

**In vitro studies of the early steps of RNA synthesis by human RNA polymerase II**

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Running title: Early steps of RNA polymerase II transcription

## Introduction

We have experimentally isolated five steps in the RNA polymerase II transcription reaction (Figure 1). These steps include preinitiation complex formation, initiation, escape commitment, promoter escape, and transcript elongation.<sup>1,2</sup> Preinitiation complex formation minimally involves RNA polymerase II and the general transcription factors binding to promoter DNA. When preinitiation complexes are provided with nucleoside triphosphates, transcription initiates and RNA polymerase II produces short 2 and 3 nt RNAs. These complexes are unstable and the RNA products are frequently released in an abortive manner. When the RNA transcript is lengthened to 4 nt, RNA polymerase II undergoes a transition referred to as escape commitment in which the ternary transcribing complex becomes stable and committed to proceeding forward through the reaction. Promoter escape then occurs as escape committed complexes transform into elongation complexes, which subsequently complete synthesis of full length RNA.

The protocols discussed here focus on methods to study the early steps in transcript synthesis (i.e. initiation, escape commitment, and promoter escape). These approaches can be used to determine how different factors affect specific steps in early RNA synthesis. Each of the steps in early transcription has the potential to be influenced by transcriptional activators and/or general transcription factors such as TFIIE and TFIIH. For example, initiation and post-initiation events,<sup>3</sup> promoter proximal pausing,<sup>4</sup> promoter clearance,<sup>5</sup> and transcript elongation (as reviewed in <sup>6-8</sup>) have been shown to be targets of transcriptional regulators. In addition, TFIIH contains two ATP-dependent helicases<sup>9,10</sup> that are thought to function during early steps in transcription (<sup>11</sup> and references contained therein). We have found that TFIIE and TFIIH increase the fraction of complexes that produce full length RNA by acting at a specific step in early transcription; namely, escape commitment.<sup>1</sup>

## **Forming preinitiation complexes**

### Theory

Preinitiation complex formation involves the assembly of general transcription factors and RNA polymerase II on the promoter DNA. The following protocols for studying promoter escape, initiation, and escape commitment all require that functional preinitiation complexes first be assembled at the promoter. The minimal components needed to form functional preinitiation complexes include: recombinant TBP, recombinant TFIIB, recombinant TFIIF, purified RNA polymerase II core enzyme, and negatively supercoiled promoter DNA. The DNA template must be negatively supercoiled to detect long RNA products in the absence of TFIIE and TFIIH.<sup>12-15</sup> If linear DNA templates are to be used, then TFIIE and TFIIH must be added to preinitiation complexes prior to initiating transcription, and the reactions must include hydrolysable ATP (either in the form of rATP or dATP). Additional factors can be added during preinitiation complex formation. For example, in this purified transcription system TBP can be replaced by purified TFIID and recombinant TFIIA to study transcriptional activation.<sup>16</sup> Similar conditions for preinitiation complex formation can be used if a nuclear extract is the source of the general transcription machinery. In addition, activators or other regulatory proteins can be included during preinitiation complex formation.

### Preparation of transcription factors and DNA templates

Methods for preparing recombinant (TBP, TFIIB, TFIIE, TFIIF, and TFIIA) and native (core RNA polymerase II, TFIIH, and TFIID) human transcription factors have been described in detail elsewhere (<sup>16, 17</sup> and references therein). The DNA template should be purified from E. coli strains under conditions that maintain the negative superhelicity. Qiagen kits can be used for the preparation of plasmids as long as RNA Guard (Amersham Pharmacia) is used in transcription reactions as described below.

## Method

Preinitiation complexes are assembled in 20  $\mu$ l reactions. The protein components are preincubated in a volume of 10  $\mu$ l (reaction tube A), and the DNA template is preincubated in a volume of 10  $\mu$ l (reaction tube B). The protein and DNA components are then combined to allow preinitiation complex assembly (add tube B to tube A). The schematic in Figure 2 provides a timeline of the method for assembling preinitiation complexes and testing activity by *in vitro* transcription. Table I shows the recipes for reaction tubes A and B for a single 20  $\mu$ l reaction; the constituents are explained in detail below.

*In vitro* transcription reactions are assembled such that half the reaction volume consists of DB(100) buffer and proteins (which are dialyzed against DB(100)). Consequently, components of the DB(100) buffer are twice as concentrated as needed for preinitiation complex assembly and *in vitro* transcription. For example, the glycerol concentration in DB(100) is 20%; the final concentration in the reaction is 10%. Therefore in the complete 20  $\mu$ l reaction, the correct concentrations of all buffer components are achieved by adding 10  $\mu$ l composed of DB(100) and proteins, and 10  $\mu$ l composed of RM4X, DNA, and water. When titrating components or adding factors to transcription reactions, it is important to maintain this buffer ratio. The following protocol explains preinitiation complex formation using a minimal transcription system consisting of negatively supercoiled promoter DNA, TBP, TFIIB, TFIIF, and RNA polymerase II. To determine the volumes of solutions to make, refer to Table I and consider the total number of reactions to be performed.

1. DB(100) buffer. Make an appropriate amount of DB(100) buffer containing 20 mM Tris (pH 7.9), 20% glycerol, 1 mM DTT, 100 mM KCl, and 100  $\mu$ g/ml BSA (New England Biolabs). Store on ice. This is the same buffer that all of the general

transcription factors are dialyzed against prior to storage, with two exceptions: 1) the dialysis buffer does not contain BSA, and 2) RNA polymerase II is unstable in KCl, therefore the KCl is replaced with 100 mM ammonium sulfate, as described.<sup>17</sup>

2. RM4X buffer. Make an appropriate amount of buffer RM4X containing 40 mM Hepes (pH 8.0), 2 mM DTT, 16 mM MgCl<sub>2</sub>, and 3 units/ $\mu$ l of RNA Guard (Amersham Pharmacia). Store on ice.
3. DNA template. Prepare a DNA solution by diluting stock plasmid DNA in water to a concentration of 20 nM. 1  $\mu$ l will be added to each reaction to obtain a final concentration of 1 nM in a 20  $\mu$ l reaction. Store on ice.
4. Reaction tubes A. Assemble reactions in 1.5 ml tubes. Each reaction will require 2 tubes (A and B) because the protein and DNA components are preincubated separately. To each tube add DB(100), RM4X, and water, as indicated in Table I. Protein mix will be added later.
5. Reaction tubes B. Add DB(100), RM4X, DNA template, and water, as indicated in Table I.
6. Protein mix. Prepare a protein mix containing all the general transcription factors diluted into DB(100) to the following concentrations: 1 ng/ $\mu$ l TBP, 3 ng/ $\mu$ l TFIIB, 2 ng/ $\mu$ l TFIIF, and 9 ng/ $\mu$ l RNA polymerase II. 3  $\mu$ l of the protein mix will be added to each reaction. Assemble the protein mix on ice in the following order: DB(100), TBP, TFIIB, TFIIF, and RNA polymerase II. Mix by flicking with finger (do not vortex).  
Special consideration must be taken when working with transcription factor proteins. All protein factors should be frozen in liquid nitrogen in small aliquots and stored at -80°C after purification. Thaw the small aliquots of proteins by warming between fingers, then quickly place on ice. Many factors can be refrozen in liquid nitrogen and thawed rapidly three or four times before loss of activity is noticed (e.g. TBP, TFIIB, and TFIIF). RNA polymerase II should never be frozen and thawed more than two times.

7. Preincubation of proteins and DNA. Add the protein mix to reaction tubes A and place at 30°C in a staggered manner such that each reaction is incubated for the same amount of time. For example, at time point 0 min add 3  $\mu$ l of protein mix to reaction tube 1A, mix by flicking with finger, and place at 30°C. At the same time place reaction tube 1B at 30°C. Repeat this for each pair of reaction tubes at staggered time intervals.
8. Formation of preinitiation complexes. Allow tubes A and B to incubate two min at 30°C. At this point, add tube B to tube A to attain a total reaction volume of 20  $\mu$ l. Mix by flicking with finger and return to 30°C. Repeat this for each pair of reaction tubes at the correct time.
9. Incubate at 30°C to allow preinitiation complexes to form. It is important to determine the amount of time required to complete preinitiation complex formation at the promoter of interest to decide on the optimal time to use. We allow 10 min under the conditions described here. At the completion of preinitiation complex formation, nucleotides are added to initiate transcription, as described in the following sections.

#### Additional comments

If TFIIE and TFIIH are to be included in reactions, they can be added directly to the protein mix. We have also found that TFIIE and TFIIH can be added after other factors are assembled into preinitiation complexes and immediately prior to nucleotides.<sup>18</sup> TFIID and TFIIA can be added in place of TBP. In this case, the final concentration of  $MgCl_2$  in reactions should be increased to 6 mM. If transcriptional activation is to be studied, the activator(s) can be prebound to the DNA template by adding it to reaction tube B and removing an equivalent amount of DB(100). If a nuclear extract is to be used, replace the protein mix with nuclear extract and adjust the volume of DB(100). If the contents of reaction tubes B are the same for every reaction performed, then a single large reaction tube B can be assembled and preincubated at 30°C. Aliquots of 10  $\mu$ l can then be added to the A tubes. By doing so, the preincubation time for tube B will vary for

each reaction. This is acceptable when reaction tube B contains just DNA and buffer components (i.e. activators are not included).

## **Monitoring the transition from preinitiation complexes to elongation complexes**

### Theory

The transition from preinitiation complexes to elongation complexes includes at least three steps: initiation, escape commitment, and promoter escape (see Figure 1). To isolate these early steps in transcript synthesis from downstream steps such as elongation, RNA synthesis must be paused after promoter escape. At the AdMLP, promoter escape is complete by synthesis of a 15 nt RNA. This point was defined through rate measurements in which we found synthesis of a 15 nt RNA to be rate limiting and kinetically distinct from elongation of a 15 nt RNA to full length RNA.<sup>1,17</sup> The point at which promoter escape is complete may vary from promoter to promoter, and can be determined using kinetic experiments as described previously.<sup>17</sup>

Initiating transcription with a limited set of nucleotides is the simplest technique for pausing RNA polymerase II at the completion of promoter escape. For example, at the AdMLP (in a G-less cassette template) transcription can be paused after synthesis of a 15 nt RNA by initiating transcription with the dinucleotide ApC (many dinucleotides can be purchased from Sigma), CTP, and UTP (Figure 3A). RNA polymerase II will pause at the thymidine at +16 (template strand) due to the lack of ATP in the reaction. These paused, ternary complexes are stable over the course of the reaction. As another example, the steps of early transcription at the human interleukin-2 (IL-2) promoter can be easily studied because the IL-2 gene does not contain any cytosines in the template strand until +28 (Figure 3B). Therefore transcription will stop after synthesis of a 28 nt RNA in the presence of ATP, CTP, UTP, and the chain terminator 3'-O-Methylguanosine 5'-Triphosphate (3'-Me-GTP), which ensures that RNA polymerase II does not read through the guanosine at position +28.<sup>18</sup>

If the promoter and gene of interest do not consist of sequences easily amenable to pausing RNA polymerase II, manipulations of the DNA template can be considered. First, the DNA can be mutated to allow pausing of RNA polymerase II at +16. Second, the promoter can be isolated and fused to a downstream sequence that is amenable to study. Third, a linear DNA template can be used such that the downstream region ends between +15 and +30; therefore, RNA polymerase II will "run-off" the end and limit transcript synthesis to a short RNA. When performing run-off transcription, two additional experimental parameters must be considered. Because the DNA template is linear, TFIIE, TFIIH, and ATP (or dATP) must be included in the reaction.<sup>12-15, 17</sup> In addition, to ensure that new preinitiation complex formation does not influence studies of the early steps of transcript synthesis, transcription must be limited to a single round of synthesis. Single round transcription can be obtained with the use of a non-specific competitor DNA such as poly(dI-dC)•poly(dI-dC).<sup>18</sup>

### Method

The following experiment describes analyzing early transcription at the AdMLP by pausing RNA polymerase II in elongation complexes containing a 15 nt RNA.

1. Nucleotide premix. Prepare a solution of 11x nucleotides by diluting stock solutions of nucleotides into TE buffer (10 mM Tris (7.9), 0.1 mM EDTA) to the following concentrations: 11 mM ApC; 7 mM UTP; 275  $\mu$ M [ $\alpha$ -<sup>32</sup>P]CTP (2.5  $\mu$ Ci/ $\mu$ l). Each reaction will get 2  $\mu$ l of the nucleotide premix. Both non-radioactive and radioactive CTP are added to the nucleotide premix to obtain the final concentration and specific activity.
2. Add 2  $\mu$ l of nucleotide premix to each reaction containing preinitiation complexes (assembled as described earlier). Mix by flicking with finger. Incubate at 30°C for

30 min. This is sufficient time to complete promoter escape at the AdMLP, however, this should be determined for the promoter of interest.

3. To stop transcription, add 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.
4. Ethanol precipitate the transcription reactions by adding 300  $\mu$ l of cold 100% ethanol. Vortex. Spin at 16,000 x g for 20 min at 4°C. Remove the ethanol. The ethanol will have a significant number of radioactive counts and should be disposed of appropriately.
5. Add 200  $\mu$ l of cold 80% ethanol. Spin at 16,000 x g for 5 min at 4°C. Remove the ethanol.
6. Dry the pellets in a speed vac.
7. Resuspend the pellets in 4  $\mu$ l of formamide loading buffer (95% formamide, 15 mM EDTA, 0.025% bromophenol blue).
8. Load the entire reaction on a 14% polyacrylamide (19:1) gel (20 cm x 20 cm; 0.4 mm spacers) containing 7 M Urea and 0.5x TBE (45 mM Tris, 45 mM Boric Acid, 1 mM EDTA).
9. Run the gel in 0.5x TBE at 17 watts until the bromophenol blue is two thirds of the way down the gel.
10. Dry gel and visualize transcript RNA by Phosphorimagery.

#### Additional considerations

Kinetic differences between the early steps of transcription make promoter escape experimentally discernable from initiation and escape commitment. Both initiation and escape commitment are complete within seconds at the AdMLP and at the IL-2 promoter.<sup>1,18</sup> Because promoter escape is the rate limiting step in early transcription at these two promoters, any factor or experimental parameter that affects the rate at which a short RNA is produced must do so by affecting promoter escape. It remains to be determined whether promoter escape is rate limiting at most promoters.

## **Monitoring the formation of functional initiation complexes by abortive initiation**

### Theory

Abortive initiation is a steady-state assay that monitors functional initiation complexes. RNA polymerases synthesize short (2 to 3 nt) aborted RNA products when preinitiation complexes are provided with limited nucleotide substrates specific to the transcription start site sequence of a given promoter.<sup>19-23</sup> These short RNA products are released by RNA polymerase II, which remains stably bound to the promoter (Figure 4A).

The choice of nucleotide substrates to use depends on the sequence around the transcriptional start site of the promoter to be studied. At many promoters, multiple different combinations of nucleotides will allow abortive initiation because RNA polymerase II can initiate at a range of sites surrounding +1. For example, at the AdMLP the following nucleotide combinations all produce 2 or 3 nt abortive products: the dinucleotide ApC and [ $\alpha$ -<sup>32</sup>P]UTP produce ApCpU; CpA and [ $\alpha$ -<sup>32</sup>P]CTP produce CpApC; and ATP and [ $\alpha$ -<sup>32</sup>P]CTP produce pppApC.

Short RNAs migrate aberrantly in polyacrylamide gels. The number of phosphates on the 5' end of the RNA dramatically affects migration; therefore, to simplify analysis we treat the abortive RNAs with a phosphatase to remove all 5' phosphates. This also better separates small RNA products from unincorporated <sup>32</sup>P-labeled nucleotides. The positions at which short RNA products migrate do not correlate with size in the predicted manner. For example, a 2 nt RNA migrates more slowly than a 3 nt RNA. Moreover, sequence affects migration. For these reasons it is important to compare all transcripts with RNA standards of identical size and sequence to the expected products, as described in the protocol.

For each nucleotide combination tested, a parallel reaction should be performed with a mutant promoter in which the start site position is changed such that the

combination of nucleotides used will not produce a transcript. Any transcript produced from the mutant template originates from non-specific start sites on the plasmid DNA and can be subtracted as background from the transcript produced from the wild-type template.

### Method

The protocol below uses the example of the AdMLP and the nucleotides CpA and CTP, but can be used for other nucleotide combinations and promoters.

1. Nucleotide premix. Prepare a solution of 11x nucleotides by diluting stock solutions of nucleotides into TE buffer (10 mM Tris (7.9), 0.1 mM EDTA) to the following concentrations: 11 mM CpA; 5.5  $\mu$ M [ $\alpha$ -<sup>32</sup>P]CTP (2.5  $\mu$ Ci/ $\mu$ l). Each reaction will get 2  $\mu$ l of the nucleotide premix. Both non-radioactive and radioactive CTP are added to the nucleotide premix to obtain the final concentration and specific activity.
2. Add 2  $\mu$ l of nucleotide mix to each reaction containing preinitiation complexes (assembled as described earlier). Mix by flicking with finger. Incubate at 30°C for 30 min.
3. Stop reactions by transferring to 70°C for 3 min.
4. Add calf intestinal alkaline phosphatase (10 Units) to each reaction and incubate at 37°C for 20 min.
5. Add 3  $\mu$ l of a stop solution containing 200 mM EDTA, 20% glycerol, and 0.025% bromophenol blue to each reaction. Vortex. Reactions can be stored at -20°C prior to electrophoresis.
6. Load 6  $\mu$ l of each reaction on a 20% polyacrylamide (19:1) gel (20 cm x 20 cm; 0.4 mm spacers) containing 7 M Urea, 0.5x TBE (45 mM Tris, 45 mM Boric Acid, 1 mM EDTA). Also load RNA size standards. Size standards are 2, 3, 4, and 5 nt RNA oligonucleotides (Dharmacon Research) with the same sequence as the expected RNA

transcripts produced from the promoter of interest. Sufficient quantities should be loaded such that the standards can be visualized by UV shadowing (1 nanomole is ample).

7. Run the gel in 0.5x TBE at 17 watts until the bromophenol blue is half way down the gel.

8. The gel can be subjected to Phosphorimagery and the RNA size standards can be visualized by UV shadowing with a 254 nm light after drying the gel or while it is still wet.

### Interpretation of results

In the experiment shown in Figure 4B we tested whether the fusion protein GAL4-VP16c, which has been shown to activate transcription *in vitro*,<sup>24</sup> could stimulate abortive initiation. When GAL4-VP16c was added to abortive initiation reactions, a 5 fold increase in the amount of 3 nt RNA product was observed (compare lane 2 to lane 1). As a control, a mutant AdMLP in which the +1 was changed from U to C (template strand) was included in lanes 3 and 4. No abortive product was observed, indicating that the CpApC transcript observed with the wild type template was produced from the AdMLP start site. Because abortive initiation only monitors those steps in transcription that occur during production of a 3 nt RNA (i.e. preinitiation complex formation and initiation), we conclude that GAL4-VP16c can activate transcription at one or both of these steps.

### **Monitoring escape commitment**

#### Theory

Escape commitment occurs during the formation of ternary complexes that are stable and committed to proceeding forward through the remainder of the transcription reaction.<sup>1,2</sup> This occurs as RNA polymerase II leaves the abortive mode of transcript synthesis, as discussed in the previous section, and synthesizes a short 4 nt RNA that

remains stably associated with the transcribing preinitiation complex (see Figure 1). Consequently, one method to experimentally monitor escape commitment is to isolate ternary complexes containing 4 nt RNAs. The use of gel filtration spin columns allows the separation of 4 nt RNAs contained in stable ternary complexes, which pass through the column, from short RNAs that are abortively synthesized and released, which are retained in the column.<sup>2,23</sup> In this way, transcripts made during escape commitment can be monitored independently from those made during initiation.

The primary consideration in monitoring escape commitment is how to limit transcript synthesis to a 4 nt RNA. The sequence of some promoters will naturally halt transcription at +5 if only a subset of nucleotides is added, as was the case with the IL-2 promoter.<sup>18</sup> When characterizing escape commitment at the AdMLP, we made a mutation at +5 (+5mt AdMLP) such that the longest RNA that could be made was 4 nt when transcription was initiated with ApC, UTP, and CTP (Figure 5A).<sup>1</sup> On the promoters studied to date, escape commitment depends on the length the transcribed RNA (4 nt) and not on the sequence of the promoter.<sup>2</sup> Therefore, mutations that limit transcript synthesis to 4 nt do not affect the characteristics of escape commitment.

### Method

The following experiment describes analyzing escape commitment at the +5mt AdMLP.

1. Nucleotide premix. Prepare a solution of 11x nucleotides by diluting stock solutions of nucleotides into TE buffer (10 mM Tris (7.9), 0.1 mM EDTA) to the following concentrations: 11 mM ApC; 1.1 mM UTP; 5.5  $\mu$ M [ $\alpha$ -<sup>32</sup>P]CTP (2.5  $\mu$ Ci/ $\mu$ l). Each reaction will get 2  $\mu$ l of the nucleotide premix. Both non-radioactive and radioactive CTP are added to the nucleotide premix to obtain the final concentration and specific activity.

2. Add 2  $\mu$ l of nucleotide premix to each reaction containing preinitiation complexes (assembled as described in an earlier section). Mix by flicking with finger. Incubate at 30°C for 30 min.
3. While transcription is occurring, equilibrate G25 gel filtration spin columns (Amersham Pharmacia). To do so, snap off the bottom of the column and place in a 1.5 ml tube. Spin at 735 x for exactly 1 min. Remove the column to a new 1.5 ml tube. Add 100  $\mu$ l of a solution consisting of 50  $\mu$ l DB(100), 25  $\mu$ l RM4X, and 25  $\mu$ l ddH<sub>2</sub>O to each column. Spin at 735 x g for exactly 1 min. Place the equilibrated column into a new 1.5 ml tube in which to collect the eluate.
4. Pipet each transcription reaction on the top of an equilibrated G25 spin column. Spin at 735 x g for exactly 2 min.
5. Heat the eluates at 70°C for 3 min.
6. Treat the eluates with calf intestinal alkaline phosphatase (10 U) at 37°C for 20 min.
7. Add 3  $\mu$ l of stop mix containing 200 mM EDTA, 20% glycerol, and 0.025% bromophenol blue. Vortex.
8. Load 8-10  $\mu$ l of reaction on a 20% polyacrylamide (19:1) gel (20 cm x 20 cm; 0.4 mm spacers) containing 7 M Urea, 0.5x TBE (45 mM Tris, 45 mM Boric Acid, 1 mM EDTA). Also load RNA size standards, as described in the abortive initiation section.
9. Run the gel in 0.5x TBE at 17 watts until the bromophenol blue is half way down the gel.
10. Dry gel and visualize transcript RNA by Phosphorimagery and the size standard RNAs by UV shadowing with 254 nm light.

### Interpretation of results

Data illustrating the above technique is shown in Figure 5B. As shown in lane 1, large quantities of 3 nt RNA are visible when the reaction was not passed through a spin column; however, 3 nt RNA is not detected in the eluate from a spin column, as shown in

lane 2. This indicates that the 3 nt RNA was abortively produced and released and not part of stable ternary complexes. When lane 2 was exposed longer, 4 nt RNA that eluted from the spin column as part of ternary complexes could be seen. This RNA was produced during escape commitment. The longer exposure was necessary because the 4 nt RNA was produced only once per actively transcribing complex, whereas the 3 nt RNA was produced and released multiple times per complex.

## **Quantitation of template usage**

### Theory

It is common that during in vitro transcription in mammalian systems only a fraction of preinitiation complexes give rise to full length RNA products. An estimation of this fraction is obtained from calculating template usage under single round transcription conditions. Template usage is defined as the moles of transcript RNA that are produced per mole of template DNA added to the reaction. Under multiple round conditions and when monitoring abortive products, it is possible for more than one RNA molecule to be produced per template DNA. Therefore under these conditions, the template usage calculation merely provides insight into the efficiency of the transcription reaction over time.

Template usage calculations are useful because they can reveal how specific factors and conditions affect the in vitro transcription reaction. For example, we have shown that TFIIE and TFIIH increase the fraction of complexes that produce a full length RNA (as determined by template usage) under single round conditions.<sup>17</sup>

### Method

The following protocol for how to calculate template usage assumes the RNA transcript is labeled with [ $\alpha$ -<sup>32</sup>P]CTP. It is important to do this for every experiment performed to account for decay of the radioactivity over time.

1. Save a portion of the nucleotide premix used to initiate transcription. Make serial dilutions of the nucleotide premix in the range of 1:1000 to 1:100,000.
2. Spot 10  $\mu$ l of each dilution on Whatman paper and expose to a Phosphorimager screen along with the transcription experiment.
3. Calculate the moles of CTP in each spot. Remember that both radioactive and non-radioactive CTP are included in the nucleotide premix.
4. Quantitate the spots using Phosphorimagery and calculate the Phosphorimager units per mole of CTP.
5. Quantitate the RNA band using Phosphorimagery.
6. Calculate the moles of CTP incorporated into transcript RNA. For this calculation, divide the Phosphorimager units in the RNA band (value from step 5) by the Phosphorimager units per mole of CTP (value from step 4).
7. Calculate the moles of RNA produced. Divide the value from step 6 (moles of CTP in the transcript) by the number of cytosines in the transcript RNA.
8. To calculate template usage, divide the moles of RNA produced in the transcription reaction (value from step 7) by the moles of DNA template added to the reaction.

### Interpretation of results

It has been widely observed that template usage values for in vitro transcription by RNA polymerase II are quite low. Values for a single round of transcription are usually below 10% and often approximate 1%. This indicates that a large fraction of the preinitiation complexes abort prior to the production of a full length RNA, and that the transcription reaction branches between functional and aborted pathways during at least one point in the reaction.<sup>1,17</sup>

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### **Figure legends**

**Figure 1.** Model depicting the steps in the RNA polymerase II transcription reaction. See text for description. Abbreviations are as follows: R, general transcription factors (e.g. TBP, TFIIB, TFIIF, and RNA polymerase II); P, promoter DNA; PIC, preinitiation complex;  $RP_I \bullet$  (3nt RNA), initiated complex containing 3 nt RNA;  $RP_{EC} \bullet$  (4nt RNA), escape committed complex containing 4 nt RNA;  $R_E \bullet$  (15nt RNA), elongation complex containing 15 nt RNA;  $R_E \bullet$  (FL RNA), elongation complex containing full length RNA. Adapted from J.F. Kugel and J.A. Goodrich. Translocation after synthesis of a four-nucleotide RNA commits RNA polymerase II to promoter escape. Mol. Cell. Biol. 22, 762-773 (2002).

**Figure 2.** Time course for assembly of preinitiation complexes in vitro. General transcription factors and DNA template are incubated separately at 30°C for 2 min prior to mixing. Preinitiation complexes form during the subsequent 10 min incubation at 30°C. Nucleotides are then added to initiate transcription.

**Figure 3.** Methods to pause RNA polymerase II soon after completion of promoter escape. **(A)** Schematic of the AdMLP. The template strand sequence in the initial transcribed region is shown. The sequence of the 15 nt RNA produced in vitro in the presence of ApC, CTP, and UTP is shown above the DNA. **(B)** Schematic of the human IL-2 promoter. The template strand sequence in the initial transcribed region is shown. Shown above the DNA is the sequence of the 28 nt RNA produced in vitro in the presence of ATP, CTP, UTP, and the chain terminator 3'-O-Methylguanosine 5'-Triphosphate (Amersham Pharmacia) (3'-Me-GTP), which ensures that RNA polymerase II does not read through the G at position +28.

**Figure 4.** GAL4-VP16c stimulates abortive initiation. **(A)** Schematic depicting the abortive initiation assay used at the AdMLP. The template strand sequence is shown along with the RNA transcript produced. See text for details. **(B)** The amount of abortive product increases in the presence of GAL4-VP16c. The transcription reactions contained TFIIA, TFIIB, TFIID, TFIIIE, TFIIF, TFIIH, core RNA polymerase II, and 200  $\mu$ M dATP in addition to the CpA and CTP, as discussed in the method. Activator was included or omitted as indicated. Reactions in lanes 1 and 2 received a plasmid containing the wildtype AdMLP. Reactions in lanes 3 and 4 received a plasmid containing the AdMLP with a mutation at the transcription start site: +1 changed from T to C on the template strand (J.A. Goodrich and R. Tjian. *Cell*. 77, 145 (1994).) 3 nt RNA product is shown.

**Figure 5.** Escape committed complexes contain 4 nt RNAs stably bound. **(A)** The sequence of the template strand of the AdMLP +5mt is shown along with the 4 nt RNA produced. **(B)** Escape commitment results in stable ternary complexes with 4 nt RNA bound. Reactions were passed over G25 spin columns as indicated. Lane 3 is a longer exposure of lane 2. The positions of 3 nt and 4 nt RNA products are indicated. Adapted

from J.F. Kugel and J.A. Goodrich. Translocation after synthesis of a four-nucleotide RNA commits RNA polymerase II to promoter escape. Mol. Cell. Biol. 22, 762-773 (2002).

Figure 1

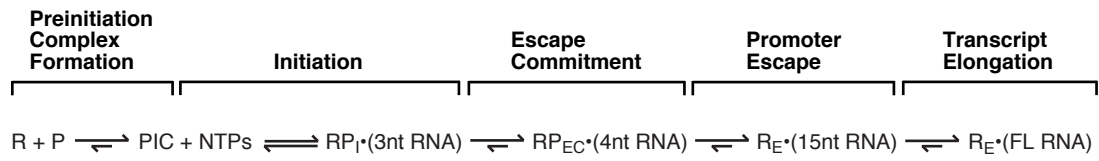


Figure 2

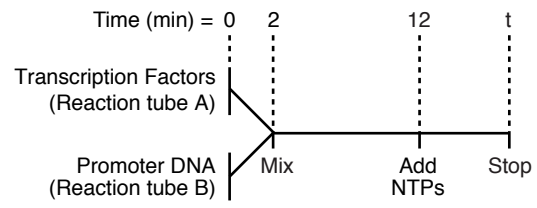
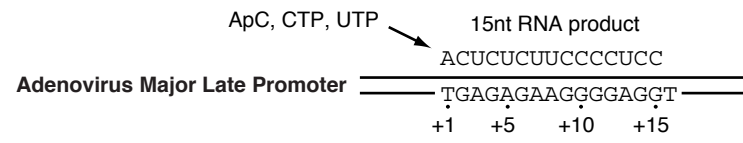


Figure 3

**A**



**B**

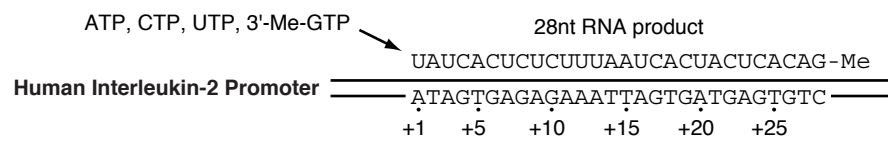


Figure 4

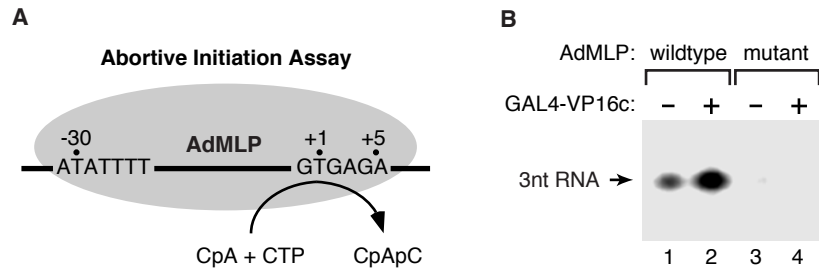


Figure 5

