data not shown).

Using this method, we measured the rates at which paused RNAs between 6 and 13 nt in length were extended to full-length RNAs. As shown in the bar plot in Figure 2B, short transcripts in complexes paused on the +7 and +8T promoters were extended with rates similar to that measured on the +5T promoter (lower dashed line), indicating that complexes containing 6 and 7 nt RNAs had not passed through the rate-limiting step. By contrast, transcripts in complexes paused on the +9, +10, +11, +12, and +14T promoters were extended with rates similar to that measured on the +16T promoter (upper dashed line). The short RNA products formed on the +8, +9, and +10T promoters, as well as representative extension rate curves for the +8 and +9T promoters are shown in Supplementary Figure 2. Notably, a five-fold jump in the extension rate constant occurred between the +8 and +9T promoters, corresponding to paused RNA lengths of 7 and 8 nt. A second, less substantial increase (approximately two-fold) occurs after synthesis of an 11 nt RNA, and may be divided between two or more steps (compare the +12, +14, and +16T promoters). These results show that the rate-limiting step occurs after synthesis of a 7 nt RNA (i.e. extension on the +8T promoter is slow) and before binding of the ninth NTP (i.e. extension on the +9T promoter is fast). This stage of the reaction minimally involves loading of the eighth NTP, catalysis to form an 8 nt RNA, and translocation of the Pol II active site to the ninth register.

To begin to explore the mechanism by which the position of the rate-limiting step is set, we took advantage of the ability of Pol II to assemble into functional transcription complexes in the absence of general transcription factors when incubated with a template DNA strand annealed to a complementary RNA oligonucleotide (Kireeva *et al*, 2000). As diagrammed in Figure 2C, a single-stranded DNA oligonucleotide comprising the AdMLP template DNA was annealed to either a 7 or 8 nt ³²P-labeled RNA oligonucleotide (complementary to the +1/+7 or +1/+8 regions), and Pol II was

added to form ternary complexes. Nucleotides were then added and the portion of the RNA oligonucleotide that was extended to full-length (29 nt) RNA was measured after 2 and 20 min. As shown in Figure 2C, extension of the 7 nt RNA was much slower than extension of the 8 nt RNA. The ratios of full-length RNA observed at 2 and 20 min were similar to those expected from the rate constants measured for extension of 7 and 8 nt RNAs using the minimal transcription system (Figure 2B). These results indicate that the position

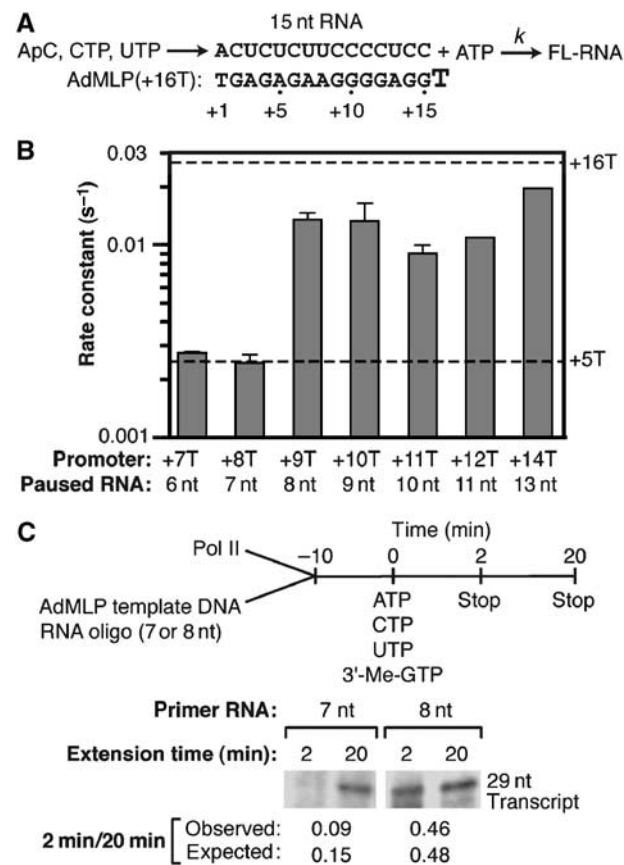


Figure 2 The rate-limiting step occurs after synthesis of a 7 nt RNA and before loading the ninth NTP. (A) The approach used to determine the position of the rate-limiting step during promoter escape. See text for description. (B) The rate-limiting step occurs at a specific position during early transcription. Rate constants for extension of short paused RNA transcripts to full-length transcripts on the +7, +8, +9, +10, +11, +12, and +14T promoters are plotted logarithmically and compared to those measured on the +16 and +5T promoters (dashed lines). Where error bars are shown, they represent one standard deviation and were obtained from the average of at least three experiments. The rate constants are as follows: +7T, $2.8 \pm 0.05 \times 10^{-3} \text{ s}^{-1}$; +8T, $2.5 \pm 0.3 \times 10^{-3} \text{ s}^{-1}$; +9T, $13 \pm 1 \times 10^{-3} \text{ s}^{-1}$; +10T, $13 \pm 3 \times 10^{-3} \text{ s}^{-1}$; +11T, $9.0 \pm 0.9 \times 10^{-3} \text{ s}^{-1}$; +12T, $11 \times 10^{-3} \text{ s}^{-1}$; +14T, $20 \times 10^{-3} \text{ s}^{-1}$. (C) The position of the rate-limiting step is independent of the general transcription factors. Ternary complexes were assembled from Pol II, AdMLP template DNA, and RNA oligonucleotides (7 or 8 nt) as shown in the schematic, and 29 nt extended RNA transcripts were monitored. The observed ratios of 29 nt transcripts at 2 and 20 min were determined and compared to the expected ratios for extension of 7 and 8 nt RNAs calculated from the rate constants in panel B. The efficiency with which the 7 nt RNA was extended was significantly less than that for the 8 nt RNA, as was expected from published work (Kireeva *et al*, 2000). Therefore, the image showing extension of the 7 nt RNA was obtained from a longer exposure than that for the 8 nt RNA.

of the rate-limiting step is set by the polymerase, DNA template, and/or transcript RNA, and is not set by the general transcription factors.

The last point in early transcription requiring negative superhelicity occurs between synthesis of a 7 nt and a 9 nt RNA

In addition to limiting the overall rate of RNA synthesis, the steps of early transcription are also sensitive to the conformation of the DNA (Parvin and Sharp, 1993; Goodrich and Tjian, 1994). This is illustrated in Supplementary Figure 3A, where in a minimal transcription system (i.e. in the absence of TFIIE and TFIIH) there is no detectable synthesis of a 15 nt RNA from preinitiation complexes assembled on linear DNA. By contrast, under the same experimental conditions, 3 nt abortive products can be produced from both linear and negatively supercoiled templates (Goodrich and Tjian, 1994; Kumar *et al*, 1998). Together, these observations indicate that in a minimal transcription system, one or more steps between synthesis of a 3 nt and a 15 nt RNA require negative superhelicity.

With the goal of identifying the last point in early transcription that requires negative superhelicity, we developed a linearization—extension assay. Transcription was paused at different positions on negatively supercoiled plasmids using the method and mutant promoters previously described. The plasmids were then digested with *HindIII*, and ATP was added to allow extension of the paused transcripts to full-length RNA. Control reactions were performed in which *HindIII* was omitted. Rapidly linearizing plasmids containing ternary complexes paused at different positions during early transcription allowed us to determine which paused transcripts were capable of being extended in the absence of negative superhelicity. A schematic depicting the experimental setup of the linearization—extension assay is shown in Supplementary Figure 3B. Supplementary Figure 3C shows that *HindIII*, which cuts upstream of the AdMLP promoter at position -179, completely linearizes plasmid DNA within 20 s under transcription conditions.

The bar plot in Figure 3 shows the ratio of full-length product formed with and without linearization (cut/uncut) for each promoter assayed. Reactions performed on the +5, +7, and +8T promoters show cut/uncut ratios much less

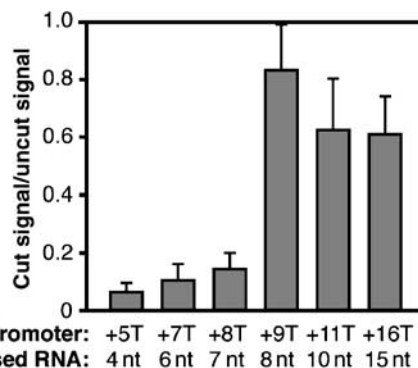


Figure 3 The last step requiring negative superhelicity occurs after synthesis of a 7 nt RNA and before loading the ninth NTP. The ratio of full-length transcript produced with and without cutting is plotted versus promoter. Each bar represents the average of at least three experiments and error bars are one standard deviation.

than 1, indicating that complexes on these promoters were paused before a step requiring negative superhelicity, and the short RNAs were not efficiently extended. By contrast, reactions performed on the +9, +11, and +16T promoters show cut/uncut ratios approaching 1, indicating that these complexes were paused after the last step requiring negative superhelicity, and extension occurred. Representative primary data on the +8 and +9T promoters are shown in Supplementary Figure 3D. Notably, a four-fold increase in the cut/uncut ratio was observed between the +8 and +9T promoters. Therefore, RNA transcripts 7 nt and shorter cannot be efficiently extended to full-length RNA in the absence of negative superhelicity; however, transcripts 8 nt and longer can be extended. We conclude that the last point in early transcription that shows a substantial requirement for negative superhelicity occurs after synthesis of a 7 nt RNA and before binding of the ninth NTP. This is the same region of the reaction in which the rate-limiting step occurs.

The positions of the rate-limiting step and the last step requiring negative superhelicity are set by the length of the RNA transcript

To further probe the mechanisms of the rate-limiting step and the last step influenced by negative superhelicity, we asked whether the positions of these steps are set by the length of the RNA transcript or the position of the polymerase active site relative to promoter DNA elements such as the TATA box. To do so, we took advantage of the ability of Pol II to initiate transcription at -1 or +2 using sequence-specific dinucleotides. We first monitored the rate of extension of short RNAs in ternary complexes paused on the +8T promoter when the start site was changed from +1 to -1 by replacing ApC with CpA (top schematic in Figure 4A). This increased the length of the paused RNA from 7 to 8 nt, without changing the position of the active site in the paused complex. The top two bars in Figure 4B show that moving the initiation site from +1 (open bar) to -1 (closed bar) on the +8T promoter caused a five-fold increase in the rate constant for extension. Hence, the position of the rate-limiting step correlated with the length of the RNA; extension of 7 nt RNA was slow and extension of 8 nt RNA was fast irrespective of the initiation site. The position of the rate-limiting step did not correlate with the position of the active site in relation to core promoter elements, because the rate of extension changed whereas the position of the active site in paused complexes did not. We also moved the initiation site on the +9T promoter from +1 to +2 by replacing ApC with CpU, which changed the length of the paused RNA from 8 to 7 nt (Figure 4A, bottom schematic). As shown in the bottom two bars of Figure 4B, doing so caused a five-fold decrease in the rate constant for extension. The short, paused RNA products formed on the +8T promoter using CpA and on the +9T promoter using CpU are shown in Supplementary Figure 4. Together, the data in Figure 4B show that the rate-limiting step is complete after synthesis of an 8 nt RNA regardless of the initiation site and the position of the Pol II active site with respect to core promoter elements. We therefore conclude that the length of the RNA (8 nt) sets the position of the rate-limiting step.

We predicted that this would also be the case for the last step requiring negative superhelicity. To test this, we performed linearization-extension assays with the same combinations of mutant promoters and dinucleotides discussed

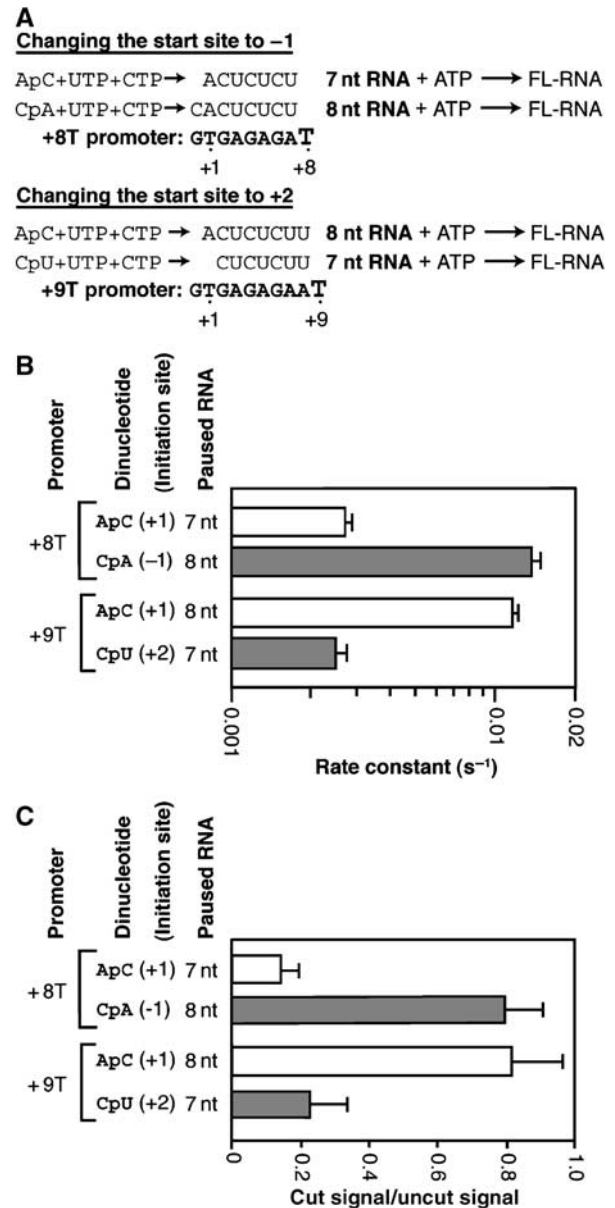


Figure 4 The positions of the rate-limiting step and the last step requiring negative superhelicity are set by the length of the RNA transcript. (A) Transcription can be initiated at -1 or +2 using the dinucleotide CpA or CpU, respectively. Shown are the sequences of the template strands of the +8 and +9T promoters and the paused RNA transcripts when transcription is initiated with ApC, CpA, or CpU. (B) The rate of extension of paused transcripts to full-length RNA is dictated by the length of the transcript in paused ternary complexes. The closed bars depict the rate constants for extension of paused transcripts initiated with CpA on the +8T promoter ($11 \pm 0.5 \times 10^{-3} \text{ s}^{-1}$) and CpU on the +9T promoter ($2.7 \pm 0.3 \times 10^{-3} \text{ s}^{-1}$). Rate constants are the average of two experiments and the error bars are the range of two rate constants. The open bars depict the rate constants for extension of paused transcripts initiated at +1 with ApC on the +8 and +9T promoters (from Figure 2B). (C) The position of the last step requiring negative superhelicity is dictated by RNA length. Transcription was initiated at -1 or +2 using the dinucleotide CpA or CpU, respectively. The closed bars depict the ratio of full-length transcript produced from paused ternary complexes initiated with CpA on the +8T promoter or CpU on the +9T promoter. The open bars depict the ratio of full-length transcript produced from paused ternary complexes initiated with ApC on either the +8T promoter or the +9T promoter (from Figure 3). All bars are an average of at least three experiments and error bars are one standard deviation.

above. Shown in Figure 4C are the cut/uncut ratios of transcripts produced on the +8 and +9T promoters when transcription was initiated at +1 with ApC (open bars), at -1 with CpA (upper closed bar), or at +2 with CpU (lower closed bar). Forcing initiation at -1 on the +8T promoter increased the length of the paused transcript from 7 to 8 nt and accordingly increased the cut/uncut ratio. By contrast, forcing initiation at +2 on the +9T promoter decreased the length of the paused transcript from 8 to 7 nt, which decreased the cut/uncut ratio. Thus, paused complexes containing 8 nt transcripts had passed the last point in the reaction at which negative superhelicity was required, irrespective of where transcription initiated and where the polymerase paused on the DNA template. This was not true for complexes containing 7 nt RNA transcripts. Taken together, the data in Figure 4 show that the RNA transcript itself plays a critical role in the mechanism of promoter escape.

The rate-limiting step occurs after synthesis of an 8 nt RNA, during the stage of translocation

Our data show that the rate-limiting step occurs after synthesis of a 7 nt RNA and before binding of the ninth NTP. As illustrated by the model in Figure 5A, this minimally encompasses three stages (each of which could consist of multiple steps): loading of the eighth NTP (N), catalysis to form the seventh phosphodiester bond (C), and translocation of the Pol II active site to the ninth register (T). To ask which of these stages is rate-limiting, we measured the rate at which 8 nt RNA is synthesized from preinitiation complexes. If synthesis of 8 nt RNA is fast, then the stage of translocation must be rate-limiting. If synthesis of 8 nt RNA is slow, then either the NTP loading or catalysis stage must be rate-limiting. As shown in Figure 5B, 8 nt RNA accumulated rapidly with a rate constant of $19 \times 10^{-3} \text{ s}^{-1}$. Thus, all steps through synthesis of the seventh phosphodiester bond occur before the rate-limiting step. We therefore conclude that the rate-limiting step occurs after synthesis of an 8 nt RNA, during the stage that encompasses translocation of the Pol II active site to the ninth register.

Two possibilities for the trigger that sets the position of the rate-limiting step are (1) RNA length itself (i.e. the physical presence of an 8 nt transcript) or (2) the presence of an 8 bp RNA:DNA hybrid. We directly tested whether an 8 bp RNA:DNA hybrid is required to get through the rate-limiting step by initiating transcription with the trinucleotide ApApC, which can anneal to the +1 and +2 positions of the AdMLP template strand. However, the 5' adenosine in this trinucleotide is not complementary to the -1 position of the template DNA. Hence, in paused ternary complexes, the maximal RNA:DNA hybrid length was 1 bp shorter than the transcript length. Rate constants were measured for extension of paused transcripts initiated with ApApC on the +5, +7, +8, and +9T promoters. Figure 5C shows that a jump in the extension rate constants occurs between the +7 and +8T promoters; a 7 nt RNA that is part of an RNA:DNA hybrid with a maximal length of 6 bp is extended slowly, whereas an 8 nt RNA that is part of an RNA:DNA hybrid with a maximal length of 7 bp is extended rapidly. We therefore conclude that an 8 bp RNA:DNA hybrid is not required to complete the rate-limiting step. Rather, our results are consistent with a model in which the physical presence of an 8 nt RNA triggers the rate-limiting step.

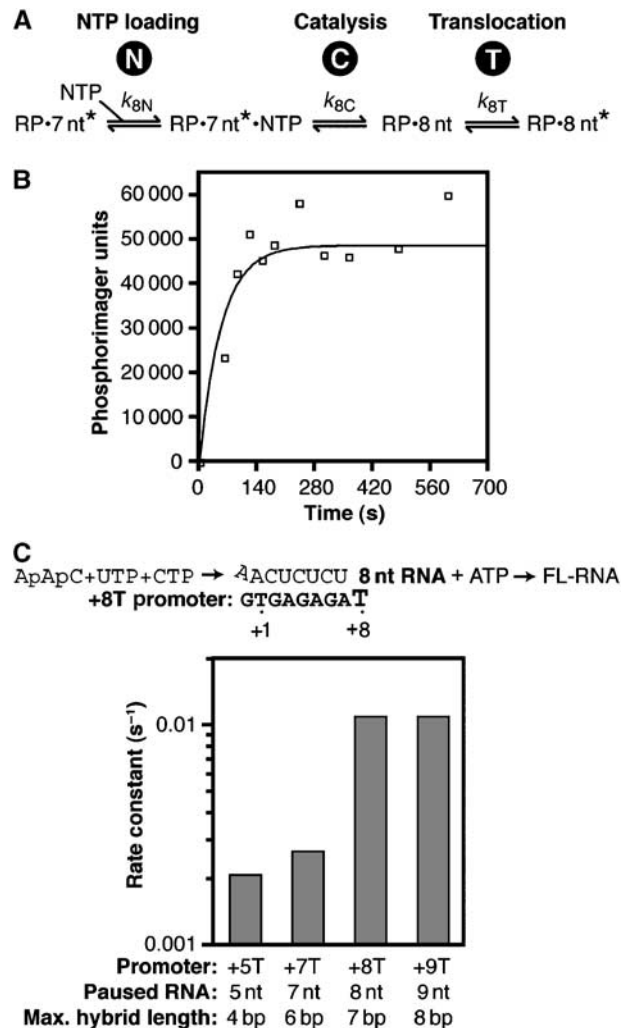


Figure 5 The rate-limiting step occurs after synthesis of an 8 nt RNA. (A) The model shows the three stages of the reaction that could be rate-limiting: N, loading of the eighth NTP; C, synthesis of the seventh phosphodiester bond; and T, translocation to the ninth register. Ternary complexes denoted with an asterisk are post-translocated, whereas those lacking an asterisk are pre-translocated. (B) An 8 nt RNA is synthesized rapidly, with a rate constant of $19 \times 10^{-3} \text{ s}^{-1}$. The +8T9C promoter and the dinucleotide CpA were used to limit read-through and slipped products. 3'-Me-GTP was not included. (C) An 8 bp RNA:DNA hybrid is not needed to complete the rate-limiting step. The nucleotides used are shown to highlight the point that the 5' ends of RNAs initiated with ApApC were not complementary to the DNA. The rate constants for extension of 5, 7, 8, and 9 nt RNAs initiated with ApApC are plotted logarithmically. The rate constants are as follows: +5T, $2.1 \times 10^{-3} \text{ s}^{-1}$; +7T, $2.7 \times 10^{-3} \text{ s}^{-1}$; +8T, $11 \times 10^{-3} \text{ s}^{-1}$; +9T, $11 \times 10^{-3} \text{ s}^{-1}$.

The last step that requires negative superhelicity is coincident with the rate-limiting step

To determine the position of the last step requiring negative superhelicity relative to the rate-limiting step, we measured the rate at which transcription becomes resistant to template linearization using the method diagrammed in Figure 6. Following the addition of nucleotides to preinitiation complexes, the plasmid containing the wild-type promoter was rapidly linearized at varying time points with HindIII. After linearization, transcription continued for 20 min, thereby allowing complexes that had proceeded beyond the last

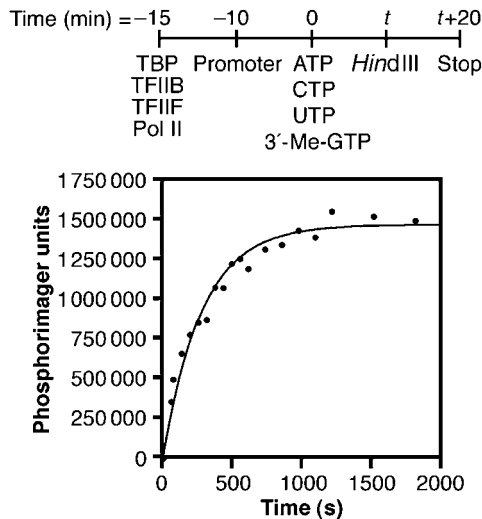


Figure 6 The last step requiring negative superhelicity is coincident with the rate-limiting step. The rate at which Pol II becomes resistant to linearization is slow, with a rate constant of $3.8 \times 10^{-3} \text{ s}^{-1}$. The diagram depicts the method used to determine the rate at which transcription becomes resistant to linearization.

point requiring negative superhelicity to complete synthesis of full-length RNA. Ternary complexes that did not reach this point before linearization were blocked, and therefore did not contribute to the full-length RNA observed. The plot in Figure 6 shows that full-length RNA accumulates slowly with a rate constant of $3.8 \times 10^{-3} \text{ s}^{-1}$, showing that the last point in the reaction at which negative superhelicity is required occurs either coincident with or after the rate-limiting step. We can distinguish between these two possibilities by considering the result in Figure 6 in the context of earlier data: the rate-limiting step occurs during translocation to the ninth register (Figure 5B) and negative superhelicity does not affect the reaction after this point (Figure 3). Therefore, the only position at which the last step requiring negative superhelicity could occur is coincident with the rate-limiting step—during the stage of translocation to the ninth register.

TFIIE and TFIIH do not change the position of the rate-limiting step and can rescue ternary complexes paused on linearized templates

Our previous data showed that TFIIE and TFIIH caused a two-fold increase in the rate constant for synthesis of 15 nt RNA at the AdMLP (Kugel and Goodrich, 1998). We asked whether these factors affected the position of the rate-limiting step by measuring (1) the rate constant for synthesis of an 8 nt RNA and (2) the rate constant for extension of an 8 nt RNA. Both would be fast if the presence of TFIIE and TFIIH does not alter the position of the rate-limiting step. As shown in Figure 7A and B, respectively, in the presence of TFIIE and TFIIH (and ATP or dATP), 8 nt RNA was both synthesized rapidly ($k = 42 \times 10^{-3} \text{ s}^{-1}$) and extended rapidly ($k = 28 \times 10^{-3} \text{ s}^{-1}$). As a control, we determined the rate of extension of 4 nt RNA in the presence of TFIIE and TFIIH and found it to be relatively slow with a rate constant of $5.8 \times 10^{-3} \text{ s}^{-1}$, as expected (Kugel and Goodrich, 1998). Because an 8 nt RNA was both synthesized and extended rapidly, whereas 4 nt

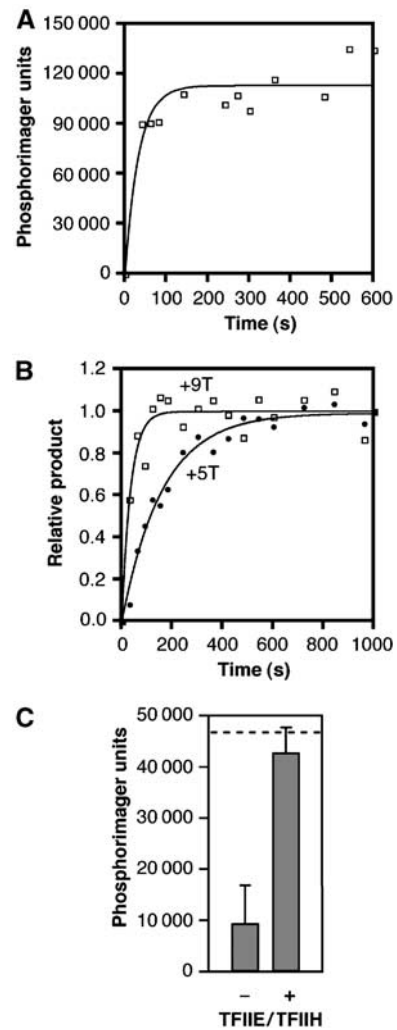


Figure 7 The effect of TFIIE and TFIIH on the rate-limiting step and the requirement for negative superhelicity. (A) An 8 nt RNA is synthesized rapidly from preinitiation complexes assembled with TFIIE and TFIIH. Reactions were performed using the +9T promoter and the dinucleotide ApC. dATP (100 μM) was included in the nucleotide mix as a source of hydrolyzable nucleotide for the TFIIH helicase. The rate constant is $42 \times 10^{-3} \text{ s}^{-1}$. (B) In the presence of TFIIE and TFIIH, 8 nt RNA is extended to full length rapidly. The rates of extension were measured in the presence of TFIIE and TFIIH for 4 and 8 nt RNAs formed on the +5 and +9T promoters, respectively. Rate constants are as follows: +5T, $5.8 \times 10^{-3} \text{ s}^{-1}$; +9T, $28 \times 10^{-3} \text{ s}^{-1}$. (C) TFIIE and TFIIH rescue paused ternary complexes containing 7 nt RNA on linearized DNA templates. Paused complexes containing 7 nt transcripts were formed on the +8T promoter, DNA was linearized with *Hind*III, TFIIE and TFIIH were added to half of the reactions, and 1 min later extension nucleotides were added. The amount of full-length RNA was quantitated. Bars are an average of at least three experiments and error bars are one standard deviation. The dashed line is the amount of full-length transcript produced in control reactions in which TFIIE and TFIIH were added to preinitiation complexes on linearized DNA templates 1 min before the pulse nucleotides.

RNA was extended slowly, we conclude that the presence of TFIIE and TFIIH does not alter the position of the rate-limiting step; it still occurs during translocation of the Pol II active site to the ninth register.

It has previously been shown that the addition of TFIIE and TFIIH to a minimal transcription system can substitute for negative superhelicity and allow full-length transcripts to

be synthesized from preinitiation complexes assembled on linear DNA templates (Goodrich and Tjian, 1994). We asked whether adding TFIIE and TFIIH to linearized templates containing ternary complexes would allow extension of the short transcripts to full-length RNA. Ternary complexes containing 7 nt RNAs were assembled on the +8T promoter. The DNA was then linearized with *Hind*III, and soon after TFIIE and TFIIH were added. After a 1 min incubation, extension nucleotides were added and full-length RNA was monitored. As shown by the bar plot in Figure 7C, adding TFIIE and TFIIH to ternary complexes containing 7 nt RNAs caused a substantial increase in the amount of full-length RNA produced during the extension reaction on the linearized template. In fact, the amount of full-length RNA produced when TFIIE and TFIIH were added to paused complexes was similar to that produced in control reactions in which TFIIE and TFIIH were added to preinitiation complexes on a linearized template before initiating transcription (indicated by the dashed line). Therefore, TFIIE and TFIIH rescued paused ternary complexes that were blocked by template linearization, showing that these factors can affect the activity of transcribing complexes that are in the midst of promoter escape.

Discussion

We found that in a minimal Pol II transcription system, both the rate-limiting step and the last step requiring negative superhelicity occur after synthesis of an 8 nt RNA, during the stage encompassing translocation of the polymerase active site to the ninth register. Importantly, the length of the growing RNA transcript sets the positions of these steps; therefore, the transcript RNA itself plays a fundamental role in the mechanism of promoter escape. Moreover, the position of the rate-limiting step is not influenced by the general transcription factors. TFIIE and TFIIH can associate with ternary complexes blocked by template linearization, thereby rescuing these complexes and allowing extension of a 7 nt RNA to full-length RNA. These studies lead to a model in which synthesis of an 8 nt RNA triggers the completion of promoter escape and formation of elongation complexes during a rate-limiting stage of the reaction that encompasses translocation of the Pol II active site to the ninth register. This conversion to elongation complexes can be facilitated by under-winding in the DNA template via negative superhelicity or the action of TFIIE and TFIIH.

Critical transformations during early transcription: forming elongation complexes from preinitiation complexes

Forming elongation complexes from preinitiation complexes involves a complex series of molecular events during early RNA synthesis, aspects of which are diagrammed in the linear reaction pathway shown in Figure 8. Highlighted are two fundamental conversions in ternary transcribing complexes that occur during distinct stages encompassing translocations to the fifth and ninth registers. After synthesis of a 4 nt RNA, the polymerase active site translocates to the fifth register, during which a rapid transformation (escape commitment) occurs that results in stable escape committed complexes (RP_{EC}) (Kugel and Goodrich, 2000). The data presented here regarding two events, the rate-limiting step and the last step requiring negative superhelicity, allowed us to identify a second critical conversion, which occurs during a stage encompassing translocation of the polymerase active site to the ninth register and marks the first point at which an elongation complex can form (R_E). The two events highlighted in Figure 8 have strikingly similar characteristics. They both occur during translocation stages, are triggered by RNA length, occur in the absence of general transcription factors, and can be facilitated by TFIIE and TFIIH. These observations suggest that translocation of the active site, which is inherent to the RNA polymerase, is the stage most amenable to structural rearrangements in the ternary complex during early transcription. Moreover, the polymerase can sense the length of the RNA transcript to trigger critical transformations.

The structure of Pol II in an elongation complex containing a 10 nt RNA (Westover *et al*, 2004) provides insight into why translocation to the ninth register triggers the formation of an elongation complex. In this structure, the polymerase active site has translocated after bond synthesis and is poised to bind the next incoming nucleotide. The stable RNA:DNA hybrid in this complex is 7 bp: nucleotides 4–10 of the RNA (counting from the 5' end) are fully hydrogen bonded to the template strand of the DNA, nucleotide 3 is beginning to peel away from the DNA, and nucleotides 1 and 2 are completely separated from the DNA (Westover *et al*, 2004). This leads to the model that the RNA:DNA hybrid in an elongation complex will oscillate between 7 and 8 bp during each cycle of catalysis and translocation. The first point in the transcription reaction at which ternary complexes could resemble an elongation complex is during translocation to the ninth

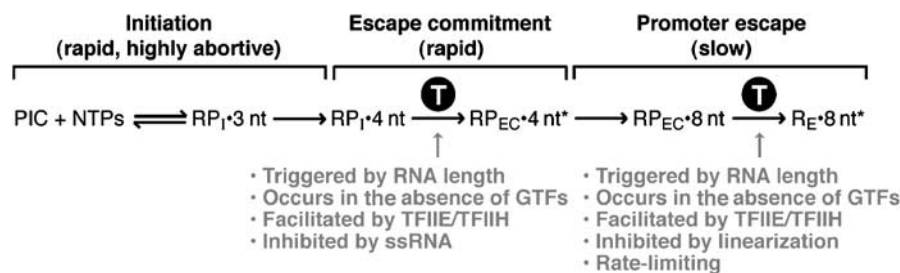


Figure 8 A model depicting two critical transformations that occur as preinitiation complexes transform into elongation complexes. Both escape commitment and promoter escape are complete during translocation stages (T), are triggered by RNAs of distinct sizes (4 and 8 nt), and occur in the absence of general transcription factors (GTFs). Ternary complexes denoted with an asterisk are post-translocated, whereas those lacking an asterisk are pre-translocated.

register after synthesis of an 8 nt RNA. At this point, the 5' end of the transcript can begin to peel away from the DNA and 7 bp of the RNA can be stably hydrogen bonded to the DNA. This is precisely the point at which the rate-limiting step occurs and when ternary complexes no longer require negative superhelicity, suggesting that the final transformation into elongation complexes happens at this critical juncture in the reaction, thereby providing a kinetic definition for the completion of promoter escape. Others have identified transformations that occur further downstream of the events we studied here that influence the clearance of the polymerase from the promoter, including collapse of the upstream region of the transcription bubble (Holstege *et al*, 1997; Pal *et al*, 2005), Pol II pausing during early elongation (Pal *et al*, 2001), and a structural transition involving downstream DNA (Dvir *et al*, 1997). These transformations most likely occur after the rate-limiting step characterized in our studies.

Our biochemical studies showed that the rate-limiting transformation occurs at the same position in the absence of all general transcription factors, as well as in the presence of TBP, TFIIB, TFIIE, TFIIF, and TFIIH. In addition, we found that an 8 nt RNA and not an 8 bp RNA:DNA hybrid is required for the rate-limiting transformation to occur. Hence, the polymerase itself senses the size of the transcript as the trigger for transforming from early transcribing complexes into elongation complexes, and the position of this critical juncture is dictated only by the Pol II/DNA/RNA complex. This suggests that the position of the rate-limiting step could be uniform across systems and at other promoters. Our previous studies of the effect of promoter sequence on the rate of transcription are consistent with this proposal. In comparative studies of the AdMLP and the human IL-2 promoter, we found that the rate of transcript synthesis can be set by the thermodynamic stability of the RNA:DNA hybrid at positions +7/+8, leading to the conclusion that the rate-limiting step could not occur before synthesis of an 8 nt RNA (Weaver *et al*, 2005). Considering our studies together, we propose a model in which promoter sequence and general factors affect the rate of promoter escape.

In the case of the rate-limiting step, the actual translocation of the active site to the ninth register might be slow or the rate-limiting step might involve a conformational change in the complex that occurs either immediately before or after translocation to the ninth register (but before binding of the ninth NTP). Our studies do not yet allow us to distinguish between these possibilities, or to favor one more than the other. Burton and colleagues performed kinetic studies of elongation complexes and propose that translocation of the polymerase active site is driven by binding of the two incoming NTPs downstream of the active site (Nedialkov *et al*, 2003; Gong *et al*, 2005). When considered in the context of this model, the rate-limiting step could not occur during the actual translocation to the ninth register, but would involve a conformational change immediately before translocation, because the rate-limiting step is complete after synthesis of an 8 nt RNA even in the absence of the ninth NTP.

Implications of a rate-limiting transformation facilitated by negative superhelicity

We found that TFIIE and TFIIH do not change the position of the rate-limiting step; however, they have a two-fold effect on its rate. In addition, the rate-limiting step is coincident with

the last point in the reaction at which negative superhelicity or TFIIE and TFIIH are required, suggesting that the helicase activity of TFIIH is not required beyond register +9. We also found that the transcriptional activity of ternary complexes blocked by linearizing the DNA template can be rescued via the addition of TFIIE and TFIIH. Together, our observations show that the slow transformation demarcating the formation of an elongation complex is influenced by the conformation of the DNA, and either under-winding of the template or TFIIE/TFIIH is required for it to occur. Our studies are consistent with others showing that dATP, and by implication the TFIIH helicase, was no longer required after bubble collapse, which occurred after synthesis of an 8 nt RNA from a promoter similar to the AdMLP used here (Pal *et al*, 2005). Moreover, the position of bubble collapse can be influenced both by the size of the RNA transcript and the distance between the TATA box and the transcription start site (Pal *et al*, 2005). It will be interesting to determine how altering the spacing between the TATA box and the start site affects the rate of transcription and the last position requiring negative superhelicity.

Structural and biochemical studies have indicated that TFIIB plays a role in the formation of elongation complexes. The yeast Pol II/TFIIB co-crystal structure shows that the N-terminus of TFIIB projects into the active center of the polymerase and occupies space that will ultimately contain the growing RNA transcript, leading to the prediction that TFIIB must vacate this space before the RNA is 9–10 nt in length (Bushnell *et al*, 2004). The N-terminal domain of TFIIB is also positioned in the active center of the polymerase in the context of preinitiation complexes (Chen and Hahn, 2003). Previous biochemical studies found that TFIIB releases from transcribing complexes by position +10 (Zawel *et al*, 1995). It has also been proposed that the removal of TFIIB from the polymerase active center is coupled to collapse of the upstream edge of the transcription bubble, and that this collapse marks the completion of the remodeling of ternary complexes that occurs during promoter escape (Pal *et al*, 2005). Our observation that the rate-limiting step occurs at a similar position with ternary complexes assembled in the absence of general transcription factors shows that TFIIB does not cause the slow step. It remains possible that ejection of TFIIB from the active center of the polymerase is triggered by the rate-limiting transformation and occurs after synthesis of an 8 nt RNA.

It is clear that events in early Pol II transcription are subject to regulation in cells. Paused ternary complexes have been observed close to the transcription start sites of some promoters (Krumm *et al*, 1995; Rasmussen and Lis, 1995). In the best studied example, the *Drosophila* HSP70 gene harbors paused elongation complexes containing approximately ~25 nt RNA transcripts (Rougvie and Lis, 1988). As this phenomenon is studied further at other genes, it will be interesting to determine whether any paused complexes contain 8 nt RNA transcripts. Given the complexity of eukaryotic nuclei, we do not propose that the transition to elongation complexes is rate or extent limiting in cells. It does, however, seem possible that this specific transformation in ternary complexes occurs at the same position in cells and could provide a means for proteins that affect DNA conformation to regulate transcription. For example, topoisomerases I and II have been shown to affect the transcriptional

activity of several genes *in vivo* (Collins *et al*, 2001); perhaps topoisomerases can target the transition between promoter escape and elongation. Activators and repressors previously shown to regulate promoter escape (Kumar *et al*, 1998; Liu *et al*, 2000, 2001; Fukuda *et al*, 2002, 2004; Weber *et al*, 2005) could target the rate or extent of steps occurring during translocation to the ninth register, possibly via TFIIF. In addition, the regulation of chromatin structure at promoters in cells could impart a localized change in DNA conformation that either facilitates or impedes the transition from initiation to elongation complexes.

Materials and methods

Preparation of transcription factors and template DNAs

Recombinant human TBP, TFIIB, TFIIE, TFIIF, and native human Pol II and TFIIF were prepared as described previously (Kugel and Goodrich, 1998; LeRoy *et al*, 1998; Weaver *et al*, 2005). The wild-type DNA template used in reactions was plasmid pBS-MLP-100G-less, which contains the AdMLP core promoter sequence from -53 to +10 fused to a 90bp G-less cassette. This construct was made using PCR to shorten the 390bp G-less cassette contained on plasmid pBS-MLP-G-less (Goodrich and Tjian, 1994) to 90bp. The PCR fragment was subcloned into the *EcoRI* and *HindIII* sites of pBluescript(KS+). Mutants in the early transcribed region were created by site-directed mutagenesis or PCR in the context of pBS-MLP-100G-less.

In vitro transcription reactions

Reactions were performed at 30°C in buffer A containing 10% glycerol, 10 mM Tris (pH 7.9), 50 mM KCl, 10 mM Hepes (pH 7.9), 4 mM MgCl₂, 1 mM DTT, 25 µg/ml BSA, 0.02% NP-40, and 15 U/ml of RNA Guard (GE Biosciences). Transcription factors and template DNA were used at the following final concentrations: 3.5 nM TBP, 10 nM TFIIB, 2 nM TFIIF, 1–3 nM Pol II, and 1–2 nM DNA template. Transcription factors were incubated for 3 min, template DNA was added, and the incubation was continued for 10 min. Transcription was initiated by addition of a pulse NTP solution resulting in final concentrations of 25 µM UTP, 1 µM [α -³²P]CTP (5 µCi/reaction), and 500 µM dinucleotide (ApC (Sigma-Aldrich), CpA (Dharmacon), or CpU (Dharmacon)). After 15 s, a chase NTP solution was added that increased the CTP concentration to 50 µM. After 20 min, an extension NTP solution was added, resulting in final concentrations of 625 µM CTP, 625 µM ATP, and 100 µM 3'-Me-GTP (GE

Biosciences). Reactions were quenched at different time points using 3.1 M ammonium acetate, 20 mM EDTA, 10 µg of carrier yeast RNA, and 15 µg proteinase K. Transcripts were ethanol precipitated and resolved by 8% PAGE. The experiments in Figure 7 contained TFIIE-34 (9 nM), TFIIE-56 (5 nM), and TFIIF (0.1–0.2 µl). Where indicated, template DNA was linearized using 10 U of *HindIII* (New England Biolabs). To measure the rate of synthesis of 8 nt transcripts (Figures 5B and 7A), the extension NTP solution was omitted, the final CTP concentration was 25 µM, and transcripts were resolved by 20% denaturing PAGE.

Ternary complex assembly

Assembly of Pol II into ternary complexes in the absence of general transcription factors was performed as described (Kireeva *et al*, 2000). Briefly, reactions were performed in buffer A and 7 nt and 8 nt RNA oligonucleotides (1.3 µM; Dharmacon) were ³²P-labeled and annealed to template strand AdMLP DNA (-10 to +28 with a G at +29) (0.67 µM, IDT) by slowly cooling from 45 to 25°C. Pol II (final concentration of ~15 nM) was then added and reactions were incubated for 10 min at 30°C. NTPs were added to final concentrations of 625 µM ATP, 625 µM CTP, 25 µM UTP, and 100 µM 3'-Me-GTP and reactions were stopped either 2 or 20 min later. RNA transcripts were ethanol precipitated and resolved by 14% denaturing PAGE.

Rate constant calculations

Transcripts were imaged via a Molecular Dynamics Typhoon™ Phosphorimager and quantitated using ImageQuant™ software. Phosphorimager units (PI) were plotted versus time in seconds and data were fit with the equation $PI = PI_{max}(1 - e^{-kt})$ using Prism™ software to solve for PI_{max} and k . For the plot in Figure 7B, data were normalized by dividing the PI units at each time point by PI_{max} .

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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