

# Kinetic and Mechanistic Analysis of the RNA Polymerase II Transcription Reaction at the Human Interleukin-2 Promoter

Heather A. Ferguson, Jennifer F. Kugel\* and James A. Goodrich\*

Department of Chemistry and Biochemistry, University of Colorado at Boulder, Campus Box 215, Boulder, CO 80309-0215, USA

Interleukin-2 (IL-2) is a cytokine critical for the proper stimulation of T-cells during the mammalian immune response. Shortly after T-cell stimulation, transcription of the IL-2 gene is upregulated. Here, we studied the kinetic mechanism of basal transcription at the IL-2 promoter using a human *in vitro* RNA polymerase II transcription system. We experimentally divided the transcription reaction into discrete steps, including preinitiation complex formation, initiation, escape commitment, and promoter escape. Using pre-steady state approaches, we measured the rate at which each of these steps occurs. We found that the rate of functional preinitiation complex formation limits the overall rate of transcription at the IL-2 promoter under the conditions described here. Furthermore, we found that the recruitment of TFIIF and RNA polymerase II to a TFIID/TFIIA/TFIIB/promoter complex dictates the rate of preinitiation complex formation. The rate of synthesis of 28 nt RNA from preinitiation complexes was rapid compared to the rate of preinitiation complex formation. Moreover, we found that the synthesis of a four nucleotide RNA was necessary and sufficient to rapidly complete the escape commitment step of transcription at the IL-2 promoter. Comparative experiments with the adenovirus major late promoter revealed that, while the overall mechanism of transcription is the same at the two promoters, promoter sequence and/or architecture dictate the rate of promoter escape. We present a kinetic model for a single round of basal transcription at the IL-2 promoter that provides insight into mechanisms by which the IL-2 gene is transcriptionally regulated.

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**Keywords:** interleukin-2; RNA polymerase II; kinetics; transcription; preinitiation complex formation

\*Corresponding authors

## Introduction

The cytokine interleukin-2 (IL-2) is a key signaling molecule in controlling the mammalian immune response to infection (reviewed by Abbas *et al.*<sup>1</sup>). Proper regulation of IL-2 expression is critical for clonal proliferation and differentiation of T-

lymphocytes after stimulation with foreign antigen. Upon T-cell stimulation, an elaborate cascade of differential gene expression begins. One of the earliest gene products detected is the cytokine IL-2, which is secreted from T-cells within 45 minutes after stimulation.<sup>2</sup> It is now well understood that the regulation of IL-2 expression is controlled primarily at the levels of transcription and mRNA stability.<sup>3–6</sup> IL-2 transcriptional regulation is the end result of signal transduction pathways that trigger the activation and nuclear localization of transcriptional activators that bind to the IL-2 promoter regulatory region (as reviewed by Jain *et al.*<sup>5</sup>). The enhancer region of the IL-2 promoter extends approximately 300 basepairs upstream of the transcription start site and contains elements that bind transcriptional activators from the NFAT, AP-1, Oct, and NF- $\kappa$ B families.<sup>7–9</sup> Signal transduction

Abbreviations used: IL-2, interleukin-2; TFIID, transcription factor of RNA polymerase II; GTF, general transcription factor, AdMLP, adenovirus major late promoter; TBP, TATA-binding protein; TAF, TBP-associated factor; PIC, preinitiation complex; RP<sub>EC</sub>, escape committed complex; R<sub>E</sub>, elongation complex; RP<sub>I</sub>, initiated complex.

E-mail addresses of the corresponding authors: james.goodrich@colorado.edu  
jennifer.kugel@colorado.edu

events controlling the IL-2 transcriptional activators are well understood; however, the mechanisms that regulate transcription at the IL-2 promoter have yet to be described. One approach to understanding how IL-2 transcription is regulated is to measure rate constants for discrete steps in the basal (unregulated) transcription reaction at the IL-2 promoter. It can then be determined how transcriptional activators influence these rates. This *in vitro* approach to studying transcription at a natural mammalian promoter will provide critical insight into how IL-2 gene expression is regulated.

The IL-2 gene is transcribed by RNA polymerase II. The core polymerase enzyme can synthesize RNA from a DNA template, but requires additional general transcription factors (GTFs, including TFIIA, TFIIB, TFIID, TFIIE, TFIIIF, and TFIIH) for promoter-specific initiation of transcription (as reviewed by Orphanides *et al.*<sup>10</sup>). In addition to these general transcription factors, many studies have shown that other coactivator complexes and chromatin remodeling factors are critical to gene regulation.<sup>11–13</sup> Even in the context of chromatin, the GTFs and RNA polymerase II must assemble at the promoter to form preinitiation complexes before transcription can initiate. *In vitro* studies have revealed that the TFIID complex, consisting of the TATA-binding protein (TBP) and associated factors (referred to as TAFs), is responsible for recognizing core promoter elements, and is aided by TFIIA.<sup>14–19</sup> In one model, the remaining general transcription factors and RNA polymerase II then sequentially assemble on promoter-bound TFIID/TFIIA in the following order: TFIIB, TFIIIF/RNA polymerase II, TFIIE, and TFIIH.<sup>20–22</sup> Alternatively, it has been proposed that TFIID and TFIIA bound to promoter DNA can recruit RNA polymerase II and the remaining general transcription factors as a single entity (see Carey<sup>23</sup> and references therein). Once preinitiation complexes form, the DNA around the transcriptional start site is melted in a reaction that can be facilitated by the helicase activity of TFIIH.<sup>24</sup> In the presence of nucleoside triphosphates, preinitiation complexes initiate phosphodiester bond synthesis and the polymerase proceeds through a series of early steps before the RNA elongates and ultimately transcription terminates. These early transcription steps minimally include initiation, escape commitment, and promoter escape.<sup>25</sup> Initiation encompasses synthesis of the first and second phosphodiester bonds. Escape commitment is a unique step that occurs during synthesis of the third phosphodiester bond and results in the formation of stable ternary complexes.<sup>25,26</sup> Promoter escape is a transition that occurs during early phosphodiester bond synthesis as escape committed complexes transform into elongation complexes. Each of these steps in the transcription reaction has the potential to limit the rate of RNA synthesis, and it is likely that different steps limit the rate of transcription at different promoters.

In previous studies of a well-characterized viral promoter (the adenovirus major late promoter, AdMLP), we measured the rates of the aforementioned steps in the RNA polymerase II reaction.<sup>25,27</sup> We found that promoter escape limits the forward rate of a single round of transcription at the AdMLP ( $k_{PE} = 2 \times 10^{-3} \text{ s}^{-1}$ ) and occurs during synthesis of the fourth through 14th phosphodiester bonds. All steps prior to promoter escape were found to be rapid (complete within ten seconds). These studies were performed in a minimal *in vitro* transcription system consisting of only TBP, TFIIB, TFIIIF, and RNA polymerase II. This was possible because not all of the general transcription factors are required under some *in vitro* conditions.<sup>28–33</sup> Studies of the AdMLP in reconstituted transcription systems have revealed much about the intrinsic mechanism of the RNA polymerase II reaction; however, few mechanistic studies of natural human promoters have been performed.

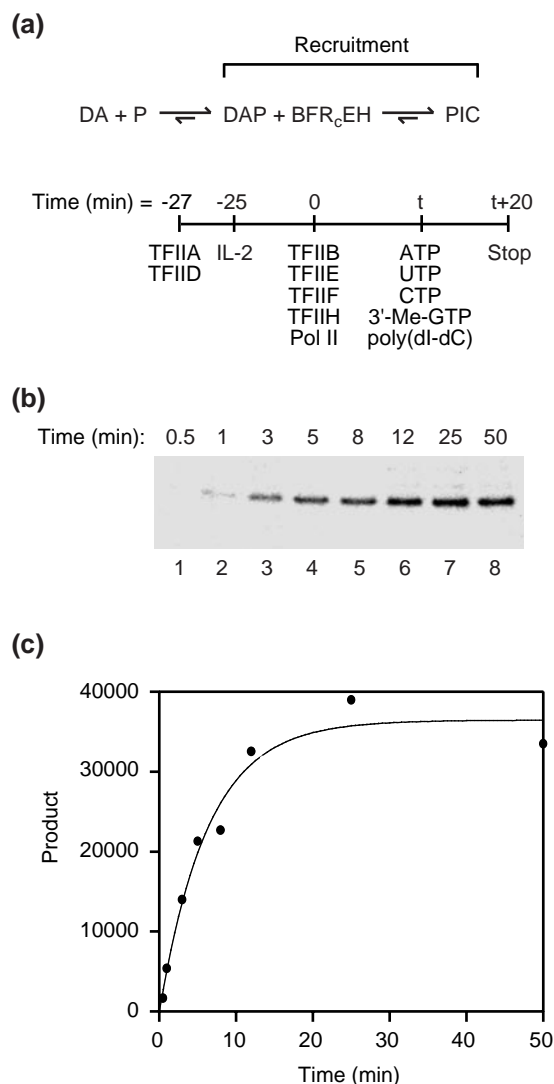
Here, we used a reconstituted RNA polymerase II transcription system to characterize the kinetics of basal transcription at the human IL-2 promoter. Using pre-steady state approaches, we experimentally isolated and measured the rates of preinitiation complex formation, escape commitment, and promoter escape. Our results reveal that recruitment of TFIIIF and RNA polymerase II to the IL-2 promoter limits the overall rate of IL-2 transcription *in vitro*, and that the early steps of RNA synthesis are relatively rapid under the conditions tested here. Notably, we found that the overall mechanism of transcription at the IL-2 promoter is the same as that previously characterized on the AdMLP, but the rate of promoter escape is dramatically different at the two promoters. We present a kinetic model for a single round of basal transcription at the IL-2 promoter that provides insight into the regulation of transcription of the IL-2 gene.

## Results

To determine the kinetic parameters of individual steps in transcription at the human IL-2 promoter, we utilized a transcription system consisting of purified recombinant (TFIIA, TFIIB, TFIIE and TFIIIF) and native (TFIID, TFIIH, and RNA polymerase II) human transcription factors. The schematic of the IL-2 promoter shown in Figure 1(a) indicates both the sequence of the template strand from +1 to +28 and the sequence of the RNA transcript (complementary to the template strand) above the promoter. The first guanosine base in the IL-2 RNA is at position +28. In most experiments we took advantage of this and performed *in vitro* transcription assays in the absence of GTP and the presence of 3'-O-methylguanosine triphosphate (3'-Me-GTP) to permanently pause transcription after incorporation of the 28th nucleotide in the RNA. Doing so provided two experimental advantages:



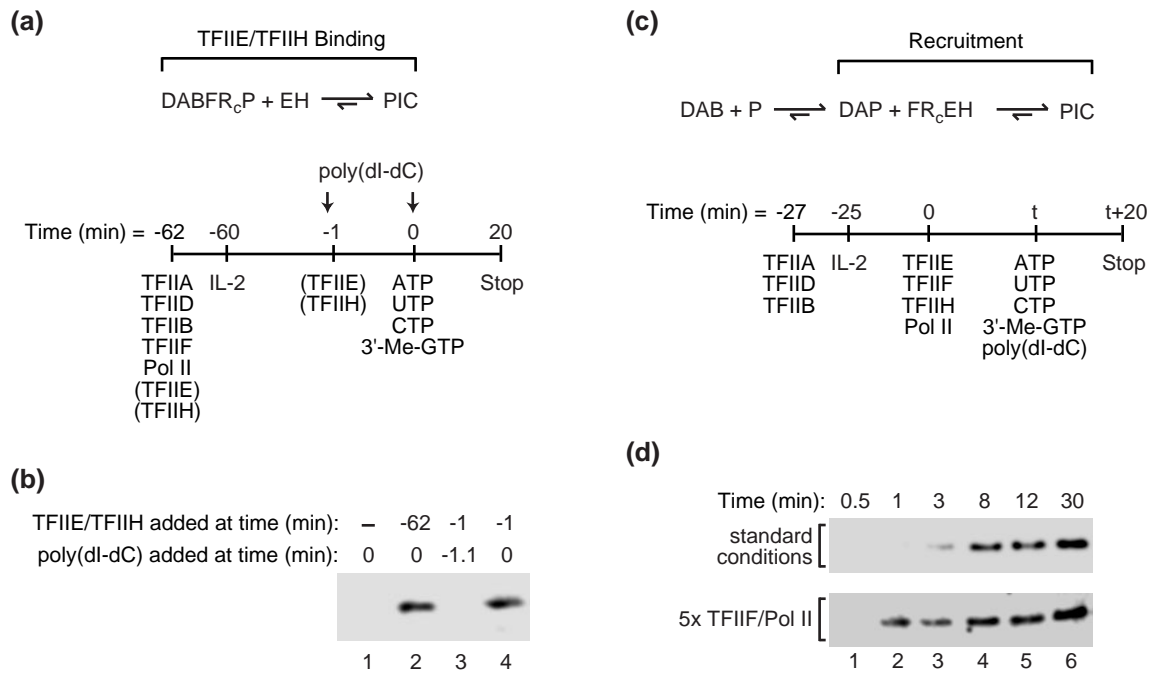




**Figure 3.** The recruitment of TFIIB, TFIIF, and RNA polymerase II to a TFIID/TFIIA/IL-2 complex is slow. (a) The model and schematic depict recruitment of transcription factors to a TFIID/TFIIA/IL-2 complex. The model at the top shows that a TFIID/TFIIA/IL-2 promoter complex (DAP) can recruit the remaining transcription factors ( $BFR_cEH$ ) to form preinitiation complexes. The schematic depicts the method used to monitor the rate of recruitment. TFIID (D), TFIIA (A) and IL-2 promoter DNA (P) were allowed to form a complex for 25 minutes at 30°C. The remaining transcription factors (TFIIB (B), TFIIE (E), TFIIF (F), TFIIH (H), and core RNA polymerase II ( $R_c$ )) were added to reactions. Nucleotides and poly(dI-dC) were added at various times and transcription was stopped 20 minutes later. (b) Rate of recruitment of transcription factors by TFIID/TFIIA bound to the IL-2 promoter. Transcription reactions were performed as described above. The 28 nt RNA products are shown. (c) Plot of the rate of recruitment of transcription factors by TFIID/TFIIA bound to the IL-2 promoter. The data in (b) were fit to a single exponential. The observed rate constant was  $2.9(\pm 0.4) \times 10^{-3} \text{ s}^{-1}$  (average of three independent experiments where the error is one standard deviation).

and TFIIH contributed significantly to the slow rate of recruitment reported above. In a model for ordered assembly, these two factors enter preinitiation complexes after the polymerase.<sup>21</sup> To monitor the rate of TFIIE and TFIIH entry, we performed the experiment diagrammed in Figure 4(a), in which preinitiation complexes lacking TFIIE and TFIIH were assembled on the promoter DNA (results are shown in Figure 4(b)). As expected for promoters contained on linear DNA, preinitiation complexes lacking TFIIE and TFIIH did not produce a transcript (lane 1).<sup>30-33</sup> In contrast, transcription was observed when TFIIE and TFIIH were present during preinitiation complex formation (lane 2). As a control, when poly(dI-dC) was added to assays immediately before TFIIE and TFIIH, the competitor blocked their entry into preinitiation complexes and no transcript was observed (lane 3). To determine whether TFIIE and TFIIH could enter preinitiation complexes quickly, they were added one minute prior to the addition of the nucleotides and poly(dI-dC). As shown in lane 4, they completely assembled into preinitiation complexes within one minute. Because TFIIE and TFIIH assembled into preinitiation complexes so quickly, we conclude that their entry does not limit the rate of recruitment. Therefore, the entry of TFIIB, TFIIF, and/or RNA polymerase II must limit the rate of formation of functional preinitiation complexes.

We next determined the rate at which TFIIF and RNA polymerase II are recruited to a pre-formed TFIID/TFIIA/TFIIB/promoter complex, as diagrammed in Figure 4(c). TFIID, TFIIA, and TFIIB were prebound to the IL-2 promoter prior to adding the remaining transcription factors and RNA polymerase II. Nucleotides and poly(dI-dC) were then added at various time-points and transcription was stopped 20 minutes later. Because we determined that TFIIE and TFIIH enter preinitiation complexes rapidly, this method monitors the rate at which TFIIF and RNA polymerase II are recruited into functional preinitiation complexes. As shown in Figure 4(d) (top panel), functional preinitiation complexes assembled on pre-bound TFIID/TFIIA/TFIIB slowly, with a rate similar to that observed for preinitiation complex assembly as a whole. This indicates that the entry of TFIIF and RNA polymerase II is rate-limiting for the formation of functional preinitiation complexes at the IL-2 promoter under our standard transcription conditions. To determine if the rate of recruitment of TFIIF and RNA polymerase II depends on the concentration of these factors, we increased the amount of TFIIF and RNA polymerase II fivefold and measured the rate at which they were recruited to a TFIID/TFIIA/TFIIB/promoter complex. As shown in the bottom panel of Figure 4(d), the rate increased as the concentration of TFIIF and RNA polymerase II increased, with an observed rate constant of  $0.010(\pm 0.003) \text{ s}^{-1}$ . This is 3.5-fold greater than the rate of recruitment we measured under our standard conditions.



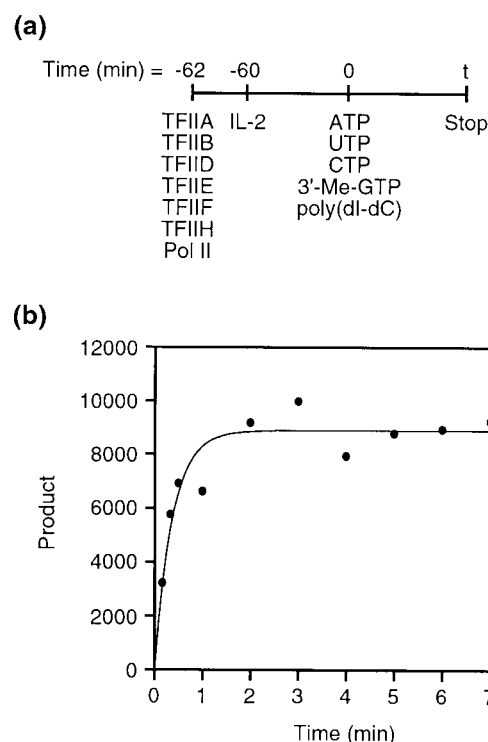
**Figure 4.** The recruitment of TFIIIF and RNA polymerase II to a TFIIID/TFIIA/TFIIIB/IL-2 complex is the slow step in preinitiation complex formation. (a) The model and schematic depict the method used to monitor the recruitment of TFIIIE and TFIIH into preinitiation complexes, as described in the text. See the legend to Figure 3(a) for abbreviations. (b) TFIIIE and TFIIH are recruited into preinitiation complexes within one minute. Transcription reactions were performed as described. The 28 nt RNA product is shown. (c) The model and schematic illustrate the method used to measure the rate at which TFIIIF and RNA polymerase II are recruited to the promoter. TFIIID, TFIIA, TFIIIB, and IL-2 promoter DNA were allowed to form a complex for 25 minutes at 30 °C. The remaining transcription factors (TFIIIE, TFIIIF, TFIIH, and core RNA polymerase II) were added to reactions. Nucleotides and poly(dI-dC) were added at variable times and transcription was stopped 20 minutes later. (d) The rate of recruitment of TFIIIF and RNA polymerase II into functional preinitiation complexes changes with respect to the concentration of these factors. Transcription reactions were performed as described above. The top and bottom panels show representative data obtained under our standard conditions or with fivefold more TFIIIF and RNA polymerase II, respectively. The 28 nt RNA product is shown.

### The rate of transcript synthesis is faster than the rate of preinitiation complex formation

We next measured the rate at which 28 nt IL-2 RNA is synthesized in a single round of transcription from preinitiation complexes. As shown in the schematic in Figure 5(a), preinitiation complexes were given 60 minutes to form, after which point nucleotides and poly(dI-dC) were added and transcription was stopped at various time-points. The plot in Figure 5(b) shows that transcript synthesis occurred with a rate constant of  $0.045(\pm 0.019) \text{ s}^{-1}$  (derived from three independent measurements). This is approximately 17-fold faster than the rate of preinitiation complex formation. Under these conditions, transcript synthesis encompassed all steps during synthesis of the first 27 phosphodiester bonds. Numerous steps in the RNA polymerase II reaction occur within this window, and the subsequent experiments investigate some of these steps.

### Escape commitment is a distinct, rapid step at the IL-2 promoter

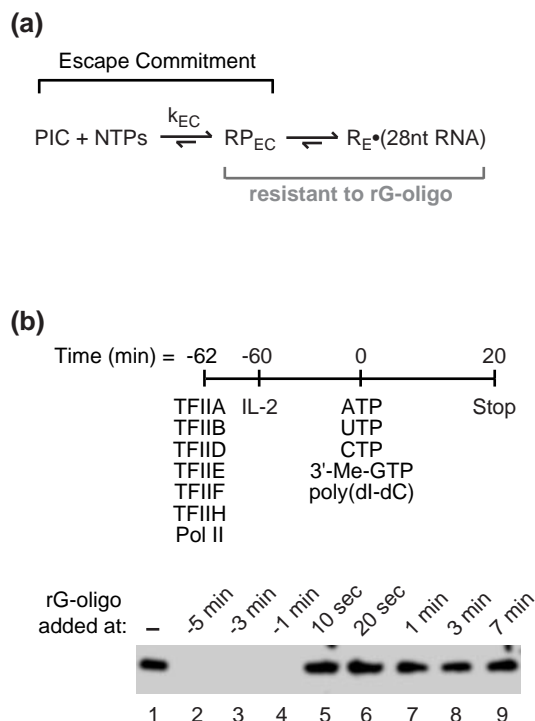
One step encompassed by transcript synthesis is escape commitment. We previously characterized escape commitment as a distinct step in early transcription at the AdMLP that results in stable ternary complexes, which are committed to proceeding forward through promoter escape and the remainder of the transcription reaction.<sup>25</sup> As illustrated in Figure 6(a), escape committed complexes ( $RP_{EC}$ ) form after preinitiation complexes are provided with nucleotides. Escape commitment can be monitored experimentally, because an oligonucleotide consisting of 20 guanosine bases (rG-oligo) inhibits escape commitment if it is added to assays prior to the nucleotides (i.e. before escape commitment occurs). In contrast, once escape commitment is complete, ternary complexes are stable and the rG-oligo has no effect on the remainder of the transcription reaction. A detailed characterization of the rG-oligo and the mechanism by which it inhi-



**Figure 5.** Transcript synthesis from preinitiation complexes is relatively rapid. (a) Schematic depicting the method used to measure the rate of transcript synthesis. RNA polymerase II and the general transcription factors were incubated for two minutes at 30°C prior to adding IL-2 promoter DNA. Preinitiation complexes formed for 60 minutes. Nucleotides and poly(dI-dC) were added and reactions were stopped at various times. (b) Plot of the rate of transcript synthesis at the IL-2 promoter. Transcription reactions were performed as described above. The 28 nt RNA products were quantified by PhosphorImagery and fit to a single exponential. The observed rate constant was  $0.045(\pm 0.019) \text{ s}^{-1}$  (average of three independent experiments where the error is one standard deviation).

bits escape commitment is discussed elsewhere.<sup>26</sup> Here, we have taken advantage of the rG-oligo to measure both the rate and the position at which escape commitment occurs at the IL-2 promoter.

To determine the rate at which escape commitment occurs, the experiment illustrated by Figure 6 was performed. The rG-oligo was added at multiple time-points either before or after the addition of nucleotides to determine the rate at which preinitiation complexes become resistant to the inhibitor. When the rG-oligo was added to assays prior to the nucleotides, escape commitment was inhibited (lanes 2-4). Within ten seconds after the addition of nucleotides, complexes were resistant to the addition of the rG-oligo and the level of transcription was similar to that observed in the absence of the inhibitor (compare lanes 5-9 to lane 1). This indicates that escape commitment is complete within ten seconds on the IL-2 promoter,



**Figure 6.** Escape commitment at the IL-2 promoter occurs within ten seconds after adding nucleotides to preinitiation complexes. (a) Model illustrating escape commitment. When nucleotides are added to preinitiation complexes (PIC), RNA polymerase II undergoes a transition termed escape commitment. Escape commitment is blocked by an inhibitory oligonucleotide consisting of 20 guanosine bases (rG-oligo). Escape committed ternary complexes ( $RP_{EC}$ ) and all subsequent complexes are resistant to the rG-oligo. (b) Escape commitment at the IL-2 promoter is complete within ten seconds. The schematic depicts the method used to measure the rate of escape commitment. RNA polymerase II and the general transcription factors were incubated for two minutes at 30°C prior to adding IL-2 promoter DNA. Preinitiation complexes formed for 60 minutes. Nucleotides and poly(dI-dC) were added and transcript synthesis proceeded for 20 minutes. The rG-oligo was added at time-points throughout the reaction as indicated above the gel. The 28 nt RNA product is shown.

which allows us to put a lower limit on the rate constant for escape commitment,  $k_{EC} > 0.1 \text{ s}^{-1}$ .

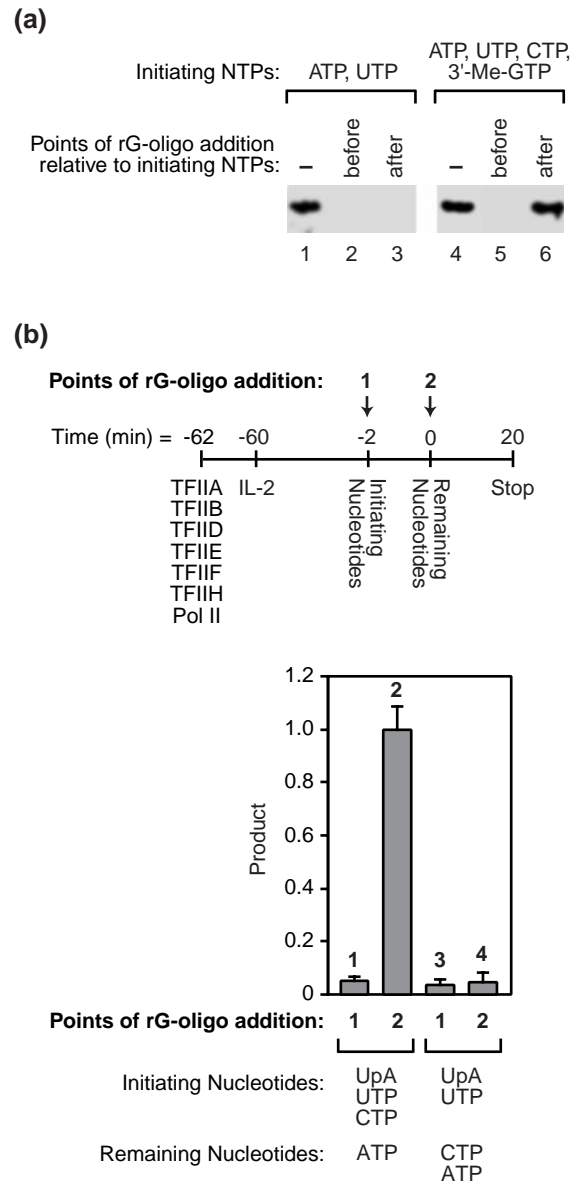
#### Escape commitment at the IL-2 promoter is complete upon synthesis of a 4 nt RNA

After determining that escape commitment occurs rapidly, we investigated the point at which escape commitment is complete at the IL-2 promoter. We previously showed on the AdMLP that a 3 nt RNA is not sufficient for escape commitment to occur, but a 4 nt RNA is sufficient.<sup>25,26</sup> We predicted this would be the case on the IL-2 promoter as well. To ask whether a 3 nt RNA was sufficient for escape commitment to occur, we performed the

experiment shown in Figure 7(a). Transcription was initiated with UTP and ATP, which allowed only a 3 nt RNA to be produced (refer to the IL-2 promoter sequence in Figure 1(a)). CTP, 3'-Me-GTP, and poly(dI-dC) were then added to lengthen the 3 nt RNA into full-length 28 nt RNA. The rG-oligo was added to experiments either one minute before or one minute after the initiating nucleotides to determine if they were sufficient for escape commitment to occur. Lane 1 shows the amount of RNA produced in the absence of the rG-oligo. As shown in lanes 2 and 3, the rG-oligo inhibited escape commitment both when it was added before or after synthesis of a 3 nt RNA, respectively. Lanes 4-6 depict a control experiment in which transcription was initiated with a complete set of nucleotides (ATP, UTP, CTP, 3'-Me-GTP, and poly(dI-dC)). Under these control conditions, escape commitment was inhibited only when the rG-oligo was added before the nucleotides (lane 5). When the inhibitor was added one minute after the nucleotides (lane 6) the level of transcript was equal to that observed in the absence of inhibitor (lane 4). Therefore, escape committed complexes form at some point after synthesis of a 3 nt RNA.

To test whether escape commitment was complete after synthesis of a 4 nt RNA, we split the addition of nucleotides into two steps, as indicated in Figure 7(b). The first set of nucleotides included UTP, CTP, and the dinucleotide UpA to initiate transcription. These were sufficient to produce a 4 nt RNA at the IL-2 promoter, but nothing longer (refer back to the promoter sequence in Figure 1(a)). The second set of nucleotides included ATP and 3'-Me-GTP to allow the 4 nt RNA to elongate into full-length 28 nt RNA. The rG-oligo was added with either set of nucleotides to determine whether a 4 nt RNA was sufficient for escape commitment to occur. As expected, when the rG-oligo was added to assays before production of a 4 nt RNA, escape commitment was inhibited and a low level of transcript was observed (Figure 7(b), bar 1). In contrast, when the rG-oligo was added after a 4 nt RNA was synthesized, a high level of transcript was observed, indicating that escape commitment was already complete (Figure 7(b), bar 2). As a control, transcription was also initiated with UpA and UTP, which produced a 3 nt RNA. The remaining set of nucleotides then included CTP, ATP, and 3'-Me-GTP to allow the 3 nt RNA to elongate to the full-length RNA. The rG-oligo was added with either the initiating or the remaining set of nucleotides. As expected, a 3 nt RNA was not sufficient for escape commitment to occur, as a low level of transcript was observed irrespective of where the rG-oligo was added (Figure 7(b), bars 3 and 4). We conclude that escape commitment is complete after synthesis of a 4 nt RNA at the IL-2 promoter.

Because escape commitment was complete within ten seconds after the addition of nucleotides, we presumed that subsequent RNA synthesis steps would limit the rate of production of the 28



nt IL-2 RNA. Therefore, we measured the rate of RNA synthesis after escape commitment was complete. We found that a 4 nt RNA lengthened to a 28 nt RNA with a rate constant of  $0.025(\pm 0.002) \text{ s}^{-1}$  (data not shown). This rate approximates the rate measured for the synthesis of 28 nt RNA from preinitiation complexes (Figure 5(b),  $0.045(\pm 0.019) \text{ s}^{-1}$ ). Thus a post-escape commitment step dictates the overall rate of synthesis of a 28 nt RNA at the IL-2 promoter under the conditions used here.

### The promoter DNA dictates the rate of transcript synthesis at the IL-2 promoter and at the AdMLP

We have shown that the rate-limiting step at the IL-2 promoter under the conditions tested here is preinitiation complex formation (dictated by the recruitment of TFIIF and RNA polymerase II), while the rate of RNA synthesis is rapid in comparison. In contrast to the IL-2 promoter, we previously found that preinitiation complex formation on the AdMLP was extremely rapid, and that RNA synthesis (namely promoter escape) was rate-limiting.<sup>27</sup> Several possible reasons for the difference in the rates of both preinitiation complex formation and RNA synthesis at the two promoters exist. In addition to the sequences of the promoters, two significant experimental conditions differed between the earlier studies with the AdMLP and those with the IL-2 promoter presented here. Namely, the former study utilized (1) a minimal transcription system consisting only of TBP, TFIIB, TFIIF, and RNA polymerase II and (2) the AdMLP was contained on a negatively supercoiled template. To begin to address the differences between the two promoters, we measured rates of preinitiation complex formation and RNA synthesis at the AdMLP in the more complex transcription system described here. In these experiments the AdMLP was still contained on a negatively supercoiled template.

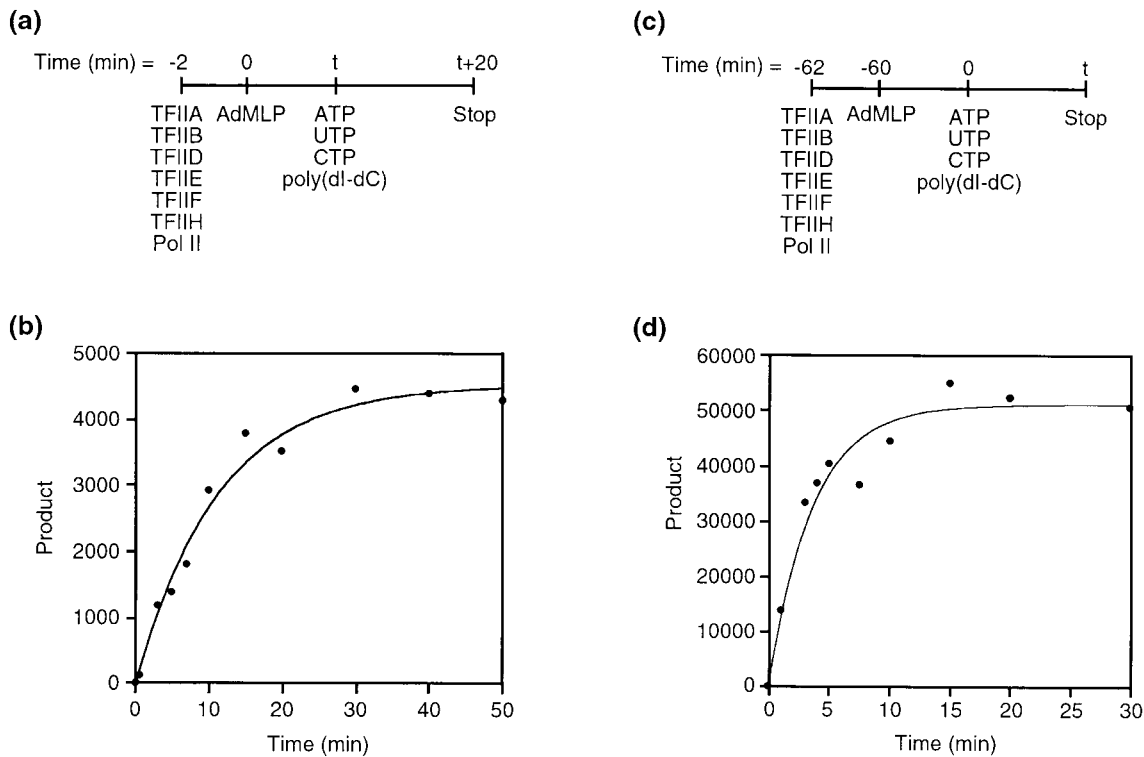
Figure 8(a) illustrates the method used to measure the rate of preinitiation complex formation at the AdMLP; it is the same method described for the IL-2 promoter in Figure 2. As shown by the plot in Figure 8(b), preinitiation complexes formed slowly with an observed rate constant of  $1.5(\pm 0.2) \times 10^{-3} \text{ s}^{-1}$ . This is similar to the rate of preinitiation complex formation we observed on the IL-2 promoter ( $2.6(\pm 1.3) \times 10^{-3} \text{ s}^{-1}$ ), indicating that the promoter itself has little to do with the rate at which preinitiation complexes form. In contrast, we previously found that preinitiation complex formation at the AdMLP in a minimal transcription system (only TBP, TFIIB, TFIIF, and RNA polymerase II) was complete within ten seconds.<sup>27</sup> Therefore, the slower rate for preinitiation complex formation at the AdMLP determined here must be due to the presence of TAFs, TFIIA, TFIIE, or TFIIH.

We next measured the rate of RNA synthesis at the AdMLP in the complex transcription system using the method depicted in Figure 8(c). As shown from the plot in Figure 8(d), RNA synthesis from the AdMLP under these conditions was slow, with a rate constant of  $4.5(\pm 2.5) \times 10^{-3} \text{ s}^{-1}$ . The rate of transcript synthesis we measured previously on the AdMLP promoter in the minimal transcription system ( $1.9(\pm 0.4) \times 10^{-3} \text{ s}^{-1}$ )<sup>27</sup> is quite similar to that determined here. In contrast, the rate constant for RNA synthesis at the IL-2 promoter ( $4.5(\pm 1.9) \times 10^{-2} \text{ s}^{-1}$ , Figure 5) is tenfold greater than at the AdMLP under any conditions. This indicates that the presence of TAFs, TFIIA, TFIIE, or TFIIH do not affect the rate of promoter escape at the AdMLP, but rather the difference between the two promoters is likely due to the promoter sequence itself or the helical state of the DNA.

### Discussion

Here, we studied the kinetics of the RNA polymerase II transcription reaction at the human IL-2 promoter *in vitro*. We divided the transcription reaction into multiple experimentally distinguishable steps and measured the rate of each step. We found that preinitiation complex formation limits the rate of a single round of IL-2 transcription *in vitro*; more specifically, the recruitment of TFIIF and RNA polymerase II is the slow step. In contrast, the rates of steps during RNA synthesis were relatively rapid. Our results provide a kinetic profile for a single round of transcription at the IL-2 promoter, as depicted in Figure 9. We discuss each step below and relate our findings to kinetic studies on the AdMLP, as well as to other studies of the RNA polymerase II reaction.

To form preinitiation complexes (PIC), the general transcription factors and core RNA polymerase II ( $R_c$ ) assemble on the promoter DNA (P). We found that the rate of preinitiation complex formation limits the overall rate of a single round of transcription under the conditions tested here. We first divided preinitiation complex formation into two steps: binding of TFIID/TFIIA (DA) to the promoter and recruitment of the remaining factors (BEFHR<sub>c</sub>). The rate we measured for recruitment of factors to a TFIID/TFIIA/promoter complex ( $2.9(\pm 0.4) \times 10^{-3} \text{ s}^{-1}$ ) was equal (within error) to the rate we measured for preinitiation complex formation as a whole ( $2.6(\pm 1.3) \times 10^{-3} \text{ s}^{-1}$ ). We also found that this rate was unaffected by pre-forming a TFIID/TFIIA/TFIIB complex on the promoter. Furthermore, we found that TFIIE and TFIIH were rapidly recruited into preinitiation complexes formed in their absence. Taken together, these results demonstrate that the binding of TFIIF and RNA polymerase II limits the rate of preinitiation complex formation under the experimental conditions tested here. Moreover, we found that the rate of recruitment of TFIIF and RNA polymerase

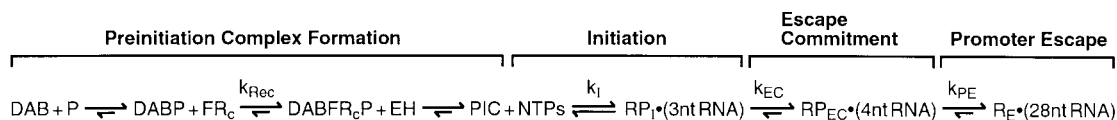


**Figure 8.** Both preinitiation complex formation and RNA synthesis at the AdMLP are slow under the conditions tested here. (a) Method used to monitor the rate of preinitiation complex formation at the AdMLP. RNA polymerase II and general transcription factors were incubated for two minutes at 30 °C prior to adding AdMLP DNA (negatively supercoiled plasmid DNA containing the AdMLP core promoter (−53 to +10) fused to a 380-bp G-less cassette). Nucleotides and poly(dI-dC) were added at various times ( $t$ ) and transcription was stopped 20 minutes later. (b) The rate of preinitiation complex formation at the AdMLP is slow. Reactions were performed as described above, 390 nt RNA was quantitated by PhosphorImagery, and data were fit to a single exponential. The observed rate constant was  $1.5(\pm 0.2) \times 10^{-3} \text{ s}^{-1}$ . (c) Method used to monitor the rate of RNA synthesis at the AdMLP. RNA polymerase II and the general transcription factors were incubated for two minutes at 30 °C prior to adding AdMLP DNA. Preinitiation complexes formed for 60 minutes. Nucleotides and poly(dI-dC) were added and reactions were stopped at various times. (d) The rate of RNA synthesis at the AdMLP is slow. Reactions were performed as described above, 390 nt RNA was quantified by PhosphorImagery, and data were fit to a single exponential. The observed rate constant was  $4.5(\pm 2.5) \times 10^{-3} \text{ s}^{-1}$ .

II increased as the concentration of the two factors was increased. This indicates that at very high concentrations of transcription factors, the rate of RNA synthesis, rather than recruitment of TFIIF and RNA polymerase II, will limit the rate of transcription at the IL-2 promoter.

Other studies have suggested mechanisms by which preinitiation complex formation can limit the rate of transcription. Pugh and colleagues have found that the dimerization of TFIID (and TBP) can affect the rate of preinitiation complex formation. Moreover, TFIIA can cause the dissociation of TFIID dimers, thereby facilitating TFIID binding to TATA boxes.<sup>35,36</sup> Our observation that recruitment of TFIIF and RNA polymerase II is the slow step at the IL-2 promoter indicates that the dissociation of TFIID dimers is not likely to be limiting the rate of IL-2 transcription under the conditions investigated here. Sundseth & Hansen found that the rate of recruitment of TFIID and TFIIB can limit the rate of transcription at the SV40

and AdMLP promoters.<sup>37</sup> We found that on the IL-2 promoter, the recruitment of TFIIB does not limit the rate of transcription. Chiang and colleagues have found that the TAF subunits of TFIID affect the rate-limiting step during preinitiation complex assembly.<sup>38</sup> We have found that when TFIID is replaced with the single subunit TBP, the rates of preinitiation complex formation and recruitment on the IL-2 promoter do not change significantly (data not shown). Other studies have found that core promoter sequences can dramatically affect rates of preinitiation complex formation and transcription *in vitro*.<sup>39–44</sup> Despite this, we found that the rates of preinitiation complex formation on the AdMLP and the IL-2 promoters, when measured in the same *in vitro* transcription system, are similar. Future experiments will address exactly which factors and/or promoter characteristics affect the rate of preinitiation complex formation at these two promoters.



**Figure 9.** Model depicting the steps in the RNA polymerase II transcription reaction at the IL-2 promoter. See Discussion for a detailed description of the model. Abbreviations are as follows: D, TFIID; A, TFIIA; P, IL-2 promoter; B, TFIIB; F, TFIIF;  $R_c$ , core RNA polymerase II; E, TFIIE; H, TFIIF; PIC, preinitiation complex;  $\text{RP}_1 \cdot (3\text{nt RNA})$ , initiated complex containing 3 nt RNA;  $\text{RP}_{\text{EC}} \cdot (4\text{nt RNA})$ , escape committed complex containing 4 nt RNA;  $\text{R}_E \cdot (28\text{nt RNA})$ , elongation complex containing 28 nt RNA.

As depicted in the model for transcription at the IL-2 promoter (Figure 9), when preinitiation complexes are provided with nucleotides, escape committed complexes ( $\text{RP}_{\text{EC}} \cdot (4\text{nt RNA})$ ) form upon synthesis of a 4 nt RNA. Escape commitment occurred within seconds after providing nucleotides to preinitiation complexes, thus we put a lower limit on the rate constant ( $k_{\text{EC}} > 0.1 \text{ s}^{-1}$ ). The characteristics of escape commitment observed on the IL-2 promoter are identical with those observed on the AdMLP where escape commitment was first characterized.<sup>25,26</sup> At both promoters, escape commitment was inhibited by the rG-oligo, was complete after synthesis of a 4 nt RNA, and occurred within ten seconds. A complete discussion of the mechanism of escape commitment and how it relates to other studies of early transcription is provided elsewhere (see Kugel & Goodrich<sup>25,26</sup> and references therein). Here, we also demonstrated that synthesis of a 3 nt RNA was not sufficient for escape commitment to occur. This distinguishes escape commitment from initiation in which 2 nt and 3 nt RNAs are produced. In the model, initiated complexes are designated as  $\text{RP}_1 \cdot (3\text{nt RNA})$ . Because escape commitment was complete within ten seconds, initiation must also occur within ten seconds. We infer then that the rate constant for initiation ( $k_i$ ) is greater than  $0.1 \text{ s}^{-1}$ . At the AdMLP, initiated ternary complexes are unstable based on the observation that 2 nt and 3 nt RNAs are abortively produced and released.<sup>45,46</sup> The abortive production of short RNAs remains to be investigated on the IL-2 promoter.

In our experiments with the IL-2 promoter we found that post-escape commitment steps limited the rate of 28 nt RNA synthesis from preinitiation complexes. These steps include promoter escape and perhaps early elongation events, and end with the 28 nt RNA transcript as part of a ternary elongation complex designated as  $\text{R}_E \cdot (28\text{nt RNA})$ . Although we do not know the exact point at which promoter escape is complete at the IL-2 promoter, for simplicity we designated the post-escape commitment steps as promoter escape. We measured a rate constant for this step of  $2.5(\pm 0.2) \times 10^{-2} \text{ s}^{-1}$ . The rate of promoter escape we previously measured on the AdMLP promoter in a minimal transcription system ( $2.0(\pm 0.4) \times 10^{-3} \text{ s}^{-1}$ )<sup>25</sup> is significantly slower than that observed at the IL-2 promoter. We began to address this difference by measuring the rate of promoter escape at the

AdMLP in the more complex transcription system used here. We found it to be similar to the rate we measured initially in the minimal system. Thus, the difference in the rates of promoter escape at the two different promoters is likely due to the nature of the promoter DNA, be it the sequence or the helical state. We believe both factors might contribute to setting the rate of promoter escape (data not shown).

With a kinetic outline for basal transcription at the IL-2 promoter in place, we are now poised to ask how the transcriptional activators that act at the IL-2 promoter affect the rates of individual steps in IL-2 transcription. It is possible that the multiple activators that bind the IL-2 promoter could simultaneously stimulate the rates of more than one step in IL-2 transcription. For example, at the IL-2 promoter activators could enhance the rate of recruitment of TFIIF and RNA polymerase II as well as the rate of promoter escape. Studies of other promoters utilizing nuclear extracts found that activators can affect the rate and extent of preinitiation complex formation as well as steps in early transcript synthesis.<sup>37,47-60</sup> Previous studies have also found that promoter sequence and transcriptional activators can affect the reinitiation of transcription.<sup>40,61-63</sup> In addition, studies have found that transcription can pause during early RNA synthesis and that activators can affect pausing.<sup>64-66</sup> It is possible that activators will need to be continuously present to maintain the high levels of multiple round IL-2 transcription that must occur after T-cells are activated.

## Materials and Methods

### Preparation of transcription templates, transcription factors, and other reagents

Plasmid pBS-IL-2-Luc was created by subcloning an *XhoI-EcoRI* fragment containing the IL-2 promoter (−326 to +45), firefly luciferase gene, and SV40 polyadenylation signal from plasmid pXIL-2-Luc (a gift from T. Hoey, Tularik, Inc.<sup>67</sup>) into the *XhoI* and *EcoRI* sites of pBluescript-KS(+). Plasmid pBS-IL-2-G-less contains that IL-2 promoter upstream of a 424 bp G-less cassette. This plasmid was generated by simultaneous ligation of two DNA fragments into the *EcoRI* and *Clai* sites of pBluescript-KS(+). One of the DNA fragments contained a 382 bp G-less cassette and was generated by PCR using pBS-MLP-G-less as a template, followed by digestion of the PCR product with *PacI* and *EcoRI*. The second frag-

ment contained the IL-2 promoter (−326 to +42) with a point mutation that changed the guanosine base at position +28 of the non-template strand to adenosine. This fragment was generated by PCR using plasmid pBS-IL-2-Luc as a template, followed by digestion of the PCR product with *Clal* and *PacI*. The region of the resulting plasmid containing the two inserts was sequenced. Plasmid pBS-IL-2 was created by subcloning the *Sall-HindIII* fragment containing the IL-2 promoter (−326 to +45) into the *Sall* and *HindIII* sites of pBluescript-KS(+). The blunt-ended DNA fragment containing the IL-2 promoter used in *in vitro* transcription studies was prepared by digesting pBS-IL-2 with *HincII* and *EcoRV*. After digestion, the DNA fragment was resolved on a 1% (w/v) agarose gel. The region of the gel containing the DNA fragment was excised (in the absence of ethidium bromide staining), crushed, and soaked in TE buffer (10 mM Tris (pH 7.9), 1 mM EDTA) for 30 minutes at 4°C. Eluted DNA was separated from agarose by filtration. The elution procedure was repeated twice, samples were combined, and DNA fragment was ethanol-precipitated.

Recombinant (TFIIB, TFIIE, and TFIIF) and native (TFIIH and RNA polymerase II) human transcription factors were prepared as described (see Kugel & Goodrich,<sup>27</sup> and references therein). Recombinant human TFIIA was prepared as described.<sup>68,69</sup> Native TFIID was purified as described.<sup>70</sup> Briefly, HeLa cell nuclear extracts were fractionated by phosphocellulose chromatography,<sup>71</sup> and TFIID was immunopurified using an  $\alpha$ -hTAF<sub>130</sub> monoclonal antibody and eluted with an epitope peptide. Poly(dI-dC)·poly(dI-dC) was purchased from Amersham Pharmacia. The RNA oligonucleotide consisting of 20 guanosine bases (rG-oligo) was purchased from Dharmacon Research.

### ***In vitro* transcription reactions**

Transcription reactions were performed in buffer A containing 10 mM Tris-HCl (pH 7.9), 10 mM Hepes (pH 8.0), 10% (v/v) glycerol, 1 mM DTT, 6 mM MgCl<sub>2</sub>, 50 mM KCl, 50  $\mu$ g/ml of BSA, and 3.5 units of RNA Guard. Reactions contained the following amounts of transcription factors: 10 ng of TFIIB, 6 ng of TFIIF, 50 ng of RNA polymerase II, 15 ng of TFIIE-34, 6 ng of TFIIE-56, ~14 ng of TFIIH, 3 ng of TFIID, and 20 ng of TFIIA. The DNA template (0.8 nM) for all experiments except that shown in Figures 1(c) and 8 was a blunt-ended 393 bp DNA fragment containing the IL-2 promoter (−326 to +45) excised from pBS-IL-2 as described above. Nucleotides were added to assays at final concentrations of 625  $\mu$ M ATP, 625  $\mu$ M UTP, 25  $\mu$ M [ $\alpha$ -<sup>32</sup>P]CTP (5  $\mu$ Ci per reaction), 100  $\mu$ M 3'-Me-GTP (Amersham Pharmacia), and 1 mM UpA where indicated in the Figures. All reactions were performed in a volume of 20  $\mu$ l. A general scheme for the transcription reactions follows, with differences indicated in Figures and Figure legends. Transcription factor proteins were mixed and incubated at 30°C for two minutes. After the addition of template DNA, preinitiation complexes formed at 30°C for times indicated in the Figures. Nucleotides and poly(dI-dC) were then added (4  $\mu$ l per reaction). Transcription proceeded for times indicated in the Figures, and reactions were stopped with 100  $\mu$ l of a stop solution containing 3.1 M ammonium acetate, 10  $\mu$ g of carrier yeast RNA, and 15  $\mu$ g of proteinase K. The samples were ethanol-precipitated and resolved by denaturing PAGE (14% (w/v) polyacrylamide).

### **Rate constant calculations**

The amount of full-length RNA produced per *in vitro* transcription reaction was quantified using a Molecular Dynamics PhosphorImager. After subtracting background, PhosphorImager units (PI) from RNA at each time-point were plotted *versus* time (*t*). Data were fit to the equation  $PI = PI_{max}(1 - e^{-kt})$ , to solve for  $PI_{max}$  (the maximal PhosphorImager units) and *k* (the rate constant).

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### **References**

1. Abbas, A. K., Lichtman, A. H. & Pober, J. S. (1994). *Cellular and Molecular Immunology*, 2nd edit., W.B. Saunders Company, Philadelphia, PA.
2. Ullman, K. S., Northrop, J. P., Verweij, C. L. & Crabtree, G. R. (1990). Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function: the missing link. *Annu. Rev. Immunol.* **8**, 421-452.
3. Crabtree, G. R. & Clipstone, N. A. (1994). Signal transmission between the plasma membrane and nucleus of T lymphocytes. *Annu. Rev. Biochem.* **63**, 1045-1083.
4. Goodbourn, S. (1994). T-cell activation. Transcriptional regulation in activated T-cells. *Curr. Biol.* **4**, 930-932.
5. Jain, J., Loh, C. & Rao, A. (1995). Transcriptional regulation of the IL-2 gene. *Curr. Opin. Immunol.* **7**, 333-342.
6. Chen, C. Y., Del Gatto-Konczak, F., Wu, Z. & Karin, M. (1998). Stabilization of interleukin-2 mRNA by the c-Jun NH2-terminal kinase pathway. *Science*, **280**, 1945-1949.
7. Durand, D. B., Bush, M. R., Morgan, J. G., Weiss, A. & Crabtree, G. R. (1987). A 275-basepair fragment at the 5' end of the interleukin 2 gene enhances expression from a heterologous promoter in response to signals from the T-cell antigen receptor. *J. Expt. Med.* **165**, 395-407.
8. Rooney, J. W., Sun, Y. L., Glimcher, L. H. & Hoey, T. (1995). Novel NFAT sites that mediate activation of the interleukin-2 promoter in response to T-cell receptor stimulation. *Mol. Cell. Biol.* **15**, 6299-6310.
9. Serfling, E., Avots, A. & Neumann, M. (1995). The architecture of the interleukin-2 promoter: a reflection of T lymphocyte activation. *Biochem. Biophys. Acta*, **1263**, 181-200.
10. Orphanides, G., Lagrange, T. & Reinberg, D. (1996). The general transcription factors of RNA polymerase II. *Genes Dev.* **10**, 2657-2683.
11. Malik, S. & Roeder, R. G. (2000). Transcriptional regulation through mediator-like coactivators in yeast and metazoan cells. *Trends Biochem. Sci.* **25**, 277-283.

12. Naar, A. M., Lemon, B. D. & Tjian, R. (2001). Transcriptional coactivator complexes. *Annu. Rev. Biochem.* **70**, 475-501.
13. Workman, J. L. & Kingston, R. E. (1998). Alterations of nucleosome structure as a mechanism of transcriptional regulation. *Annu. Rev. Biochem.* **67**, 545-579.
14. Nakajima, N., Horikoshi, M. & Roeder, R. G. (1988). Factors involved in specific transcription by mammalian RNA polymerase II: purification, genetic specificity, and TATA box-promoter interactions of TFIID. *Mol. Cell Biol.* **8**, 4028-4040.
15. Hoey, T., Dynlacht, B. D., Peterson, M. G., Pugh, B. F. & Tjian, R. (1990). Isolation and characterization of the Drosophila gene encoding the TATA box binding protein, TFIID. *Cell*, **61**, 1179-1186.
16. Kaufmann, J. & Smale, S. T. (1994). Direct recognition of initiator elements by a component of the transcription factor IID complex. *Genes Dev.* **8**, 821-829.
17. Weis, L. & Reinberg, D. (1992). Transcription by RNA polymerase II: initiator-directed formation of transcription competent complexes. *FASEB J.* **6**, 3300-3309.
18. Verrijzer, C. P., Chen, J.-L., Yokomori, K. & Tjian, R. (1995). Binding of TAFs to core elements directs promoter selectivity by RNA polymerase II. *Cell*, **81**, 1115-1125.
19. Burke, T. W. & Kadonaga, J. T. (1997). The downstream core promoter element, DPE, is conserved from Drosophila to humans and is recognized by TAF<sub>II</sub>60 of Drosophila. *Genes Dev.* **11**, 3020-3031.
20. Reinberg, D., Horikoshi, M. & Roeder, R. (1987). Factors involved in specific transcription by mammalian RNA Polymerase II. Functional analysis of initiation factors IIA and IID and identification of a new factor operating at sequences downstream of the initiation site. *J. Biol. Chem.* **262**, 3322-3330.
21. Flores, O., Lu, H. & Reinberg, D. (1992). Factors involved in specific transcription initiation by RNA polymerase II: identification and characterization of factor IIIH. *J. Biol. Chem.* **267**, 2786-2793.
22. Buratowski, S., Hahn, S., Guarente, L. & Sharp, P. A. (1989). Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell*, **56**, 549-561.
23. Carey, M. F. (1995). A holistic view of the complex. *Curr. Biol.* **5**, 1003-1005.
24. Holstege, F. C. P., van der Vliet, P. C. & Timmers, H. T. M. (1996). Opening of an RNA polymerase II promoter occurs in two distinct steps and requires the basal transcription factors IIE and IIIH. *EMBO J.* **15**, 1666-1677.
25. Kugel, J. F. & Goodrich, J. A. (2000). A kinetic model for the early steps of RNA synthesis by human RNA polymerase II. *J. Biol. Chem.* **275**, 40483-40491.
26. Kugel, J. F. & Goodrich, J. A. (2001). Translocation after synthesis of a four nucleotide RNA commits RNA polymerase II to promoter escape. *Mol. Cell Biol.* **In the press**.
27. Kugel, J. F. & Goodrich, J. A. (1998). Promoter escape limits the rate of transcription from the adenovirus major late promoter on negatively supercoiled templates. *Proc. Natl Acad. Sci. USA*, **95**, 9232-9237.
28. Peterson, M. G., Tanese, N., Pugh, B. F. & Tjian, R. (1990). Functional domains and upstream activation properties of cloned human TATA binding protein. *Science*, **248**, 1625-1630.
29. Kao, C. C., Lieberman, P. M., Schmidt, M. C., Zhou, Q., Pei, R. & Berk, A. J. (1990). Cloning of a transcriptionally active human TATA binding factor. *Science*, **248**, 1646-1650.
30. Parvin, J. D., Timmers, H. T. M. & Sharp, P. A. (1992). Promoter specificity of basal transcription factors. *Cell*, **68**, 1135-1144.
31. Tyree, C. M., George, C. P., Lira-DeVito, L. M., Wampler, S. L., Dahmus, M. E., Zawel, L. & Kadonaga, J. T. (1993). Identification of a minimal set of proteins that is sufficient for accurate initiation of transcription by RNA polymerase II. *Genes Dev.* **7**, 1254-1265.
32. Parvin, J. D. & Sharp, P. A. (1993). DNA topology and a minimal set of basal factors for transcription by RNA polymerase II. *Cell*, **73**, 533-540.
33. Goodrich, J. A. & Tjian, R. (1994). Transcription factors IIE and IIIH and ATP hydrolysis direct promoter clearance by RNA polymerase II. *Cell*, **77**, 145-156.
34. Hoopes, B. C., LeBlanc, J. F. & Hawley, D. K. (1992). Kinetic analysis of yeast TFIID-TATA box complex formation suggests a multi-step pathway. *J. Biol. Chem.* **267**, 11539-11547.
35. Coleman, R. A., Taggart, A. K. P., Benjamin, L. R. & Pugh, B. F. (1995). Dimerization of the TATA binding protein. *J. Biol. Chem.* **270**, 13842-13849.
36. Coleman, R. A., Taggart, A. K., Burma, S., Chicca, J. J. 2nd & Pugh, B. F. (1999). TFIIA regulates TBP and TFIID dimers. *Mol. Cell*, **4**, 451-457.
37. Sundseth, R. & Hansen, U. (1992). Activation of RNA polymerase II transcription by the specific DNA-binding protein LSF. Increased rate of binding of the basal promoter factor TFIIB. *J. Biol. Chem.* **267**, 7845-7855.
38. Wu, S.-Y. & Chiang, C.-M. (2001). TATA-binding protein-associated factors enhance the recruitment of RNA polymerase II by transcriptional activators. *J. Biol. Chem.* **276**, 34235-34243.
39. Jacob, G. A., Kitzmiller, J. A. & Luse, D. S. (1994). RNA polymerase II promoter strength *in vitro* may be reduced by defects at initiation or promoter clearance. *J. Biol. Chem.* **269**, 3655-3663.
40. Yean, D. & Gralla, J. (1997). Transcription reinitiation rate: a special role for the TATA box. *Mol. Cell Biol.* **17**, 3809-3816.
41. Wolner, B. S. & Gralla, J. D. (2001). TATA-flanking sequences influence the rate and stability of TATA-binding protein and TFIIB binding. *J. Biol. Chem.* **276**, 6260-6266.
42. Wolner, B. S. & Gralla, J. D. (2000). Roles for non-TATA core promoter sequences in transcription and factor binding. *Mol. Cell Biol.* **20**, 3608-3615.
43. Zenzie-Gregory, B., O'Shea-Greenfield, A. & Smale, S. T. (1992). Similar mechanisms for transcription initiation mediated through a TATA box or an initiator element. *J. Biol. Chem.* **267**, 2823-2830.
44. Hoopes, B. C., LeBlanc, J. F. & Hawley, D. K. (1998). Contributions of the TATA box sequence to rate-limiting steps in transcription initiation by RNA polymerase II. *J. Mol. Biol.* **277**, 1015-1031.
45. Luse, D. S. & Jacob, G. A. (1987). Abortive initiation by RNA polymerase II *in vitro* at the adenovirus major late promoter. *J. Biol. Chem.* **262**, 14990-14997.
46. Luse, D. S., Kochel, T., Kuempel, E. D., Copploa, J. A. & Cai, H. (1987). Transcription initiation by RNA polymerase II *in vitro*: at least two nucleotides must be added to form a stable ternary complex. *J. Biol. Chem.* **262**, 289-297.

47. Horikoshi, M., Carey, M. F., Kakidani, H. & Roeder, R. G. (1988). Mechanism of action of a yeast activator: direct effect of GAL4 derivatives on mammalian TFIID-promoter interactions. *Cell*, **54**, 665-669.
48. Wang, W., Gralla, J. D. & Carey, M. (1992). The acidic activator GAL4-AH can stimulate polymerase II transcription by promoting assembly of a closed complex requiring TFIID and TFIIA. *Genes Dev.* **6**, 1716-1727.
49. Sauer, F., Hansen, S. K. & Tjian, R. (1995). Multiple TAF<sub>II</sub>s directing synergistic activation of transcription. *Science*, **270**, 1783-1788.
50. Lin, Y.-S. & Green, M. R. (1991). Mechanism of action of an acidic transcriptional activator *in vitro*. *Cell*, **64**, 971-981.
51. Johnson, F. B. & Krasnow, M. A. (1992). Differential regulation of transcription preinitiation complex assembly by activator and repressor homeodomain proteins. *Genes Dev.* **6**, 2177-2189.
52. Lieberman, P. M. & Berk, A. J. (1994). A mechanism for TAFs in transcriptional activation: activation domain enhancement of TFIID-TFIIA-promoter DNA complex formation. *Genes Dev.* **8**, 995-1006.
53. Farrell, S., Simkovich, N., Wu, Y., Barberis, A. & Ptashne, M. (1996). Gene activation by recruitment of RNA polymerase II holoenzyme. *Genes Dev.* **10**, 2359-2367.
54. Jiang, Y., Yan, M. & Gralla, J. D. (1996). A three-step pathway of transcription initiation leading to promoter clearance at an activation RNA polymerase II promoter. *Mol. Cell. Biol.* **16**, 1614-1621.
55. Narayan, S. & Wilson, S. H. (2000). Kinetic analysis of Sp1-mediated transcriptional activation of a TATA-containing promoter. *Biochemistry*, **39**, 818-823.
56. Yie, J., Senger, K. & Thanos, D. (1999). Mechanism by which the IFN-beta enhanceosome activates transcription. *Proc. Natl Acad. Sci. USA*, **96**, 13108-13113.
57. Liu, J., Akoulitchev, S., Weber, A., Ge, H., Chuikov, S., Libutti, D. *et al.* (2001). Defective interplay of activators and repressors with TFIIH in xeroderma pigmentosum. *Cell*, **104**, 353-363.
58. Kumar, K. P., Akoulitchev, S. & Reinberg, D. (1998). Promoter-proximal stalling results from the inability to recruit transcription factor IIIH to the transcription complex and is a regulated event. *Proc. Natl Acad. Sci. USA*, **95**, 9767-9772.
59. Narayan, S., Widen, S. G., Beard, W. A. & Wilson, S. H. (1994). RNA polymerase II transcription: rate of promoter clearance is enhanced by a purified activating transcription factor cAMP response element-binding protein. *J. Biol. Chem.* **269**, 12755-12763.
60. Wolner, B. S. & Gralla, J. D. (1997). Promoter activation via a cyclic AMP response element *in vitro*. *J. Biol. Chem.* **272**, 32301-32307.
61. Sandaltzopoulos, R. & Becker, P. B. (1998). Heat shock factor increases the reinitiation rate from potentiated chromatin templates. *Mol. Cell. Biol.* **18**, 361-367.
62. Yean, D. & Gralla, J. D. (1999). Transcription reinitiation rate: a potential role for TATA box stabilization of the TFIID:TFIIA:DNA complex. *Nucl. Acids Res.* **27**, 831-838.
63. Yudkovsky, N., Ranish, J. A. & Hahn, S. (2000). A transcription reinitiation intermediate that is stabilized by activator. *Nature*, **408**, 225-229.
64. Krumm, A., Hickey, L. B. & Groudine, M. (1995). Promoter-proximal pausing of RNA polymerase II defines a general rate-limiting step after transcription initiation. *Genes Dev.* **9**, 559-572.
65. Lis, J. (1998). Promoter-associated pausing in promoter architecture and postinitiation transcriptional regulation. *Cold Spring Harbor Symp. Quant. Biol.* **63**, 347-356.
66. Rasmussen, E. B. & Lis, J. T. (1993). *In vivo* transcriptional pausing and cap formation on three Drosophila heat shock genes. *Proc. Natl Acad. Sci. USA*, **90**, 7923-7927.
67. Hoey, T., Sun, Y. L., Williamson, K. & Xu, X. (1995). Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins. *Immunity*, **2**, 461-472.
68. Ozer, J., Moore, P. A., Bolden, A. H., Lee, A., Rosen, C. A. & Lieberman, P. M. (1994). Molecular cloning of the small (gamma) subunit of human TFIIA reveals functions critical for activated transcription. *Genes Dev.* **8**, 2324-2335.
69. Sun, X., Ma, D., Sheldon, M., Yeung, K. & Reinberg, D. (1994). Reconstitution of human TFIIA activity from recombinant polypeptides: a role in TFIID-mediated transcription. *Genes Dev.* **8**, 2336-2348.
70. Galasinski, S. K., Lively, T. N., Grebe de Barron, A. & Goodrich, J. A. (2000). Acetyl-CoA stimulates RNA polymerase II transcription and promoter binding by TFIID in the absence of histones. *Mol. Cell. Biol.* **20**, 1923-1930.
71. Dignam, J. D., Martin, P. L., Shastry, B. S. & Roeder, R. G. (1983). Eukaryotic gene transcription with purified components. *Methods Enzymol.* **101**, 582-598.

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