A Role for ATP and TFIIH in Activation of the RNA Polymerase II Preinitiation Complex Prior to Transcription Initiation*

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A requirement for an ATP cofactor in synthesis of the first 8–10 bonds of promoter-specific transcripts by RNA polymerase II is well established. Whether ATP is required for synthesis of the first phosphodiester bond or at a slightly later stage in synthesis of nascent transcripts, however, remains controversial. Goodrich and Tjian (Goodrich, J. A., and Tjian, R. (1994) Cell 77, 145–156) recently proposed that synthesis of the first phosphodiester bond of promoter-specific transcripts by RNA polymerase II is independent of ATP and general transcription factors TFIIE and TFIIF. Here we investigate this model. Taken together, our findings indicate that ATP, TFIIE, and TFIIF can have a profound effect on the efficiency of transcription initiation. First, we observe that synthesis of the first phosphodiester bond of transcripts initiated at the adenovirus 2 major late promoter depends strongly on ATP, TFIIE, and TFIIF. In a transcription system reconstituted with RNA polymerase II, TFIIF, and recombinant TBP, TFIIB, TFIIE, and TFIIF. Second, we demonstrate that, in this enzyme system, ATP-dependent activation of transcription initiation can occur immediately prior to synthesis of the first phosphodiester bond of nascent transcripts. Finally, we demonstrate that the activated initiation complex is unstable and decays rapidly to an inactive state in the presence of the inhibitor ATP·S (adenosine 5′-O-(thio)triphosphate), even during reiterative synthesis of abortive transcripts.

Promoter-specific transcription by RNA polymerase II on linear or relaxed DNA templates is a complex biochemical process requiring the general transcription factors TFIIB, TFIID, TFIIE, TFIIF, and TFIH and a hydrolyzable ATP cofactor. A role for ATP as a cofactor in transcription by RNA polymerase II was first suggested in studies carried out with crude or partially fractionated transcription systems. Using HeLa cell extracts, Weinmann and co-workers (1) initially observed that AMP-PNP1 could not replace ATP in synthesis of AdML promoter-specific transcripts, even though AMP-PNP is a substrate for elongation by RNA polymerase II; these findings were subsequently corroborated and extended by Shakhtin and co-workers (2). In experiments carried out with partially fractionated HeLa cell extracts, Sawadogo and Roeder (3) obtained evidence that ATP is required at an early stage in transcription by demonstrating that ATP is essential for synthesis of the first 8 phosphodiester bonds of transcripts initiated at the AdML promoter. In an elegant series of experiments, Luse and Jacob (4) presented convincing evidence that, in HeLa cell extracts, ATP is essential for synthesis of the first phosphodiester bond of promoter-specific transcripts by demonstrating that hydrolyzable ATP is required for synthesis of dinucleotide-primed, abortive AdML-specific trinucleotide transcripts. These findings were recently confirmed and extended by Gralla and co-workers (5), who demonstrated that ATP is required for synthesis of dinucleotide-primed, abortive trinucleotide transcripts initiated at the adenovirus E4 promoter in HeLa cell extracts.

Although the function of ATP has not been unequivocally established, several lines of evidence have led to the proposal that ATP is utilized by a DNA helicase activity associated with TFIH to promote unwinding of the DNA template at the transcriptional start site prior to initiation. First, using K2MnO4 as a probe for DNA melting, Gralla and co-workers (5–7) demonstrated that, in HeLa cell extracts, ATP is needed for unwinding of a short stretch of DNA surrounding the transcriptional start site prior to initiation. Second, among the general transcription factors, only TFIH has detectable ATP-hydrolyzing activities. TFIH has been shown to possess closely associated DNA-dependent ATPase/dATPase (8, 9), DNA helicase (10, 11), and protein kinase (9, 12, 13) activities. The TFIH protein kinase activity has been shown to be dispensable for transcription under conditions where ATP is needed (14–16), arguing that it does not mediate the essential ATP-requiring step in transcription by RNA polymerase II. Finally, recent biochemical studies indicate that the ATP cofactor is not required for transcription under a limited set of conditions where TFIH is dispensable for initiation; these conditions include transcription from promoters on negatively supercoiled DNA templates (17–19) or promoters containing a short stretch of mismatched base pairs surrounding the transcriptional start site (20, 21).

Recently, a requirement for ATP in transcription initiation by RNA polymerase II has been called into question. In experiments carried out with a transcription system reconstituted with RNA polymerase II and TFIH purified from HeLa cells and recombinant TBP, TFIIB, TFIIE, and TFIIF, Goodrich and Tjian (22) observed synthesis of dinucleotide-primed, abortive AdML-specific trinucleotide transcripts in the absence of added ATP, TFIIE, and TFIH. In light of this finding, they proposed that ATP and TFIH are not essential for synthesis of the first

1 The abbreviations used are: AMP-PNP, adeny1-5′-yl imidodiphosphate; AdML, adenovirus 2 major late; ATP·S, adenosine 5′-O-(thio)triphosphate.

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phosphodiester bond of nascent transcripts but, instead, are required at a later transcriptional stage referred to as promoter clearance. To explain the discrepancy between their findings and those of earlier studies, they suggested that the previously observed requirement for ATP in transcription initiation might be an artifact resulting from impurities present in crude transcription systems.

In this report, we have investigated the role of ATP and TFIIE in transcription initiation using an enzyme system reconstituted with RNA polymerase II, TFIIH, and recombinant TBP, TFIIB, TFIIE, and TFIIF. As we describe below, our findings argue strongly that ATP and TFIIH can have a profound effect on the efficiency of transcription initiation by RNA polymerase II, and they shed new light on the role of ATP in this process.

EXPERIMENTAL PROCEDURES

Materials—Unlabeled ultrapure ribonucleoside 5′-triphosphates and dATP were purchased from Pharmacia Biotech Inc. Dinucleotides CpA, CpU, ApC, ApG, GpA, UpU, and UpG, α-amanitin, and polyvinyl alcohol (type II) were obtained from Sigma. ATP-S was from Boehringer Mannheim. (α-32P)CTP (>400 Ci/mmol) was purchased from Amer- sham Corp. Bovine serum albumin (Pentex fraction V) was obtained from ICN Immunobiologicals. Human placental ribonuclease inhibitor (RNasin) was from Promega.

Preparation of RNA Polymerase II and Transcription Factors—RNA polymerase II (12) and TFIIH (23) were purified as described from rat liver nuclear extracts. Recombinant yeast TBP (24, 25) and recombinant rat TFIIB (26) were expressed in Escherichia coli, and purified as described. Recombinant TFIIE was prepared as described, except that the 56-kDa subunit was expressed in BL21(DE3)pLysS (27). Recombinant TFIIF was expressed in JM109(DE3) using the M13mpET expression system and purified as described previously (28).

Assay of Transcription Initiation—Preinitiation complexes were assembled at the AdML promoter at 28 °C by either a 20-min (Figs. 1B, 28, and 4A) or a 45-min (Figs. 1C, 2A, and 3) preincubation of 60-μl reaction mixtures containing 20 mM Hepes-NaOH (pH 7.9), 20 mM Tris-HCl (pH 7.9), 60 mM KCl, 7 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, 2% (w/v) polyvinyl alcohol, 7% (v/v) glycerol, 6 units of the EcoRI-Ndel fragment from pDN-AdML (29), 100 ng of recombinant TFIIH, ~20 ng of recombinant TFIIF, ~20 ng of recombinant TFIIIE and TFIIH (~150 ng of rat δ TSK DEAE 5-PW fraction in Figs. 1B, 1C, 2A, 3, and 4B or ~40 ng of rat δ TSK SP 5-PW fraction in Figs. 2B and 4A), ~50 ng of yeast TBP (Aca44 fraction), and ~0.01 unit of RNA polymerase II. As indicated in the figure legends, transcription was initiated by addition of dinucleotide primer, ribonucleoside triphosphates, and α-32P-labeled ribonucleoside triphosphates. Transcription was carried out at 28 °C for the times indicated in the figure legends. Transcription was stopped by addition of 8 μl of reaction mix to 2 μl of a stop solution containing 100 mM EDTA and 0.5 mg/ml proteinase K. Following an incubation of at least 15 min at room temperature, 10 μl of 9 M urea containing 0.025% bromphenol blue and 0.025% xylene cyanol FF was added, and trinucleotide transcripts were analyzed by electrophoresis through 25% (w/v) acrylamide, 3% (w/v) bisacrylamide, 7 M urea gels as described (30). Trinucleotide synthesis was quantitated by PhosphorImager analysis and is expressed in arbitrary units that represent the phosphorescence intensity measured in a given exposure time.

RESULTS AND DISCUSSION

In this report, we have used the abortive initiation assay to investigate the requirements for synthesis of the first phosphodiester bond of AdML promoter-specific transcripts in a transcription system reconstituted with recombinant TBP, TFIIH, TFIIIE, and TFIIF, and RNA polymerase II and TFIIH purified from rat liver. As shown previously (4, 5, 22, 29, 31) RNA polymerase II will utilize dinucleotides to prime synthesis of promoter-specific transcripts; depending on the dinucleotide primer provided, initiation can occur over an approximately nine-base region centered around the normal transcription start site (31). If only a dinucleotide primer and the next nucleotide encoded by the template are provided as substrates for RNA synthesis, polymerase will efficiently synthesize abortively initiated, trinucleotide transcripts (4, 5, 22, 30, 32). The dinucleotide-primed abortive initiation assay has been widely used in studies analyzing synthesis of the first phosphodiester bond of nascent transcripts by prokaryotic (33, 34) and eukaryotic (4, 5, 22, 30, 32) RNA polymerases.

To assess the specificity of trinucleotide synthesis in our reconstituted transcription system, abortive initiation reac-
tions with RNA polymerase II, all five general transcription factors, and dATP were carried out in the presence of \([\alpha-32P]CTP\) and various dinucleotide primers. As expected from the sequence of the AdML non-template strand in the region of the transcription start site (Fig. 1A), we observe efficient trinucleotide synthesis when CpA or CpU but not ApC, ApG, UpU, and UpG are used as the dinucleotide primers (Fig. 1B). In addition, trinucleotide synthesis is strongly inhibited by 1 \(\mu\)g/ml \(\alpha\)-amanitin, which inhibits RNA polymerase II but not bacterial RNA polymerases or mammalian RNA polymerases I or III; thus the observed trinucleotide synthesis does not result from a contaminating polymerase activity (Fig. 1C, compare lanes 1 and 2).

To determine the requirements for synthesis of the first phosphodiester bond of AdML promoter-specific transcripts in our reconstituted transcription system, the abortive initiation reaction was carried out in the presence of various combinations of RNA polymerase II and general transcription factors, in the presence or absence of dATP. As expected, trinucleotide synthesis was strongly dependent on the presence of RNA polymerase II but not bacterial RNA polymerases or mammalian RNA polymerases I or III, thus the observed trinucleotide synthesis does not result from a contaminating polymerase activity (Fig. 1C, compare lanes 1 and 2).

ATP, TFIIE, and TFIIH can have a profound effect on the efficiency of synthesis of the first phosphodiester bond of nascent transcripts when transcription initiation is carried out in a highly purified, reconstituted transcription system.

To investigate in greater detail the function of ATP in synthesis of abortively initiated transcripts, we exploited the nucleotide analog ATP\(_{\gamma}S\). In a previous study, we observed that ATP\(_{\gamma}S\) is a potent inhibitor of ATP/dATP-dependent transcription by RNA polymerase II in a partially fractionated transcription system from rat liver, and we obtained evidence that ATP\(_{\gamma}S\) inhibits a reversible, ATP/dATP-requiring step that occurs prior to synthesis of 4–9 nucleotide, Sarkosyl-resistant transcripts in this system (29). Here we demonstrate that ATP\(_{\gamma}S\) is also a potent inhibitor of dATP-dependent trinucleotide synthesis in the purified, reconstituted transcription system. When added to reaction mixtures either with dATP, CpA, and \([\alpha-32P]CTP\) (see Fig. 4A) or with dATP and prior to addition of CpA and \([\alpha-32P]CTP\), trinucleotide synthesis is very strongly inhibited.
inhibited (Fig. 3, first two lanes of inset). The ATP-dependent step can occur prior to initiation and is reversible, since preincubation of template, RNA polymerase II, and the general transcription factors with dATP for 1 min prior to addition of CpA and \([\alpha-32P]CTP\) renders trinucleotide synthesis resistant to inhibition by ATP-\(Y\); in the absence of initiating nucleotides, the activated preinitiation complex decays with a half-life of 30–60 s. To determine whether ATP-\(Y\) inhibits transcription once trinucleotide synthesis has been initiated, we carried out the experiment shown in Fig. 4. In this experiment ATP-\(Y\) was added to reaction mixtures 10 min after addition of CpA and \([\alpha-32P]CTP\); this resulted in the immediate cessation of trinucleotide synthesis (Fig. 4B), indicating that the activated initiation complex is unstable and decays rapidly to an inactive state in the presence of the inhibitor ATP-\(Y\), even during reiterative synthesis of abortive transcripts. Inhibition of trinucleotide synthesis was completely reversible; following addition of excess dATP to an ATP-\(Y\)-inhibited reaction, trinucleotide synthesis resumed immediately, and the rate of trinucleotide synthesis following dATP addition was the same as in a control reaction containing no ATP-\(Y\).

In summary, in this report we have investigated the role of ATP and general transcription factors TFII E and TFIIH in the synthesis of the first few phosphodiester bonds of promoter-specific transcripts by RNA polymerase II in a transcription system reconstituted with purified TFII H and recombinant TBP, TFII B, TFII E, and TFII F. Our findings indicate that ATP, TFII E, and TFIIH can have a dramatic effect on the efficiency of transcription initiation. In addition, we observe that ATP-dependent activation of transcription initiation can occur immediately prior to synthesis of the first phosphodiester bond of nascent transcripts and, further, that the activated initiation complex is unstable and decays rapidly to an inactive state in the presence of the inhibitor ATP-\(Y\), even during reiterative synthesis of abortive transcripts. Our finding that the activated initiation complex is unstable during abortive transcription, which involves multiple rounds of initiation by the same RNA polymerase II molecules (4, 5), suggests that, unlike elongating polymerase, RNA polymerase II in the initiation complex is either unable to maintain the DNA template in an “open” configuration or incompetent to catalyze synthesis of phosphodiester bonds in the absence of continuing ATP hydrolysis. At the present time, we do not know whether a separate ATP activation event is required for each round of initiation or whether a single ATP activation event is sufficient to promote multiple rounds of initiation, perhaps until the activated initiation complex decays to an inactive state. Finally, our findings do not exclude the possibility that additional ATP-requiring steps may be required after synthesis of the first phosphodiester bond of nascent transcripts but prior or concomitant to entry of RNA polymerase II into the elongation stage of transcription. Future experiments addressing these issues will be essential for a complete understanding of the role of ATP in transcription by RNA polymerase II.

Note Added in Proof—While this manuscript was under review, we learned that Holstege et al. (Holstege, F. C. P., van der Vliet, P. C., and Timmers, H. T. M. [1996] EMBO J. 15, in press) had obtained similar findings.

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