Transcription initiation by RNA polymerase II (pol II) requires a helicase within TFIIH to generate the unpaired template strand. However, pol II preinitiation complexes (PICs) lose the ability to synthesize RNA very rapidly upon exposure to ATP alone in the absence of other NTPs. This inactivation is not caused by the TFIIH kinase activity, the loss of transcription factors or pol II from the PIC, or the collapse of the initially formed transcription bubble. TFIIE is necessary for PIC formation, but TFIIE is not retained as a stable component in PICs prepared by our protocol. Nevertheless, activity can be at least partially restored from the PIC, or the collapse of the initially formed transcription bubble. TFIIE is necessary for PIC formation, but TFIIE is not retained as a stable component in PICs prepared by our protocol. Nevertheless, activity can be at least partially restored to ATP-treated PICs by the readdition of TFIIE. PICs formed on premelted (bubble) templates require TFIIH for effective transcription elongation to +20. Incubation of bubble template PICs with ATP caused reduced yields of 20-mers, but this effect was partially reversed by the addition of TFIIE. Our results suggest that once the open complex is formed, TFIIH decays into an inactive configuration in the absence of nucleotides for transcription. Although TFIIH does not play a role in transcript initiation itself, inactivation resulting from ATP preincubation can be reversed by a remodeling process mediated by TFIIE. Finally, we have also uncovered a major role for TFIIH in the earliest stages of transcript elongation that is unique to bubble templates.

In the canonical path for assembly of the pol II3 PIC at promoters containing a TATA element, TBP (or TFIID) and TFIIB initially associate with the template, followed by TFIIH and pol II and finally by TFIIIE and TFIIH (1). In this scheme, TFIIE is primarily a partner for loading TFIIH (2–5). However, there are a number of indications that TFIIE is not simply a TFIIH chaperone. TFIIE alone is strongly stimulatory (6) or required (7) for transcription of some supercoiled templates, on which TFIIH is not needed for initiation. Holstege et al. (7) hypothesized that TFIIE facilitates promoter melting. A similar role has been suggested for TFE, the archaeal analog of TFIIH, which is essential for initiation by archaeal RNA polymerase (8). It has been reported that potential promoters in yeast can recruit most of the general transcript initiation factors, including TFIIH, but these promoters remain inactive because they fail to recruit both pol II and TFIIH (9). Some yeast genes apparently do not require TFIIH for expression. These are genes that lack TATA elements (10) (see also Ref. 11), which is interesting in the context of gene expression in higher eukaryotes because, for example, most mammalian promoters lack a TATA box (reviewed in Ref. 12).

Within yeast PICs, the site of TFIIH interaction with pol II has been localized to the pol II clamp region (13), consistent with the idea that TFIIH could function to stabilize the nontemplate strand once promoter melting has occurred (14) (see also Ref. 15). TFE interacts with an analogous region of the RNA polymerase in the archaeal PIC (8). If the interactions of TFIIE and the nontemplate strand are functionally significant, it should be informative to explore further the effect of TFIIH on the open complex and the earliest stages of transcript elongation. Formation of the pol II transcription bubble requires ATP to support the TFIIH helicase activity (16, 17). Interestingly, the newly formed open complex is functionally unstable (i.e. if NTPs are not quickly supplied, the transcription complex becomes inactive) (18, 19). We investigate this phenomenon in the present paper and show that inactivation from ATP preincubation does not result from the loss of TFIIH or other factors from the PIC, nor is it caused by the kinase activity of TFIIH. We demonstrate that TFIIH addition can partially rescue the transcriptional activity of pol II complexes inactivated by exposure to ATP. Both ATP inactivation and TFIIH rescue can be observed with premelted templates. Our results suggest that if template melting occurs in the absence of NTPs for transcription, TFIIH cannot remain in an active configuration. TFIIH promotes remodeling of this inhibited complex to return it to the active state.
In Experimental Procedures

Reagents—NTPs were obtained from Amersham Biosciences, [α-32P]CTP was from PerkinElmer Life Sciences, dATP was from Fermentas, and 3′-deoxy-GTP was from TriLink Biotechnologies. ApC dinucleotide, AMP-PNP, DMSO, and anti-FLAG M2 antibody (for detection of FLAG-tagged TFIIEα) were obtained from Sigma. RNasin was purchased from Promega, CpA dinucleotide was custom synthesized by Dharmacon, and casein kinase 2 was obtained from New England Biolabs. The antibody against the p62 subunit of TFIIE (sc-292) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibody against Rbp7 was kindly provided by Erica Golemis. The antibody specific for phosphoserine 5 of the pol II C-terminal repeat domain (CTD) (MMS-134R, from Covance) and the kinase inhibitors 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and flavopiridol (originally obtained from Sigma) were kindly provided by Richard Padgett.

Plasmid Constructions and Immobilized Template Preparations—All promoters were based on the pML20-40 adenovirus major late promoter (20). The pML-8gW, pML-8g2D, and pML-8A2D-75g constructs were described previously (20–22). The double-stranded templates used in transcription reactions (~210 bp) were generated by PCR amplification. They contained a PvuII restriction site and the transcription start site at 21 and 100 bp from the upstream (5′-biotinylated) and the downstream (3′-biotinylated) end of the fragment, respectively. The bubble template, premelted from −9 to −1 relative to the transcription start site, was derived from pML20–40(6g) (21). The bubble template was generated by annealing two complementary oligonucleotides mispaired from −9 to −1, with the nontemplate strand 5′-biotinylated. Transcription templates were resolved on agarose gels and purified using the QIAquick gel extraction kit (Qiagen; for double-stranded templates) or by elution from polyacrylamide gels (for bubble templates). All templates were immobilized on streptavidin-coupled Dynabeads M-280 (Invitrogen) as described by the manufacturer.

Protein Preparations—Recombinant general transcription factors TBP, TFIIB, TFIIE, and TFIIF were expressed and purified as described (21). TFIIE was isolated from HeLa nuclear extract as reported (21), and RNA polymerase II was purified from HeLa nuclear pellets as described (23, 24). TFIIF was phosphorylated in vitro with casein kinase 2 and subsequently purified by the method of Újvári et al. (25) with minor modifications as described (22).

Assembly of PICs and ATP Pretreatment—Assembly was performed at 30°C for 20 min as described (22). Typically, 10 μl reactions contained 558 fmol of Dynabead-attached template, 95 fmol of TBP, 3.3 fmol of TFIIE, 72 fmol of TFIIF, 46 fmol of TFIIF, either unmodified or phosphorylated (P-TFIIF), 13 fmol of pol II, and 1 μl of TFIIE in 20 mM Tris-HCl, pH 7.9, 65 mM KCl, 8 mM MgCl2, 1 mM DTT, 0.05 mM EDTA, 5% glycerol, 0.1% Nonidet P-40, and 1 mg/ml BSA. Our TFIIE is only partially purified (21); typically, 1 μl supports about 80% of the maximum transcription signal in a TFIIE titration for a 10 μl reaction. The PICs were washed at 30°C with BC100 buffer (20 mM Tris-HCl, pH 7.9, 100 mM KCl, 20% glycerol, 0.25 mM EDTA, 1 mM DTT) containing 0.5 mg of BSA/ml and 0.1% Nonidet P-40 and subjected to two equilibrium washes at 30°C with transcription buffer M5 (20 mM Tris-HCl, pH 7.9, 65 mM KCl, 10 mM β-glycerophosphate, 10 mM MgCl2, 5% glycerol, 0.25 mM EDTA, 1 mM DTT), containing 0.25 mg/ml BSA and 0.1% Nonidet P-40 (M5/BSA/Nonidet P-40). Preincubation of PICs with ATP or AMP-PNP was performed at 30°C with the indicated ATP or AMP-PNP concentrations and for the times indicated in the figure legends.

In Vitro Transcription and Abortive Initiation—The sequence of the nontemplate strand of our promoters near transcription start is ...CACT... , where the underlined A residue is +1. Transcription reactions were initiated with 0.5 mM ATP, 1 mM ApC, or 1 mM CpA; 25 μM dATP was also added as the energy source with the dinucleotides. Nascent RNAs were advanced to the first downstream G or A stop with 2 μM [α-32P]CTP and a 0.5 mM concentration of the appropriate NTPs in the presence of RNasin (0.4 unit/μl) at 30°C for 5 min. Reactions in Fig. 18 contained the chain terminator 3′-dGTP at 0.1 mM. Abortive initiation assays were run for 5 min with 1 mM CpA, 25 μM dATP, and 1 μM [α-32P]CTP. RNAs were resolved on denaturing 15% (for 20-mers), 20% (for 7–9-mers), or 28% (for trinucleotides) polyacrylamide gels. The gels were imaged with a Storm Imager and quantified with ImageQuant software (GE Healthcare).

Analysis of Proteins Bound to Template after PIC Assembly—To generate complexes for immunoblotting assays, PIC assembly was scaled up 3 times from the procedure described above and by Čabart et al. (22). The template-associated proteins were released immediately (Fig. 2A) or after a relevant treatment (Fig. 1D) with PvuII (New England Biolabs; 3.3 units/μl) for 10 min at 30°C in a 15 μl volume. Following the addition of herring sperm DNA (1 ng/3 ng of template) for 1 min at room temperature, the supernatants from the PvuII digest were collected, the beads were washed with 5 μl of M5/BSA/Nonidet P-40, and the combined supernatants were used for analysis. Proteins were resolved on discontinuous 6–12% SDS-polyacrylamide gels, which were transblotted onto polyvinylidene fluoride membranes (Millipore). Immunodetection was performed by enhanced chemiluminescence (Millipore) as recommended by the manufacturer.

Results

Exposure of Pol II Preinitiation Complexes to ATP Results in Their Rapid Inactivation for RNA Synthesis—Earlier studies indicated that the pol II PIC is destabilized upon template melting (18, 19). In the course of our recent investigations of the composition of minimal pol II PICs assembled on double-stranded templates (22), we noted that TFIIB, TFIIF, and TBP are all fully retained 5 min after an energy source (ATP or dATP) is supplied to generate the open complex. This raised the question of why transcriptional activity is lost under these circumstances. To investigate this issue, we first confirmed that in our current system, pol II PICs do lose the ability to make RNA after exposure to ATP in the absence of other NTPs for transcription. Inactivation was largely complete after 1 min of incubation with ATP (Fig. 1A), and inhibition was observed even for the formation of the first phosphodiester bond (Fig. 1C). Inhibition also occurred upon preincubation with dATP.
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(A) PICs in the presence of two different kinase inhibitors did not affect inhibition (Fig. 1B). These inhibitors were effective, as shown by the loss of phosphorylation of the pol II CTD at the serine 5 position when the inhibitors were included in the ATP treatment of PICs (Fig. 1E). Additionally, phosphatase treatment of ATP-inhibited complexes did not restore their activity (data not shown). The results in Fig. 1D show that TFIIH was retained in the PICs upon ATP addition. Pol II levels did not change with ATP addition (as judged by the Rpb7 subunit), and as expected, ATP supported phosphorylation of the pol II CTD at the serine 5 position (Fig. 1, D and E).

The formation of an active PIC with our system is completely dependent on each of the general transcript initiation factors, including TFIIE (22). However, when we attempted to confirm that TFIIE is retained in open complexes, we were surprised to find that we could not detect any TFIIE in our PICs prior to ATP treatment (Fig. 2A), even after deliberate overexposure of the blots. It should be noted that our PICs were repeatedly washed with transcription buffer before assaying for either RNA synthesis or factor content. Thus, TFIIE must participate in PIC formation but is only loosely associated with our complete PICs.

Addition of TFIIE Alone Can Partially Reverse ATP Inhibition—It appears that no transcript initiation factors are lost specifically at pol II open complex formation. One could imagine that the initially generated transcription bubble collapses in the absence of NTPs for transcription. However, this seems unlikely based on earlier work, which demonstrated that the pol II bubble survives for 5 min after the ATP addition (26) and remains open for more than 1 min when helicase activity is inhibited by γ-thio–ATP (27). It is intriguing that TFIIE is not stably retained within our PICs. In addition to functioning as a chaperone for TFIIH (2–5), TFIIIE may also be important in stabilizing the newly unpaired nontemplate strand in the open complex (7, 8, 14). If the bubble formed in the absence of NTPs is not stabilized, perhaps the open complex isomerizes into a functionally inactive configuration. This model suggests that ATP-induced inactivation of the PIC might be reversed by re-exposure of the PIC to TFIIE. To test this, PICs were initially treated with ATP for 5 min, washed, and then incubated further with various amounts of TFIIE. Reactions were split, with half receiving an additional wash. All reactions were then challenged with the necessary substrates to synthesize an 8-mer transcript, including dATP as an energy source. A schematic of this experimental procedure is shown in Fig. 2B. Roughly half of the initial ATP-dependent activity loss could be restored by incubation with TFIIIE using this procedure (Fig. 2, C and D). The TFIIE-mediated rescue was nearly complete when about 2.5 fmol of the factor was supplied to our standard reaction (Fig. 2, C and D), which is roughly the amount of TFIIE initially needed to support PIC assembly in the same reaction volume. Note that the addition of TFIIE followed by a gentle wash eliminated the rescue (Fig. 2, C and D).

We found that the transcriptional activity rescue procedure must include an energy source. Essentially no recovery of activity was seen if dATP or ATP was absent from the transcription reaction (data not shown). All of the results in Fig. 2 are consistent with the idea that TFIIIE restores activity by reassociation with the transcription complex. This association, as was
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FIGURE 2. Addition of TFIIE alone can partially reverse ATP-mediated inactivation. A, PICs assembled on the 8a2D-75g template were analyzed for retention of TFIIE (subunit α) and pol II (Rpb7) by immunoblotting (lane 2). Lane 1 contained 20 fmol of TFIIE and 1 fmol of Rpb7 as standards. The asterisk indicates an unknown protein that cross-reacted with the antibody. B, the schematic timeline indicates, in minutes, the order of additions and washes for the experiment in C. PIC-IT, initial preinitiation complex; NTPs/*C, NTPs including 32P-labeled CTP. C, PICs assembled on the 8g2D template were incubated without (lane 1) or with (lanes 2–5) 0.1 mM ATP and washed. ATP-treated reactions were supplemented with 0, 2.5, 12.5, or 50 fmol of TFIIE as indicated (lanes 2–5), and all reactions were incubated. Each reaction was separated into two parts, with one portion receiving an additional wash (+). Transcription reactions were then run to generate labeled 8-mer RNAs. D, RNA levels in C were quantified and plotted with the lane 1 values, with or without (+/−) wash, set to 1. Black bars, no wash; gray bars, wash after incubation of PICs with TFIIE.

the case with TFIIE binding to the original PIC, is apparently not stable because washing prior to transcription eliminates the rescue effect (Fig. 2C). However, unlike the case of the initial function of TFIIE in supporting PIC assembly, the rescue of ATP-inhibited complexes requires energy. We can therefore suggest that recovery from ATP inhibition involves a TFIIE-mediated remodeling of the inactive PIC, returning the complex to its active form.

PICs Formed on Premelted Templates Are Also Subject to Inactivation by Exposure to ATP—We have extended our analysis of the ATP preincubation effect to PICs assembled on premelted (bubble) templates. The preformed bubble we used extends from −9 to −1 relative to transcription start. We have shown that synthesis of 7-mer transcripts on this particular bubble template does not require TFIIE, TFIIF, or an energy source, but these factors and ATP or dATP are needed for efficient extension of the nascent RNA to +20 (21). We asked whether PICs assembled on the −9/−1 template with the full complement of initiation factors are inhibited for transcription to +7 or +20 by preincubation with ATP. As expected from our earlier study (21), efficient 7-mer synthesis on the −9/−1 template was not strongly dependent on TFIIE (Fig. 3A, 7-mer panel, lanes 1 and 4; results quantified in Fig. 3B). The production of 7-mer transcripts was not inhibited by preincubation of the bubble template PICs with ATP (Fig. 3A, 7-mer panel, lanes 1–6). Also as expected, efficient 20-mer synthesis did require TFIIE (Fig. 3A, 20-mer panel, lanes 1 and 4; results quantified in Fig. 3B). Most importantly, TFIIE-dependent 20-mer production by these bubble template PICs was inhibited by ATP preincubation but not by preincubation with AMP-PNP (Fig. 3A, 20-mer panel, lanes 4–6; results quantified in Fig. 3B).
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Exposing pol II PICs to an energy source without the addition of nucleotides for transcription causes rapid inactivation of RNA synthesis capability. PICs preincubated with ATP do not lose RNA synthesis activity because of loss of pol II or transcript initiation factors that are otherwise stably associated with the complex. Earlier studies indicated that inactivation should not result from rapid reclosure of the initial transcription bubble (26, 27), which is consistent with our finding that ATP-dependent inactivation occurs with premelted templates. Inactivation must involve one of the helicase activities in TFIIH because ATP and dATP cause inactivation, AMP-PNP does not inactivate, and kinase inhibitors do not block the inactivation. The xeroderma pigmentosum B (XPB), or p89, helicase is the most likely candidate for the critical activity because XPB drives both template opening and promoter clearance (29–32). In particular, it was shown that transcription complexes assembled on bubble templates are unable to advance effectively to +18 when XBP is defective (31). We find that the readdition of TFIIE to the ATP-treated complexes can (along with an energy source) partially restore activity for both conventional and bubble templates.

DISCUSSION

To reassociate with inactivated PICs to mediate restoration of transcriptional activity.

FIGURE 3. PICs formed on premelted templates are inhibited for synthesis of 20-mer RNAs by preincubation with ATP. A, the schematic timeline indicates, in minutes, the order of additions and washes for the experiment in the bottom half of the panel. PIC-IT, initial preinitiation complex; NTPs(°C), NTPs including °P-labeled CTP. PIC assembly reactions were performed on bubble templates with positions −9 to −1 unpaired, with or without TFIIH as indicated. In the reactions shown in lanes 7–9, P-TFIIF was substituted for unmodified TFIIF. Each PIC was incubated with no addition, 0.1 mM ATP, or 0.1 mM AMP-PNP, as indicated, followed by transcription to generate labeled 7-mer (top) or 20-mer (bottom) RNAs. B, transcript levels for 7- or 20-mers were quantified with the AMP-PNP, TFIIH-containing reaction set to 1 for each group of reactions. Open bars, mock treatment; black bars, ATP treatment; gray bars, AMP-PNP treatment of PICs.

FIGURE 4. ATP inhibition of 20-mer synthesis by PICs on premelted templates is partially reversed by addition of TFIIE. A, the schematic timeline indicates, in minutes, the order of additions and washes for the experiment in the bottom half of the panel. PIC-IT, initial preinitiation complex; NTPs(°C), NTPs including °P-labeled CTP. PICs were assembled on the −9/−1 bubble template with TFIIH and P-TFIIF and treated with ATP, AMP-PNP, or no addition as described in the legend to Fig. 3. One ATP-treated reaction was supplemented with 2.5 fmol of TFIIE (lane 3); the remaining reactions were not supplemented. All reactions were incubated as indicated, followed by transcription to generate labeled 20-mer RNAs. B, results from two experiments as in A were quantified, with the value for the untreated, unsupplemented reactions (as in A, lane 1) set to 1. Black bar, mock treatment; gray bar, ATP treatment; open bar, AMP-PNP treatment of the PICs. Error bars, range of values.

quantified in Fig. 3B). The synthesis of 20-mer transcripts from PICs assembled with TFIIH and P-TFIIF was strongly reduced by ATP preincubation but not by preincubation with AMP-PNP (compare lanes 2 and 4 in Fig. 4A), as we observed with PICs containing TFIIH and unmodified TFIIF (Fig. 3).

ATP Inactivation of Bubble Template PICs Can Also Be Reversed by Addition of TFIIE—Because we could demonstrate ATP inhibition of 20-mer synthesis on bubble templates, we were interested to see if this effect could be influenced by the readdition of TFIIE, as was the case with PICs on double-stranded templates. As mentioned above, the results in Fig. 4 show that only one-third of 20-mer synthesis capacity was retained by −9/−1 bubble template PICs (in this case, assembled with P-TFIIF) that were incubated with ATP, whereas there was no effect of incubation with AMP-PNP. We found that about half of this inactivation could be relieved by adding only TFIIE to the ATP-treated PICs (Fig. 4, A and B). The amount of TFIIE used for rescue in Fig. 4 (which gave maximal recovery, as determined by preliminary titration experiments not shown) was similar to the amount used for the initial PIC assembly. This is again consistent with a requirement for TFIIE...
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It seems paradoxical that ATP-treated PICs lose transcriptional activity but retain TFIIH and the general transcript initiation factors other than TFIIF. (It is well established that TFIIH is not required for initiation (6, 7, 33–35).) An important clue to the inhibitory mechanism comes from earlier observations of open complex formation at the adenovirus major late promoter. The addition of ATP alone drives the unpairing of a template segment extending from −9 to −2 relative to transcription start (26), leaving the initiation site itself double-stranded. Crucially, it was shown that the bubble only expands downstream when the NTPs necessary to support transcription are added (26). This group also showed that transcription from a supercoiled adenovirus E4 promoter does not require TFIIH but is significantly stimulated by TFIIIE; this stimulation is lost when E4 templates premelted from −8 to +2 are used (7). The ability of TFIIH to drive initial template melting is presumably dependent on TFIIH-DNA contacts that extend for over 20 bp downstream of transcription start in both the PIC and the open complex (36). Dvir and colleagues (37) provided mechanistic insight into the function of TFIIH in the earliest stages of transcription by determining that at least 28 bp of DNA must be present downstream of the point of bond formation for TFIIH to act, either at initiation or at promoter clearance. These authors inferred that TFIIH must translocate downstream as it assists pol II in the initial stages of transcription.

Based on these earlier results, we speculate that after the TFIIH helicase initially acts to generate the −9 to −2 bubble, TFIIH begins its downstream translocation as part of the process of extending the bubble through and beyond the transcription start site. NTPs and TFIIE presumably facilitate the additional melting necessary to reveal the unpaired template strand at the initiation site, in agreement with the location proposed for TFIIE within the PIC (36, 38). If NTPs for transcription and TFIIE are both absent when the −9 to −2 bubble is formed, TFIIH may be unable to begin translocation. This would prevent TFIIH from supporting either initiation or downstream traversal by the RNA polymerase, consistent with the ATP-mediated transcription inactivation that we observed on both the double-stranded and bubble templates.

It was reported earlier (28, 39) that transcript initiation and promoter clearance are stimulated by TFIIF. These experiments relied on premelted templates in order to study transcription in the presence or absence of TFIIF. We recently demonstrated that TFIIF phosphorylated by casein kinase 2 supports PIC formation (25) but is not retained within the PIC (22). Thus, by comparing PICs assembled with P-TFIIF and unmodified TFIIF, we could test whether TFIIF affects the transcriptional potential of the PICs. We found that on double-stranded templates, there is no difference in initiation or clearance regardless of the presence or absence of TFIIF within the PIC (22). However, when we tested for production of 7-mer RNAs on bubble templates in the present study, we found that using P-TFIIF supports considerably reduced transcription compared with unmodified TFIIF (Fig. 3). We presume, based on our recent work with conventional templates (22), that bubble template PICs assembled with P-TFIIF do not retain TFIIF. Our results with bubble templates are therefore in general agreement with the earlier work (28, 39). Why should the presence of TFIIF within PICs have very different effects on double-stranded versus bubble templates? We showed that PICs that lack TFIIF (because of assembly with P-TFIIF) may recruit TFIIH less effectively and tend to lose TFIIH at open complex formation (22). Because TFIIB is essential for initiation, we hypothesize that the poor activity of bubble template transcription complexes in the absence of TFIIF could result from ineffective recruitment or retention of TFIIH.

The results we report here may have broader implications for the control of gene expression. We propose that TFIIH decays into an essentially inactive state after supporting the assembly of the open preinitiation complex, a process that can be modulated by TFIIH. The FUSE-binding protein (FBP) transcription factor, a known regulator of c-myc expression, has been shown to directly stimulate the XBP helicase activity of TFIIH (40). Our model suggests that the FBP-TFIIH interaction could be important in guaranteeing that PICs assembled at FBP-regulated promoters successfully advance through open complex into transcription elongation. As an extension of this point, if initiation is slow for open complexes at some promoters, retention of TFIIE in these complexes could be important to prevent inactivation. Recent work indicates that the Spt5 subunit of the DRB sensitivity-inducing factor complex should compete with TFIIH for a common binding site on the pol II clamp domain (8, 15, 41). Thus, loading of DRB sensitivity-inducing factor into the nascent transcription complex could also influence survival of the open complex. Finally, it has been shown (42) that p53 competes with TFIIE for its binding site on TFIIH. This may represent another case, along with FBP, in which the stabilization and propagation of the transcription bubble near the initiation site can be directly controlled by a regulatory factor.

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