2016

Transcription start site scanning and the requirement for ATP during transcription initiation by RNA polymerase II

James Fishburn
Fred Hutchinson Cancer Research Center

Eric Galburt
Washington University School of Medicine

Steven Hahn
Frank Hutchinson Cancer Research Center

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 291, NO. 25, pp. 13040–13047, June 17, 2016

Transcription Start Site Scanning and the Requirement for ATP during Transcription Initiation by RNA Polymerase II*

Received for publication, February 27, 2016, and in revised form, April 12, 2016 Published, JBC Papers in Press, April 17, 2016, DOI 10.1074/jbc.M116.724583

James Fishburn[‡], **Eric Galburt**[§], **and Steven Hahn**[‡1]

From the [‡]*Fred Hutchinson Cancer Research Center, Seattle, Washington 98109 and the* [§]*Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110*

Saccharomyces cerevisiae **RNA polymerase (Pol) II locates transcription start sites (TSS) at TATA-containing promoters by scanning sequences downstream from the site of preinitiation complex formation, a process that involves the translocation of downstream promoter DNA toward Pol II. To investigate a potential role of yeast Pol II transcription in TSS scanning, *HIS4* promoter derivatives were generated that limited transcripts in the 30-bp scanned region to two nucleotides in length. Although we found that TSS scanning does not require RNA synthesis, our results revealed that transcription in the purified yeast basal system is largely ATP-independent despite a requirement for the TFIIH DNA translocase subunit Ssl2. This result is rationalized by our finding that, although they are poorer substrates, UTP and GTP can also be utilized by Ssl2. ATPγS is a strong inhibitor of rNTP-fueled translocation, and high concentrations of ATPγS make transcription completely dependent on added dATP. Limiting Pol II function with low ATP concentrations shifted the TSS position downstream. Combined with prior work, our results show that Pol II transcription plays an important role in TSS selection but is not required for the scanning reaction.**

Following assembly of the metazoan RNA polymerase (Pol)[2] II transcription preinitiation complex (PIC), productive transcription initiation involves at least three steps (1–9): 1) formation of the open complex state containing an ~11-base DNA bubble. In this state, the unwound DNA template strand is positioned within the Pol II active site cleft. 2) Synthesis of short RNAs (≤ ~9 bases) that initiate within the unwound DNA. This initial transcribing complex can contain a DNA bubble of up to ~18 bases resulting from downstream DNA scrunched into the Pol II active site. 3) Promoter escape, where Pol II releases contacts with the basal transcription factors and promoter DNA while transitioning to a processive elongating form. Linked to this release is the collapse of the DNA bubble to the size observed in transcription elongation complexes. Alternatively, during the initial transcription of short RNAs, Pol II can enter a nonproductive state where short abortive RNA products are repeatedly synthesized.

Saccharomyces cerevisiae (yeast) Pol II follows a similar but distinct initiation pathway (10). At yeast TATA-containing promoters, most Pol II initiation occurs downstream from the site of PIC formation rather than a fixed distance from the TATA element (11, 12). Initiation typically begins within a window between ~50–100 bases downstream from TATA, with TSS recognition determined in part by the DNA sequence surrounding the TSS (13–16). During the initiation process, yeast Pol II scans downstream DNA for the TSS because Pol II was shown to preferentially utilize the first TSS motif encountered within the 50- to 100-bp window (17). Here we define scanning as the translocation of downstream promoter DNA with respect to Pol II, a process that precedes recognition of the TSS.

ATP or dATP hydrolysis is required at several stages of the initiation pathway (4, 18, 19). Initial DNA opening requires the Ssl2/XPB (yeast/human) subunit of TFIIH (5, 6, 20), an ATP-dependent DNA translocase (21). Ssl2 is thought to reel downstream DNA into the Pol II cleft, leading to torsional strain and DNA opening (22–25). Single-molecule studies using an optical trap have observed the ATP-dependent translocation of downstream promoter DNA toward yeast Pol II before the onset of transcription initiation (26). In some mammalian systems, XPB has been implicated in promoter escape because ATP or dATP is required for efficient transition from the initial transcribing complex and/or abortive state to the elongating form (8, 9, 27, 28). Finally, the TFIIH Pol II C-terminal domain (CTD) kinase Kin28/Cdk7 is required *in vivo* for an early step in transcription initiation (29–31). This kinase-dependent step has not yet been observed with purified systems.

Although there is good evidence that yeast TSS scanning requires Ssl2 function, there is conflicting evidence regarding whether Pol II RNA synthesis also contributes to scanning. Utilizing an *in vitro* transcription system with the yeast *ADH1* promoter fused to a G-less cassette, Khaperskyy *et al.* (32) showed that transcription initiated normally without added GTP. Because five guanine residues are located in the *ADH1* non-template strand between the presumed PIC location and the TSS, this result showed that continuous RNA synthesis is not required for Pol II to transverse the scanned DNA. However, because of the spacing of guanine residues in this region, short RNAs of 15–17 bases could theoretically have been made and contributed to scanning. Other evidence suggests that Pol

* This work was supported by National Institute of General Medical Sciences, National Institutes of Health Grant 2RO1GM053451 (to S. H.) and National Science Foundation Molecular and Cellular Biosciences Grant 1243918 (to E. G.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

[1] To whom correspondence should be addressed: Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N., Mailstop A1-162, Seattle, WA 98109. Tel.: 206-667-5261; E-mail: shahn@fredhutch.org.

[2] The abbreviations used are: Pol, polymerase; PIC, preinitiation complex; TSS, transcription start site(s); AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; TFO, triplex-forming oligonucleotide; ATPγS, adenosine 5′-*O*-(thiotriphosphate).

II and/or basal factors influence either scanning or TSS recognition. First, many mutations have been identified in basal transcription factors that alter yeast TSS location (10, 33). These include mutations in TFIIB, TFIIF, and the Ssl2 translocase (24). Mutations that alter TSS usage have also been identified within Pol II. For example, Kaplan *et al.* (34) showed that Pol II mutations affecting elongation kinetics can alter the TSS *in vivo*. One possible explanation for these results is that RNA synthesis by Pol II contributes to the scanning and/or TSS selection process.

To examine the involvement of Pol II RNA synthesis in the scanning process, we devised a system where transcripts of no longer than 2 bases could be synthesized within a scanned promoter region. Although we found that transcription initiated normally under these conditions, eliminating a required role for Pol II RNA synthesis in scanning, we surprisingly found that yeast transcription initiated in the absence of hydrolyzable ATP or dATP. From the series of experiments presented here, we conclude that the yeast system does not have a strict requirement for ATP/dATP and that other NTPs can fulfill the energy requirement for DNA opening and TSS scanning. Finally, our results point to a role for Pol II transcription in TSS selection.

Experimental Procedures

Triplex Disruption Assay—Triplex templates and disruption reactions were performed as described previously (21) with the following modifications: 30 fmol of holo-TFIIH (Rad3-E236Q) was added to 10-μl reactions containing 30 fmol of triplex DNA and allowed to bind for 10–30 min at room temperature. NTPs were added to 400 μM each unless otherwise indicated, and reactions were incubated for 1–15 min or 4 h at 26 °C degrees. Reactions were stopped with 3 μl 5× GSMB (15% glucose, 3% SDS, 250 mM MOPS (pH 5.5), and 0.04% bromphenol blue) and analyzed on 10% acrylamide gels in TAM buffer (40 mM Tris acetate (pH 5.5), 5 mM sodium acetate, and 1 mM magnesium chloride). The gels were dried, and products were visualized using by PhosphorImager (Molecular Dynamics). Because the displaced oligonucleotide showed a tendency to stick to the sides of the reaction tubes, we found that the most reliable method of quantitation was comparing the amount starting triplex with that remaining after incubation with TFIIH and NTP(s).

GTPase Assay—The DNA-dependent GTPase activity of TFIIH (Rad3-E236Q) was measured using a colorimetric assay kit (Innova, 601-0120). Reactions were carried out as described previously for ATPase function (21) with the following modifications. Reactions contained 80 fmol holo-TFIIH and 2.5 fM to 1.5 μM DNA. After 30 min at room temperature, purified GTP (Innova, 602-9999) was added to 0.5 mM, and reactions were incubated for 20 min at 26 °C. Pi-lock Gold Mix was added to stop reactions and chased with stabilizer, and absorbance at 635 nm was measured after 30 min at room temperature. Standard curves were established using the included phosphate standard and used to determine GTPase activity. These values were plotted against DNA concentration and fit with the Michaelis-Menten expression to determine V_{max} and K_m.

In Vitro Transcription—Reactions were performed as described previously (12, 21) using recombinant yeast TATA-binding protein (TBP), TFIIB, and TFIIF and highly purified TFIIE, TFIIH, and RNA Pol II with the following modifications. 20-μl reaction mixtures contained 10 mM HEPES (pH 7.6), 100 mM potassium glutamate, 10 mM magnesium acetate, 3 mM DTT, 2 μg BSA, 4 units RNase OUT (Invitrogen), 0.05% Nonidet P-40 substitute (USB Corp.), and 150 ng of plasmid template. Reactions also contained 0.03 units of creatine phosphokinase and 38 mM phosphocreatine (Sigma, P7936) or 38 mM creatine (Sigma, C0780) as indicated. Purified factors and DNA were mixed to form preinitiation complexes for 30 min at room temperature. Transcription was initiated by adding nucleoside triphosphates (GE Healthcare, 27202501) to ~400 μM each and, where indicated, the dinucleotide primer GpA (Dharmacon) to ~200 μM. After 30 min at room temperature, reactions were stopped or supplemented with 1 mM calcium chloride and treated with 1 unit RQ1 DNase (Promega, M6101) for 15 min at 37 °C degrees to eliminate the promoter DNA. Primer extension was performed as described using fluorescent or ^{32}P-labeled lacI or ^{32}P-labeled transcript-specific primers for the A-less and G + A-less transcripts. ATPγS and AMP-PNP were from Roche.

Results

Transcription of an A-less Cassette in the Absence of ATP/dATP—As a first step in testing the contribution of RNA synthesis during yeast Pol II scanning and TSS selection, we modified the yeast *HIS4* promoter so that the transcript lacked adenine (Fig. 1*A*). *In vitro* transcription from wild-type *HIS4* primarily initiates 63 bp downstream from the beginning of the TATA and at several other downstream positions (12). A small fraction of the total initiation begins 34 bp downstream from TATA, in a position analogous to where metazoan Pol II initiates with respect to the PIC (11). To generate the A-less *HIS4* transcript, we changed the base at the primary upstream initiation site from adenine to guanine (Fig. 1*A*, *+1*) because yeast Pol II strongly prefers to initiate with a purine. We replaced other adenine residues in the transcribed region with thymines. In this modified promoter, there are 10 adenine residues located between the metazoan TSS position (−29) and the most upstream *HIS4* TSS (+1) with five continuous adenine residues in one segment. The longest RNA that could be produced in the scanned region in the absence of ATP is 10 residues.

Fig. 1*B* shows a primer extension assay of *in vitro* transcription products from wild-type and A-less *HIS4* using purified and recombinant yeast basal factors and Pol II. As expected, transcription from WT *HIS4* requires the basal factors TBP and TFIIH and all four NTPs (Fig. 1*B*, *lanes 1–5*). Synthesis of the A-less transcript also required TBP and TFIIH (Fig. 1*B*, *lanes 6–8*), and it was efficiently transcribed with 400 μM of the Pol II substrates GTP, CTP, and UTP (Fig. 1*B*, *lane 9*). In this reaction, 400 μM dATP was included to fulfill the expected requirement for Ssl2 function. Surprisingly, transcription was reduced by only 20% when dATP was omitted (Fig. 1*B*, *lane 10*). Combined, the results agree with an earlier study that continuous transcription is not required for TSS scanning (32). However, transcription in the yeast system displays an unexpected independence from ATP/dATP.

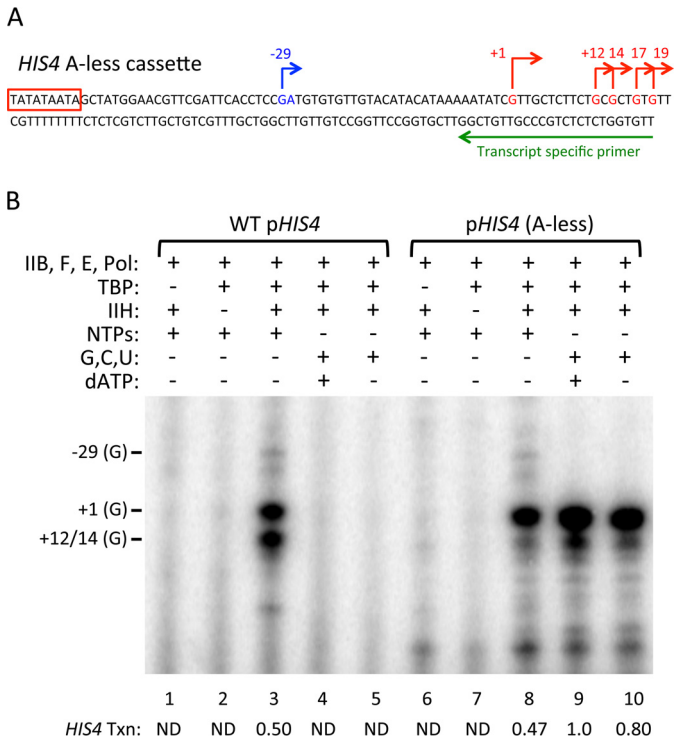


FIGURE 1. **Nucleoside triphosphate requirements for *in vitro* transcription.** *A*, the non-template DNA sequence is shown for the modified *HIS4* A-less transcription template. The promoter is identical to *HIS4* between TATA (*red box*) and the +1 transcription start site. ATP is not required for RNAs transcribed from the A-less cassette when initiated from +1 (*red arrow*) through the RT primer-recognized sequence (*green arrow*). *Red arrows* and bases indicate WT *HIS4* TSS, and the *blue arrow* (−29) indicates the small fraction of transcription initiating from the metazoan position with respect to the TATA. *B*, *in vitro* basal transcription using the yeast-purified transcription system with either wild-type *HIS4* or A-less transcription templates. Reactions contained Pol II and basal factors as indicated. NTPs indicates 400 μM of all four rNTPs were added. *G,C,U* indicates that ATP was omitted. dATP was added to 400 μM where indicated. RNA products were visualized by primer extension, and the TSSs used are indicated. Transcription was quantified for one of four independent experiments and is expressed as a fraction of maximal combined activity observed from the +1 and +12/14 start sites. *ND*, not determined.

One possible explanation for the lack of a strong ATP requirement might be the absence of a requirement for Ssl2 function in our transcription system. In contrast to this model, a transcription reaction containing TFIIH with the Ssl2 ATPase mutation E489Q shows no detectable *HIS4* transcription (Fig. 2). Because transcription of the A-less cassette is only slightly less efficient when ATP/dATP is omitted, our combined results suggest that NTPs other than ATP can promote Ssl2 activity.

However, another possible explanation for the apparent ATP independence of transcription is that our NTPs are contaminated with ATP. Our experiments use commercial preparations of NTPs that are >99.5% pure (see "Experimental Procedures"). As a test of ATP contamination, we added 0.4 μM ATP (0.1% final) to transcription reactions to determine whether this altered the transcription pattern (Fig. 3). We found that addition of this trace amount of ATP, and the dinucleotide GpA, promoted robust transcription initiation from the metazoan TSS position (−29 in Fig. 3, *lane 3*). The longer RNA contains nine adenines in the first 30 nucleotides of the transcript. Depleting the added ATP by addition of creatine and creatine phosphokinase significantly reduced the synthesis of

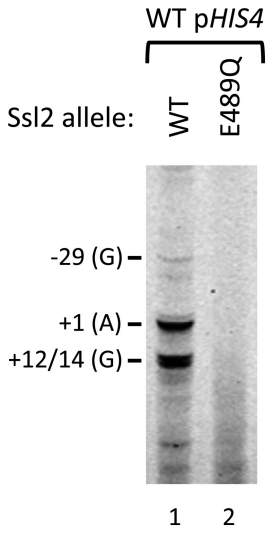


FIGURE 2. ***In vitro* transcription requires catalytically active Ssl2.** Reactions contained a wild-type *HIS4* transcription template, purified and recombinant factors as in Fig. 1, and equivalent amounts of holo-TFIIH containing an active (*WT*) or inactive (*E489Q*) Ssl2 subunit (21). The inactive variant of Ssl2 was assayed in transcription once.

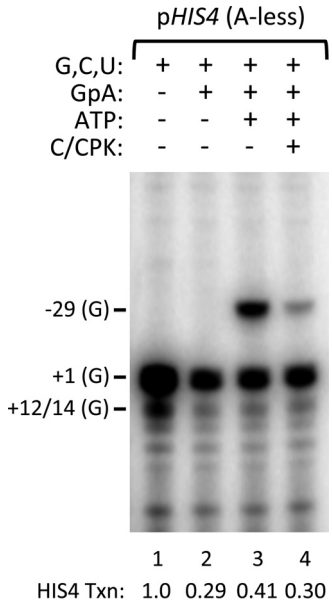


FIGURE 3. **Transcription assay to probe ATP contamination of nucleoside triphosphates.** *In vitro* transcription (*Txn*) of the *HIS4* A-less cassette using purified factors and minimally required NTPs. The −29 TSS dinucleotide primer (*GpA*) and ATP were included as indicated. Creatine and creatine phosphokinase (*C/CPK*) were included where indicated as an ATP-depleting system. Transcription was quantified for one of two independent experiments and is expressed as a fraction of maximal combined activity from the −29, +1, and +12/14 start sites.

this long transcript (Fig. 3, *lane 4*). Because the combination of CTP, UTP, and GTP does not promote the synthesis of this long transcript, we conclude that the ATP independence of yeast transcription is not due to contaminating ATP.

Ssl2 Can Utilize GTP and UTP as Substrates but with Lower Efficiency Than dATP—DNA helicases have been observed to utilize non-adenosine NTPs as substrates but with reduced processivity compared with ATP (35, 36). As a test of whether TFIIH/Ssl2 can hydrolyze alternative NTPs, we examined its GTPase activity. These assays used purified yeast TFIIH con-

A DNA-dependent ATP, GTPase activity of Ssl2

Lattice	Vmax (ATP/Ssl2/sec)	ATPase Km (μM bp)	Vmax (GTP/Ssl2/sec)	GTPase Km (μM bp)
Plasmid	11.08 ± 0.24	ND	1.75 ± 0.1	0.045 ± 0.013
60 bp	8.82 ± 0.17	1.56 ± 0.28	1.56 ± 0.06	3.96 ± 0.62
No DNA	0	ND	0	ND

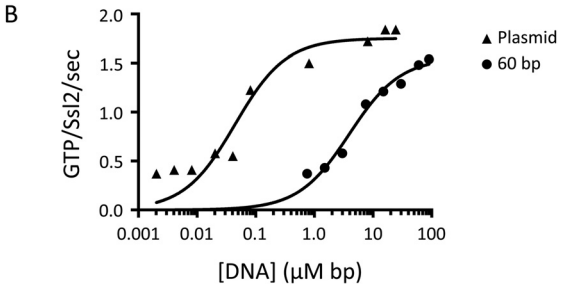
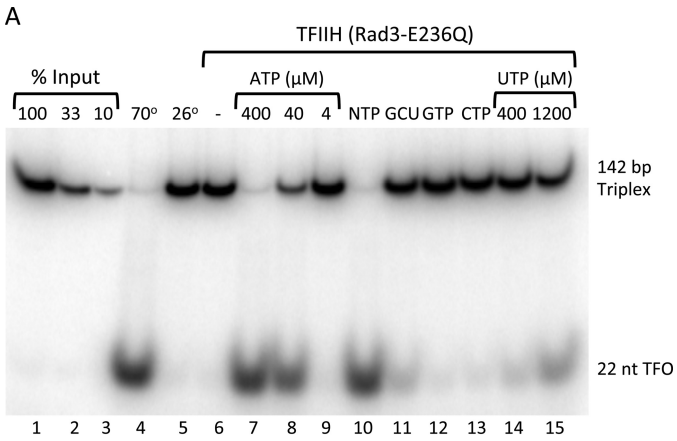


FIGURE 4. **GTP is a substrate of Ssl2.** *A*, DNA-dependent GTPase activity of TFIIH (Rad3-E236Q) was measured utilizing two templates of different length over a wide range of DNA concentrations. V_{max} and K_m values are given for GTPase and ATPase activities of Ssl2 with the ATPase values from Fishburn *et al.* (21). No hydrolysis of either nucleoside triphosphate was observed in the absence of DNA. GTPase data were compiled from two independent experiments and used to determine values for V_{max} and K_m. *B*, the steady-state rate of GTP hydrolysis as a function of DNA length and concentration. DNA concentration is plotted as micromolar base pairs, and the data are fit to Michaelis-Menten curves allowing the extraction of K_m and V_{max} parameters. *ND*, not determined.



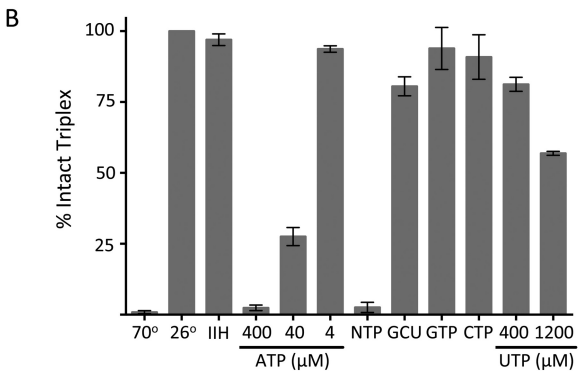


FIGURE 5. **Triple helix disruption by the nucleoside triphosphate-driven TFIIH translocase.** *A*, triplex templates were incubated for 4 h at 26 °C with TFIIH (Rad3-E236Q) in the presence of the indicated NTPs. Products were separated on a 10% acrylamide gel, and intact triplexes were quantified and plotted against a standard curve. In *lanes 10–13*, NTPs were added as indicated to a concentration of 400 μM each. ATP and UTP were added to reactions in *lanes 7–9* and *14* and *15*, respectively, at the indicted concentrations. Reactions in *lanes 8*, *9*, and *15* were performed twice, and all others were performed three times. *B*, the fraction of intact triplexes normalized to the 26 °C control reaction (*lane 5* in *A*). Means ± S.D. (*error bars*) calculated from three independent experiments are shown.

taining a mutation that eliminates the ATPase function of the DNA helicase subunit RAD3 (21) and measured phosphate released upon GTP hydrolysis. CTP and UTP hydrolysis could not be measured by this assay using commercially available nucleotides because they contain levels of phosphate that obscure phosphate release from NTP hydrolysis. We found that TFIIH/Ssl2 can hydrolyze highly purified GTP in a dsDNA-dependent manner (Fig. 4). GTPase activity was completely dependent on added DNA, with a short 60-bp DNA substrate promoting a 12% lower V_{max} of GTP hydrolysis compared with plasmid DNA. Although these results show that GTP is a substrate for Ssl2, the V_{max} of hydrolysis is 5.6-fold lower compared with ATP hydrolysis using the 60-bp DNA substrate. Furthermore, the K_m for GTP is 2.5-fold higher compared with ATP using the 60-bp DNA substrate.

We next assayed whether GTP, CTP, or UTP can promote displacement of a triple-stranded oligonucleotide by TFIIH, which we showed previously is a measure of TFIIH/Ssl2 DNA translocation (21). In these assays, we utilized individual or combinations of NTPs at 400 μM, the levels added to our standard *in vitro* transcription reactions. Disruption was quantitated by comparing the amount of triplex with or without NTP addition. We found that measurement of triplex remaining after TFIIH and NTP addition, rather than the amount of displaced oligonucleotide, was more reproducible (see "Experimental Procedures").

Consistent with earlier results, we found that, in the presence of TFIIH, either 400 μM ATP or a combination of all four NTPs promote 98% displacement of the 22 base triplex-forming oligonucleotide (TFO) (Fig. 5*A*, *lanes 6*, *7*, and *10*). The combination of GTP, CTP, and UTP promoted 17% TFO displacement (Fig. 5*B*, *lane 11*). When individual NTPs were tested, UTP promoted 16% TFO displacement, whereas GTP and CTP had minimal effects. Increasing the concentration of UTP 3-fold led to 41% TFO displacement. (Fig 5*A*, *lanes 12–15*). Because GTP can be hydrolyzed by Ssl2 in a DNA-dependent manner (Fig. 4), it was surprising that GTP had little or no activity in the TFO displacement assay. However, it seems probable that TFO displacement requires higher Ssl2 processivity compared with translocation on double-stranded DNA, and this may account for the differences in the two assays. Together, our results show that GTP and UTP can both be utilized as Ssl2 substrates but with lower efficiency compared with ATP.

Non-hydrolyzable ATP Analogs and the Requirement for ATP/dATP in Transcription—Transcription in the mammalian system is inhibited by the non-hydrolyzable analog ATPγS. This nucleotide was presumed to function as an inhibitor of the Ssl2/XPB translocase because it rapidly reverses open complex formation (6, 37, 38). We used the triplex disruption assay to test whether ATPγS inhibits translocation (Fig. 6). Under the conditions of the assay, a combination of all four NTPs results in 86% displacement of the TFO within 15 min, and substitution of ATPγS for ATP blocks this reaction (Fig. 6*A*, *lanes 5–9*). Furthermore, ATPγS added at 1/3 or 1/9 the concentration of ATP inhibits TFO displacement, with the 1/9 and 1/3 ratios

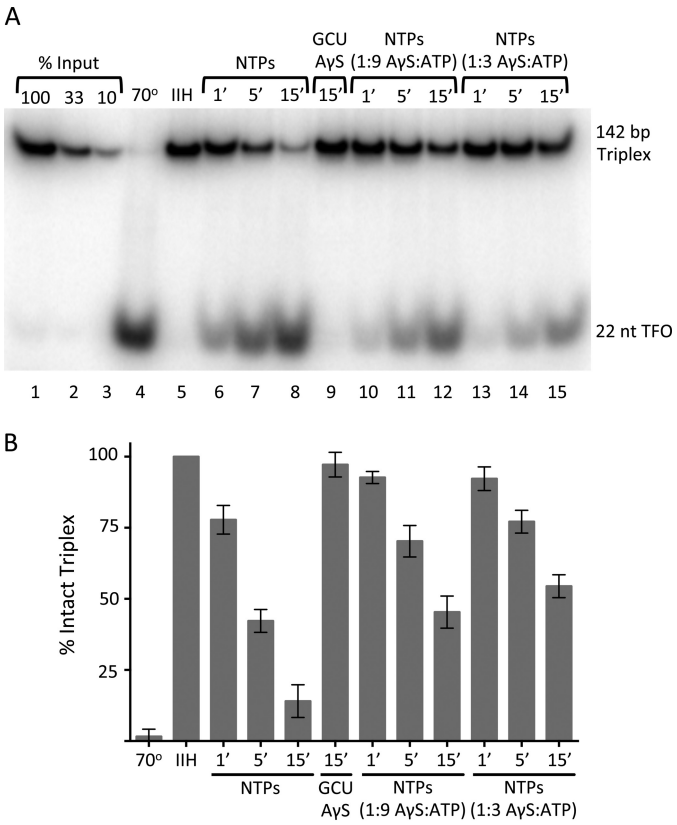


FIGURE 6. **ATPγS inhibition of TFIIH translocation and transcription.** *A*, TFIIH triplex disruption assay with 400 μM NTP mixtures containing ATP (*lanes 6–8*), ATPγS (*lane 9*), or combinations of the two (*lanes 10–15*). Templates were prebound with TFIIH for 30 min before initiating reactions with NTPs and stopped after 1–15 min at 26 °C. Products were separated on a 10% acrylamide gel, and intact triplexes were quantified and plotted against a standard curve. Triplex disruption was quantified from three independent experiments. *B*, the fraction of intact triplexes normalized to the no-NTPs control reaction (*lane 5* in *A*). Means ± SD (*error bars*) calculated from three independent experiments are shown.

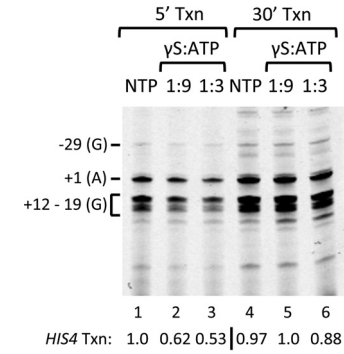


FIGURE 7. ***In vitro* transcription of a wild-type *HIS4* plasmid template using purified factors.** Reactions included the same NTP mixes used in Fig. 6 and were stopped after 5 or 30 min at room temperature. Primer extension was performed using fluorescently labeled lacI primer. Transcription (*Txn*) was quantified for one of two independent experiments and is expressed as a fraction of combined maximal activity from the +1 and +12–19 start sites.

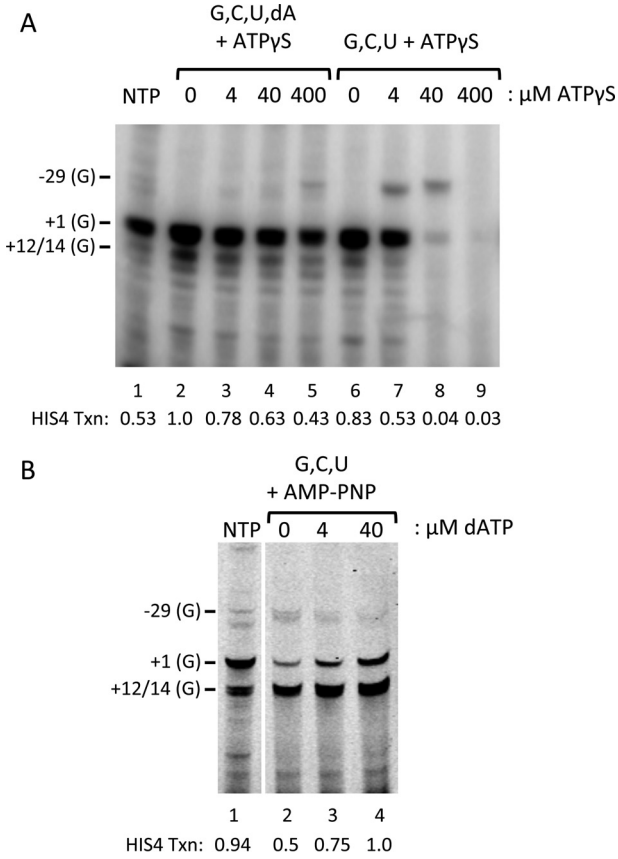


FIGURE 8. **Effects of AMP-PNP and ATPγS on transcription and dATP-dependence.** *A*, *in vitro* transcription of the *HIS4* A-less cassette using purified factors and 400 μM NTP mixtures containing ATP (*lane 1*), ATPγS and dATP (*lanes 2–5*), or ATPγS (*lanes 6–9*). Reactions were stopped after 30 min and assayed by primer extension using a ^{32}P-labeled primer specific for the A-less transcript. *B*, *in vitro* transcription of a *HIS4* template with a +1 guanine nucleotide. Reactions included purified factors and NTP mixtures containing ATP (*lane 1*) or AMP-PNP and increasing concentrations of dATP (*lanes 2–5*). Reactions were stopped after 30 min and assayed by primer extension using fluorescently labeled lacI. Both *A* and *B* show transcription (*Txn*) quantified from one of two independent experiments and expressed as a fraction of combined maximal activity from the +1 and +12/14 start sites.

resulting in 55% and 46% displacement after 15 min (Fig. 6*A*, *lanes 10–15*).

When ATPγS was added to transcription reactions, we found that a single round of transcription from *HIS4* was inhibited ~2-fold at the 1/3 ATPγS/ATP ratio and multiround transcription inhibited <12% (Fig. 7). Although Ssl2 function is clearly essential for transcription (Fig. 2), these results show that Ssl2 activity can be significantly reduced with only modest effects on transcription. This is consistent with our results that near-normal transcription can occur in the absence of ATP/dATP (Fig. 1) and that non-adenosine NTPs are not optimal Ssl2 substrates.

Our findings on the lack of a specific requirement for ATP hydrolysis in yeast transcription are surprising because many studies using mammalian systems have shown that both initiation and promoter clearance are ATP/dATP-dependent. However, all experiments assaying the initiation of transcripts longer than a few nucleotides required use of a non-hydrolyzable ATP analog such as ATPγS or AMP-PNP as substrates for RNA synthesis (5, 12, 18, 19, 27). These analogs may have created the appearance of strict ATP dependence when they are inhibitors of XPB function. To test whether these analogs can create a dependence on dATP in the yeast system, we performed transcription in the presence of 0–400 μM ATPγS (Fig. 8*A*, *lanes 6–9*). In reactions containing 400 μM GTP, CTP, and UTP and the A-less transcription template, addition of 40 and 400 μM ATPγS severely inhibits transcription from the +1 and other

downstream TSS. This inhibition is largely blocked by addition of 40–400 μM dATP (Fig. 8*A*, *lanes 4* and *5*). Thus, transcription in the presence of a high concentration of ATPγS creates a dependence for added dATP, presumably because these two NTPs bind Ssl2 with higher affinity compared with GTP and UTP. At the two lowest concentrations of ATPγS, we observed weak transcription from the upstream −29 position. We speculate that, at these ATPγS concentrations, Ssl2 is not completely inhibited and that this allows transcription initiation at −29. ATPγS can then be incorporated into this adenine-containing RNA because ATPγS is a substrate for RNA synthesis by Pol II.

Using a similar approach, we performed transcription of the *HIS4* promoter in the presence of 400 μM GTP, CTP, UTP, and AMP-PNP (Fig. 8*B*). Compared with transcription in the presence of all four NTPs, transcription with AMP-PNP preferentially initiated at +12G, indicating a preferential use of a downstream TSS. This behavior is similar to that observed when the activity of Pol II is limited by low ATP concentrations (see below). The total level of transcription with AMP-PNP was 2-fold lower compared with transcription with all four NTPs. Addition of dATP to the AMP-PNP reactions restored the total transcription level to that observed without AMP-PNP, although the system retains the *preference* for initiation at the downstream TSS (Fig. 8*B*, *lanes 3* and *4*). Because the overall level of transcription can be restored by dATP, a specific substrate for Ssl2 but not Pol II, this indicates that AMP-PNP is a modest inhibitor of Ssl2 function.

A Stringent Test of Pol II Function during TSS Scanning—To further limit the ability of Pol II to synthesize RNA during the TSS scanning phase, we modified the A-less *HIS4* template to also eliminate guanines in the transcribed region (Fig. 9*A*, *G+A-less cassette*). With this promoter, the longest RNAs that can be theoretically generated in the scanned region between the metazoan and yeast TSS positions are two nucleotides in length, with segments of 8 and 17 bp where no RNA can be synthesized. Transcription was initiated with CTP, UTP, and the dinucleotide GpA. RQ1 DNase was added after transcription was complete to reduce the potential background from primer extension of the DNA template. Transcription efficiently initiated from the expected +1 start site and was stimulated ~30% by dATP. Addition of phosphocreatine and creatine phosphokinase to regenerate any potential contaminating ATP had no effect (Fig. 9, *lane 5*). Because transcription initiates normally using the G + A-less cassette in the absence of GTP and ATP, we conclude that RNA synthesis is not required for yeast Pol II TSS scanning of downstream DNA.

Transcription Is Involved in TSS Selection—Previous results have demonstrated that mutations in the Pol II machinery can shift the TSS either upstream or downstream (10, 34). We explored the role of transcription in this process by decreasing the level of ATP while keeping the total ATP + dATP concentration at 400 μM to allow normal Ssl2 function but reduce Pol II activity. At the wild-type *HIS4* promoter, with the +1 RNA initiating with an adenine (Fig 10*A*), decreasing the ATP concentration from 400 to 4 μM shifted nearly all initiation downstream to +12 (Fig 10*B*, *lanes 1–4*). To test whether this strong sensitivity to ATP concentration was due to the first base of the

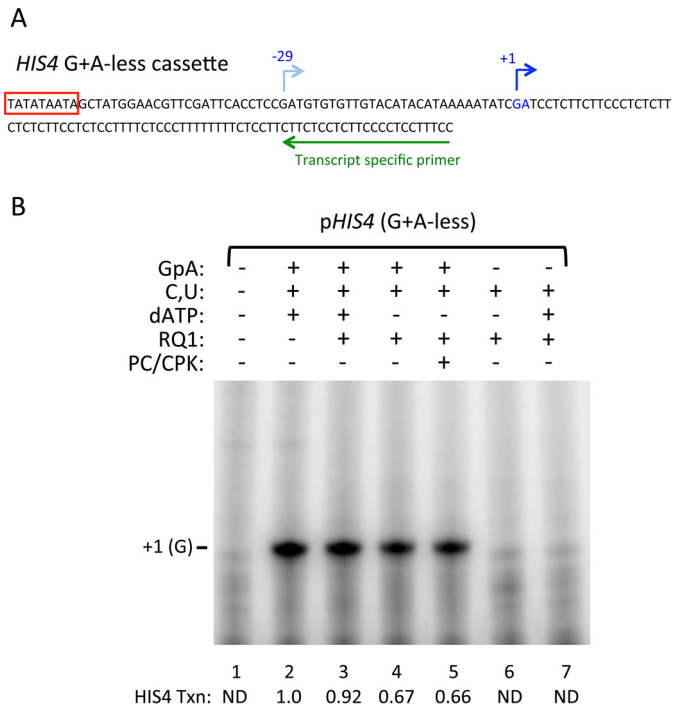


FIGURE 9. **NTP requirements for transcription of a G + A-less cassette.** *A*, the non-template DNA sequence is shown for the modified *HIS4* G + A-less transcription template. From the G + A-less cassette, neither GTP nor ATP are required for RNAs that initiate with the dinucleotide primer GpA (*dark blue arrow* and *bases*). The *faint blue arrow* (−29) indicates the metazoan TSS that is never utilized in transcription reactions lacking GTP and ATP. *B*, reactions include the *HIS4* G + A-less template with no additions (*lane 1*) and with purified factors dATP, CTP, and UTP and the dinucleotide primer GpA (*lane 2*). RQ1 DNase was added following transcription (*lanes 3–7*) to confirm primer extension of mRNA. Reactions lacking dATP for energy were performed (*lanes 4* and *5*) and supplemented with the ATP-regenerating creatine phosphokinase and phosphocreatine (*lane 5*). Negative controls lacking GpA are shown (*lanes 6* and *7*). Transcription (*Txn*) was quantified for one of three independent experiments and is expressed as a fraction of maximal activity observed from the +1 start site.

+1 RNA beginning with adenine, we changed this base to guanine. At this modified promoter, the TSS gradually shifted downstream with decreasing ATP concentration, and the strongest effect was observed at 4 μM ATP (Fig 10*B*, *lanes 5–8*). At this concentration, the ratio of downstream/upstream initiation shifted by 10-fold compared with 400 μM ATP. Our combined results point to an important role for Pol II transcription in TSS selection.

Discussion

Transcription initiation from yeast Pol II TATA-containing promoters follows a pathway distinct from metazoan systems. Instead of initiating transcription at a focused location centered on the site of PIC formation, yeast Pol II scans downstream sequences for an appropriate TSS, typically ~50–100 bp downstream from the TATA element. Although a previous study showed that continuous transcription of this scanned region is not required (32), mutations in Pol II and several other basal factors that alter TSS location point to a role for transcription in either scanning or TSS selection (10, 33, 34).

We initially designed two promoter derivatives to re-examine whether transcription of the scanned region, perhaps by synthesis of short RNA products, plays a role in scanning. Using

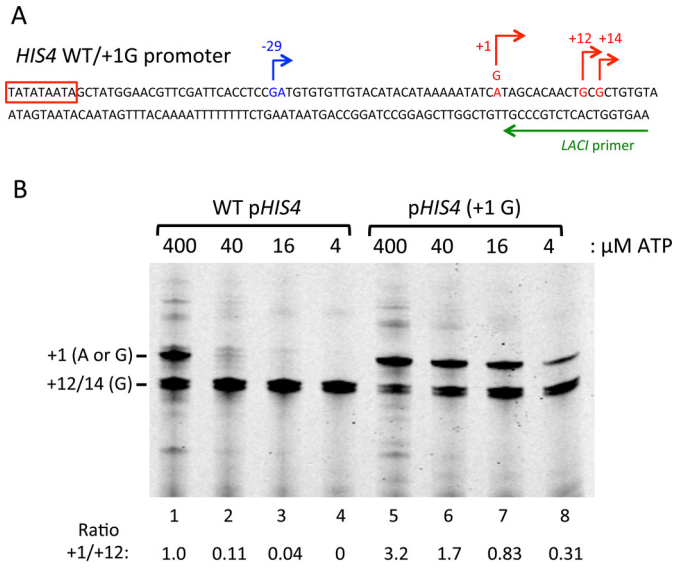


FIGURE 10. **Limiting Pol II function with low ATP concentrations shifts the TSS downstream.** *A*, sequence of the *HIS4* WT and modified promoters are shown along with the *lacI* primer used in primer extension assays. The TSS are indicated by *arrows*, with the primary TSS colored *red* as in Fig. 1. *B*, *in vitro* transcription of wild-type or *HIS4* modified to substitute a guanine at +1 of the non-template strand. The concentration of total ATP + dATP was 400 μM, and the concentration of ATP in each reaction is indicated. The ratios of initiation from the upstream/downstream TSS are listed. Transcription of the modified *HIS4* template under these conditions was tested once.

promoter derivatives lacking either adenine or adenine + guanine residues in the transcribed region, we showed that RNA synthesis is not required for TSS scanning. We found that transcription initiated normally at these promoters, which contain large regions where no transcription can occur and where the longest RNAs that could theoretically be synthesized in the 30-bp scanned region are two nucleotides. These results, combined with previous work, strongly suggest that movement of the scanned DNA with respect to Pol II is due solely to the action of the Ssl2 translocase.

The fact that mutations in Pol II and several basal factors can alter TSS location suggests that initial RNA synthesis plays a role in TSS selection. By this model, Pol II and associated basal factors scan the downstream sequence and, after scanning a minimum distance, initiate transcription at the first sequence compatible with efficient initiation. Mutations that alter TSS usage may affect recognition of the TSS consensus (39), the kinetics of initial RNA synthesis, and/or the stability of the initial transcribing complex. Consistent with this model, we found that limiting the concentration of the Pol II substrate ATP, while maintaining a high level of the Ssl2 substrate dATP, led to shifting the preferred TSS downstream.

Examination of the WT *HIS4* promoter sequence (Fig 10*A*) shows that RNA initiating from +1 has three adenines within the first seven bases, whereas there are no adenines in the first seven bases of downstream RNAs initiating from +12/14. Because low ATP will cause a slowdown of Pol II only when transcribing RNAs containing adenines, this suggests that TSS usage is sensitive to the rate of transcription of the first few nucleotides of RNA. These results are consistent with previous findings that slowing Pol II elongation shifts TSS usage downstream (34) and point to an important role for Pol II transcription in TSS selection.

During the course of this work, we discovered that transcription with our purified basal transcription system is not specifically ATP/dATP-dependent despite a requirement for Ssl2 function. Using *HIS4* promoter derivatives, we found that transcription was decreased less than 2-fold by omitting ATP or dATP. This result is surprising because nearly every study with the human transcription system has observed an ATP requirement for open complex formation and/or promoter escape.

To explain these results, we used a DNA-dependent NTPase assay and found that GTP can be used as a substrate for Ssl2/TFIIH, although the V_{max} of hydrolysis and the K_m for DNA binding are weaker than when ATP is used as a substrate. For technical reasons we were unable to measure the ability of Ssl2 to utilize UTP or CTP in this assay. Although this finding is unexpected, Jiang and Gralla (37) have reported that high concentrations of UTP, CTP, or GTP can lead to DNA unwinding at the E4 promoter in a human nuclear extract system. Many studies using the human system have also found an XPB and ATP requirement for promoter escape, requiring the synthesis of RNAs longer than 4–9 nucleotides (8, 9, 28, 40). It is possible that GTP and/or UTP may also promote yeast Pol II escape via their activation of Ssl2 or that escape is not a limiting step in the yeast system. Perhaps the strain of looped out DNA between the site of PIC formation and the TSS provides energy for efficient Pol II release when the initial transcribing complex is formed. Our combined results demonstrate that NTPs other than ATP can function as substrates the for initial DNA unwinding and TSS scanning in the yeast system.

To further compare the properties of the yeast and human systems, we examined yeast transcription in the presence of the non-hydrolyzable ATP analogs ATPγS and AMP-PNP. In earlier work, addition of these two derivatives to the human system was necessary to measure the role of ATP hydrolysis in transcription initiation of RNAs longer than a few nucleotides. Our modified promoter lacking alanines in the transcribed region allowed us to directly examine the effect of ATPγS and AMP-PNP addition. As expected from previous studies, we found that ATPγS is an inhibitor of Ssl2 function. Furthermore, addition of high concentrations of ATPγS to transcription reactions generated a strong requirement for dATP in transcription. It seems likely that dATP is required under these conditions because it has a higher affinity for Ssl2 compared with GTP/UTP and more effectively competes with ATPγS. AMP-PNP, the most commonly used derivative in studies of ATP-dependence for mammalian transcription, inhibits yeast transcription ~2-fold in single-round reactions, and this inhibition is overcome by dATP. One possibility to reconcile results in the yeast and human systems is that AMP-PNP is a stronger inhibitor of XPB compared with yeast Ssl2. We also found that AMP-PNP caused a preferential use of the downstream TSS. This is similar to the situation observed when low ATP concentration was found to shift the TSS downstream. Although AMP-PNP is a Pol II substrate, it is possible that Pol II elongation kinetics are slower compared with ATP, leading to the downstream shift in TSS usage.

In conclusion, our results have demonstrated that TSS scanning by yeast Pol II does not require synthesis of RNAs but that TSS selection likely requires transcription. We speculate that, as the scanning reaction proceeds, the basal machinery may be probing for sequences that permit efficient transcription of the first few nucleotides in the transcript. Events that may influence TSS utilization could include recognition of sequences in the template DNA strand by the TFIIB reader, the preference of Pol II for initiation at specific bases, and/or efficient formation and stabilization of an initial transcribing complex.

Author Contributions—J. F. performed all experiments. J. F., E. G., and S. H. designed the study, analyzed the data, and wrote the paper.

Acknowledgments—We thank Eric Tomko and members of the Hahn laboratory for advice and suggestions throughout the course of this work.

References

1. Sainsbury, S., Bernecky, C., and Cramer, P. (2015) Structural basis of transcription initiation by RNA polymerase II. *Nat. Rev. Mol. Cell Biol.* **16,** 129–143
2. Grünberg, S., and Hahn, S. (2013) Structural insights into transcription initiation by RNA polymerase II. *Trends Biochem. Sci.* **38,** 603–611
3. Luse, D. S. (2013) Promoter clearance by RNA polymerase II. *Biochim. Biophys. Acta* **1829,** 63–68
4. Luse, D. S., and Jacob, G. A. (1987) Abortive initiation by RNA polymerase II *in vitro* at the adenovirus 2 major late promoter. *J. Biol. Chem.* **262,** 14990–14997
5. Wang, W., Carey, M., and Gralla, J. D. (1992) Polymerase II promoter activation: closed complex formation and ATP-driven start site opening. *Science* **255,** 450–453
6. Holstege, F. C., Fiedler, U., and Timmers, H. T. (1997) Three transitions in the RNA polymerase II transcription complex during initiation. *EMBO J.* **16,** 7468–7480
7. Yan, M., and Gralla, J. D. (1997) Multiple ATP-dependent steps in RNA polymerase II promoter melting and initiation. *EMBO J.* **16,** 7457–7467
8. Kugel, J. F., and Goodrich, J. A. (1998) Promoter escape limits the rate of RNA polymerase II transcription and is enhanced by TFIIE, TFIIH, and ATP on negatively supercoiled DNA. *Proc. Natl. Acad. Sci. U.S.A.* **95,** 9232–9237
9. Pal, M., Ponticelli, A. S., and Luse, D. S. (2005) The role of the transcription bubble and TFIIB in promoter clearance by RNA polymerase II. *Mol. Cell.* **19,** 101–110
10. Hahn, S., and Young, E. T. (2011) Transcriptional regulation in *Saccharomyces cerevisiae*: transcription factor regulation and function, mechanisms of initiation, and roles of activators and coactivators. *Genetics* **189,** 705–736
11. Murakami, K., Mattei, P.-J., Davis, R. E., Jin, H., Kaplan, C. D., and Kornberg, R. D. (2015) Uncoupling promoter opening from start-site scanning. *Mol. Cell* **59,** 133–138
12. Fishburn, J., and Hahn, S. (2012) Architecture of the yeast RNA polymerase II open complex and regulation of activity by TFIIF. *Mol. Cell. Biol.* **32,** 12–25
13. Hahn, S., Hoar, E. T., and Guarente, L. (1985) Each of three "TATA elements" specifies a subset of the transcription initiation sites at the CYC-1 promoter of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 8562–8566
14. Nagawa, F., and Fink, G. R. (1985) The relationship between the "TATA" sequence and transcription initiation sites at the HIS4 gene of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U.S.A.* **82,** 8557–8561
15. Chen, W., and Struhl, K. (1985) Yeast mRNA initiation sites are determined primarily by specific sequences, not by the distance from the TATA element. *EMBO J.* **4,** 3273–3280
16. Zhang, Z., and Dietrich, F. S. (2005) Mapping of transcription start sites in *Saccharomyces cerevisiae* using 5′ SAGE. *Nucleic Acids Res.* **33,** 2838–2851
17. Kuehner, J. N., and Brow, D. A. (2006) Quantitative analysis of *in vivo* initiator selection by yeast RNA polymerase II supports a scanning model. *J. Biol. Chem.* **281,** 14119–14128
18. Bunick, D., Zandomeni, R., Ackerman, S., and Weinmann, R. (1982) Mechanism of RNA polymerase II-specific initiation of transcription *in vitro*: ATP requirement and uncapped runoff transcripts. *Cell* **29,** 877–886
19. Sawadogo, M., and Roeder, R. G. (1984) Energy requirement for specific transcription initiation by the human RNA polymerase II system. *J. Biol. Chem.* **259,** 5321–5326
20. Tirode, F., Busso, D., Coin, F., and Egly, J. M. (1999) Reconstitution of the transcription factor TFIIH: assignment of functions for the three enzymatic subunits, XPB, XPD, and cdk7. *Mol. Cell.* **3,** 87–95
21. Fishburn, J., Tomko, E., Galburt, E., and Hahn, S. (2015) Double-stranded DNA translocase activity of transcription factor TFIIH and the mechanism of RNA polymerase II open complex formation. *Proc. Natl. Acad. Sci. U.S.A.* **112,** 3961–3966
22. Grünberg, S., Warfield, L., and Hahn, S. (2012) Architecture of the RNA polymerase II preinitiation complex and mechanism of ATP-dependent promoter opening. *Nat. Struct. Mol. Biol.* **19,** 788–796
23. Kim, T. K., Ebright, R. H., and Reinberg, D. (2000) Mechanism of ATP-dependent promoter melting by transcription factor IIH. *Science* **288,** 1418–1422
24. Goel, S., Krishnamurthy, S., and Hampsey, M. (2012) Mechanism of start site selection by RNA polymerase II: interplay between TFIIB and Ssl2/XPB helicase subunit of TFIIH. *J. Biol. Chem.* **287,** 557–567
25. He, Y., Fang, J., Taatjes, D. J., and Nogales, E. (2013) Structural visualization of key steps in human transcription initiation. *Nature* **495,** 481–486
26. Fazal, F. M., Meng, C. A., Murakami, K., Kornberg, R. D., and Block, S. M. (2015) Real-time observation of the initiation of RNA polymerase II transcription. *Nature* **525,** 274–277
27. Goodrich, J. A., and Tjian, R. (1994) Transcription factors IIE and IIH and ATP hydrolysis direct promoter clearance by RNA polymerase II. *Cell* **77,** 145–156
28. Dvir, A., Conaway, R. C., and Conaway, J. W. (1997) A role for TFIIH in controlling the activity of early RNA polymerase II elongation complexes. *Proc. Natl. Acad. Sci. U.S.A.* **94,** 9006–9010
29. Wong, K. H., Jin, Y., and Struhl, K. (2014) TFIIH phosphorylation of the Pol II CTD stimulates mediator dissociation from the preinitiation complex and promoter escape. *Mol. Cell* **54,** 601–612
30. Jeronimo, C., and Robert, F. (2014) Kin28 regulates the transient association of mediator with core promoters. *Nat. Struct. Mol. Biol.* 10.1038/nsmb.2810
31. Coin, F., and Egly, J. M. (1998) Ten years of TFIIH. *Cold Spring Harb. Symp. Quant. Biol.* **63,** 105–110
32. Khaperskyy, D. A., Ammerman, M. L., Majovski, R. C., and Ponticelli, A. S. (2008) Functions of *Saccharomyces cerevisiae* TFIIF during transcription start site utilization. *Mol. Cell. Biol.* **28,** 3757–3766
33. Hampsey, M. (1998) Molecular genetics of the RNA polymerase II general transcriptional machinery. *Microbiol. Mol. Biol. Rev.* **62,** 465–503
34. Kaplan, C. D., Jin, H., Zhang, I. L., and Belyanin, A. (2012) Dissection of Pol II trigger loop function and Pol II activity-dependent control of start site selection *in vivo*. *PLoS Genet.* **8,** e1002627
35. Zhou, X., Zolotukhin, I., Im, D. S., and Muzyczka, N. (1999) Biochemical characterization of adeno-associated virus rep68 DNA helicase and ATPase activities. *J. Virol.* **73,** 1580–1590
36. Tuteja, N., and Tuteja, R. (2004) Prokaryotic and eukaryotic DNA helicases. *FEBS J.* 10.1111/j.1432–1033.2004.04093.x
37. Jiang, Y., and Gralla, J. D. (1995) Nucleotide requirements for activated RNA polymerase II open complex formation *in vitro*. *J. Biol. Chem.* **270,** 1277–1281
38. Dvir, A., Garrett, K. P., Chalut, C., Egly, J. M., Conaway, J. W., and Conaway, R. C. (1996) A role for ATP and TFIIH in activation of the RNA polymerase II preinitiation complex prior to transcription initiation. *J. Biol. Chem.* **271,** 7245–7248
39. Kostrewa, D., Zeller, M. E., Armache, K.-J., Seizl, M., Leike, K., Thomm, M., and Cramer, P. (2009) RNA polymerase II-TFIIB structure and mechanism of transcription initiation. *Nature* **462,** 323–330
40. Jiang, Y., Yan, M., and 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

Transcription Start Site Scanning and the Requirement for ATP during Transcription Initiation by RNA Polymerase II

James Fishburn, Eric Galburt and Steven Hahn

J. Biol. Chem. 2016, 291:13040-13047.
doi: 10.1074/jbc.M116.724583 originally published online April 17, 2016