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A Three-Step Pathway of Transcription Initiation Leading to Promoter Clearance at an Activated RNA Polymerase II Promoter

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The progress of transcription bubbles during initiation in vitro was followed in order to learn how RNA polymerase II begins transcription at the activated adenovirus E4 promoter. The issues addressed include the multiple roles of ATP, the potential effect of polymerase C-terminal domain phosphorylation, and the ability of polymerase to clear the promoter for reinitiation. The results lead to a three-step model for the transition from closed complex to elongation complex, two steps of which use ATP independently. In the first step, studied previously, ATP is hydrolyzed to open the DNA strands over the start site. In a second step, apparently independent of ATP, transcription bubbles move into the initial transcribed region where RNA synthesis can stall. In the third step, transcripts can be made as polymerase is released from these stalled positions with the assistance of an ATP-dependent process, likely phosphorylation of the polymerase C-terminal domain. After this third step, the promoter becomes cleared, allowing for the reinitiation of transcription.

In order to understand the regulation of transcription, one needs to define the series of steps leading from external signaling to the formation of mRNA in the nucleus. The late steps in these signal transduction pathways involve events occurring at promoters. Promoter utilization by RNA polymerase II is known to proceed through several sequential stages. In a collective first stage, general transcription factors, activators, and RNA polymerase interact with the promoter to form a stable closed complex (40). In a second step, this closed complex is converted into an open complex in which the template DNA strand at the transcription start site is exposed (20, 60). Subsequently, the polymerase must initiate transcription with the exposed template strand and enter the transcription elongation mode.

The transition from transcription initiation to elongation is not yet very well understood for RNA polymerase II. If nucleotides are provided to allow RNA polymerase to open the DNA and read only the first few bases of the template, several processes can be observed. Short complementary "abortive RNA products" which come from transcription of the initial transcribed region appear (15, 22). The melted DNA bubble moves downstream from the start site of transcription (60). The transition of polymerase from this abortive initiation mode to the elongation mode has not been followed systematically by footprinting. The point at which this transition occurs has been the subject of discussion, with one report suggesting that it occurs before a 16-nucleotide-long RNA has been made (13). The mechanism of this transition is receiving increased attention (reviewed in references 14 and 26), because some promoters appear to be regulated by controlling the release of initiated polymerases stalled in proximal positions (*Drosophila hsp70* [31, 37] and mammalian c-*myc* [27, 28, 50]; for material on transcription regulation by human immunodeficiency virus type 1 *tat*, see references 12, 23, and 29).

Sometime during this transition, the promoter should become cleared as the polymerase escapes into the elongation mode. Clearance is also important because it allows for transcription reinitiation; reinitiation has the potential to be a very rapid process compared with the first round of transcription and thus has the potential to dominate the production of mRNA (17). Clearance has not been measured directly by viewing the ability of a new polymerase to enter a previously used and cleared promoter. However, indirect experiments have suggested that clearance may occur coincidentally with the attainment of the elongation mode and may require ATP and the helicase activity of TFIIH (13).

Much recent work has focused on the possible role of factor TFIIH in this pathway. TFIIH has two activities that hydrolyze ATP, a DNA helicase (44, 47) and a protein kinase (10, 35, 45, 46). The kinase activity is intriguing, because it phosphorylates the C-terminal domain (CTD) of the large subunit of Pol II and because it is associated with cell cycle proteins (11, 33, 41, 48, 49, 51). This phosphorylation is known to accompany transcription, and it has been speculated that it plays a role in the release of the polymerase into the elongation mode (30, 34, 39, 58). However, CTD phosphorylation is not required for transcription in certain purified systems (18, 47) or for the opening of the DNA in both purified and unpurified systems (18) (also see data shown below), indicating that it is not absolutely required for this transition. It has also been postulated that the ATP-dependent helicase plays a role in promoter clearance (reviewed in reference 8), but direct evidence is still lacking.

Such uncertainty is not unique to studies of clearance, as the factor requirements and the mechanism of a number of steps during transcription complex assembly and initiation appear to vary, depending on which experimental system is used. In general, the use of supercoiled DNA and more highly purified transcription systems leads to the requirement for fewer factors (13, 38, 52, 56, 57). One unsettled requirement is that for ATP hydrolysis (1, 6, 13, 19, 20, 43, 52, 56, 60), which is important because it is intimately related to the mechanism by which factors catalyze various substeps in this transition.

In this paper, we extend prior studies to propose that three

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separate and sequential steps are involved in converting a closed complex to an elongation complex. These steps are strand opening, bubble movement, and polymerase escape. With regard to the role of ATP, it is first used to form a transcription bubble, this bubble then moves during initiation, apparently without the need to hydrolyze ATP, and then ATP is used again, probably to phosphorylate the polymerase CTD, promoting the escape of the polymerase from the initial transcribed region. After the escape of the polymerase, the promoter is cleared, allowing the reinitiation of transcription. The three-step model provides an improved context for identifying steps potentially subject to the physiological regulation of transcription.

MATERIALS AND METHODS

High-pressure liquid chromatography-purified ribonucleoside triphosphates and deoxyribonucleoside triphosphates were purchased from Pharmacia LKB Biotechnology Inc. α-Amanitin was from Sigma. CTD kinase inhibitor H8 [*N*-(2-(methylamino)ethyl)-5-isoquinoline sulfonamide dihydrochloride] was from Seikagaku American Inc. Nuclear extract was prepared as described previously (7). The DNA template contained nine GAL4 binding sites upstream of a truncated adenovirus E4 promoter (3, 4, 17). Supercoiled plasmid templates and activator GAL-VP16 were used in all experiments save one, which used *Bgl*I-digested linear DNA and activator GAL-AH as indicated.

Potassium permanganate footprinting was done as described previously (17, 20, 21, 60, 61). First, the transcription cocktail, which contains 25 μl of HeLa nuclear extract (6 mg of protein per ml in D buffer [20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.9), 100 mM KCl, 0.1 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride]), 8.25 mM magnesium chloride, 200 ng of carrier plasmid DNA, and 10 ng of supercoiled DNA template or 12 ng of linearized DNA template, was set up. GAL4-VP16 was present at this stage. This mixture was incubated for 30 min to allow the assembly of preinitiation complexes. When appropriate, inhibitor α-amanitin or H8 was added at this stage. dATP or ATP was added for 2 min to drive promoter opening. Potassium permanganate at 6 mM was added for 3 min to probe the single-stranded region of DNA. 2-Mercaptoethanol was used to quench the potassium permanganate. Proteinase K was added to digest proteins, and this step was followed by phenol, phenol-chloroform, and chloroform extraction. DNA was precipitated and redissolved in water and was passed through a Sephadex G50-80 spin column to desalt it. *Taq* polymerase was used to extend a radiolabeled primer repetitively, and the products were resolved on a 6% sequencing gel. Potassium permanganate hypersensitive sites, indicative of DNA melting, were revealed on the autoradiograph.

Transcription assays were done as described previously (17, 20, 21) with minor modifications. Preinitiation complexes were assembled for 30 min as described above, and then combinations of nucleotides were added for 4 min. RNAs were isolated and reverse transcribed with a radiolabeled primer. The products were resolved on a gel (17).

Immunoprecipitation (42) of polymerase was done as follows. Preinitiation complexes were assembled for 30 min, as described above. Then, 6.25 μM dATP was added to open the DNA, and [γ-^{32}P]ATP (150 μCi and 0.625 μM) was added to attempt to label the polymerase CTD. Thirty minutes later, the reaction mixture was brought to 0.5 ml with NET-GEL solution (50 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, and 0.25% gelatin). One microliter of monoclonal antibody 8WG16 (54, 55) at 1 mg/ml was added and incubated for 1 h at 4°C. Twenty microliters of protein A-Sepharose slurry (Pharmacia) was then added and incubated on a rocking platform at 4°C overnight. The polymerase-antibody-protein A-Sepharose complex was collected with a microcentrifuge and washed three times with NET-GEL and finally with 10 mM Tris (pH 7.5)–0.1% Nonidet P-40. The protein was solubilized with hot sodium dodecyl sulfate (SDS) loading buffer and subjected to SDS–5% polyacrylamide gel electrophoresis (PAGE). The gel was autoradiographed to detect labeled polymerase II. The identity of the band was confirmed by loading purified polymerase in parallel lanes which were subjected to Western blotting (immunoblotting). Parallel experiments with and without H8 being added to the transcription reaction mixtures were run in adjacent lanes of the gel.

RESULTS

The initial experiments were designed to monitor the progress of the transcription bubble as RNA synthesis begins. They used the reagent potassium permanganate, which reacts selectively with single-stranded DNA. The reaction, primarily with thymines, leads to stops in a primer extension assay of modified DNA and gave rise to a series of hyperreactive bands on a DNA sequencing gel. Previously, we showed that hyperreactivity to permanganate occurs within preinitiation complexes assembled at several promoters in both crude and purified systems (18, 20, 60). The reactive positions include thymines near the start site of transcription. In experiments with a HeLa transcription (nuclear) extract, formation of this open complex required ATP, although higher concentrations of other nucleoside triphosphates (NTPs) could substitute (19). These open complexes form prior to RNA synthesis, as demonstrated by their detection in the absence of initiating nucleotides and their ability to form in the presence of the initiation inhibitor α-amanitin.

The permanganate assay was used to monitor events that occur as the open complexes begin transcription. The template assayed was G9E4T, which contains nine GAL4 DNA binding sites for activator GAL4-AH or GAL4-VP16 upstream of a truncated adenovirus E4 promoter (3, 4). This template has six consecutive thymines in the transcription start site region, which facilitates the detection of the open complex. The functionality of the open complexes detected by the permanganate assay at this promoter has been described previously (17, 20).

Transcription bubble movement can occur prior to promoter clearance. In the initial experiments, transcription bubbles were characterized under conditions in which they were restricted to the initial transcribed region. We showed previously that the promoter used forms an open complex in which the stretch of six thymines over the initial transcribed region is sensitive to permanganate. In these experiments, a high concentration of UTP was added to closed transcription complexes, which we showed previously (19) can trigger open complex formation even in the absence of ATP. UTP is also a substrate for initial transcription, because it is complementary to the six adenines within the initiation region.

In the first experiment, open complexes were produced in the presence of UTP, but transcription initiation was prevented by the inclusion of α-amanitin. Lane 1 of Fig. 1A shows the permanganate pattern under these conditions. The brackets designate the start site region where permanganate reactivity was very strong. When α-amanitin was left out (Fig. 1A, lane 2), additional strong bands occurred downstream as far as +18, where the leading edge of the transcription bubbles may occur (considering that the transcription bubble is 14 to 17 base pairs long [19]), and weak bands occurred to +28, as also observed elsewhere (19). These strong bands reflect the initial RNA synthesis that is expected to occur in the absence of α-amanitin, because the polymerase may use UTP to copy the up to six adenines in the initiation sequence. The presence of heterogeneous transcription complexes downstream from the start site during initiation has been inferred for polymerase II (16) and has been studied systematically in prokaryotic systems, in which weak readthrough to positions farther downstream has also been observed (5). However, the main experimental point is that adding the substrate for transcription initiation causes transcription bubbles to appear within the initial transcribed region.

The question then arises as to whether this bubble movement beyond the start site was accompanied by promoter clearance. We define promoter clearance as a step in which polymerase leaves the promoter in a way that allows a new polymerase to enter and form a reinitiation complex (36). The assay used to determine clearance involved the detection, via permanganate probing, of the new open complexes that formed. Previously, we used a form of this assay to show that treating open complexes with all four NTPs for 2 min caused promoter clearance (17, 20). Clearance was demonstrated by a

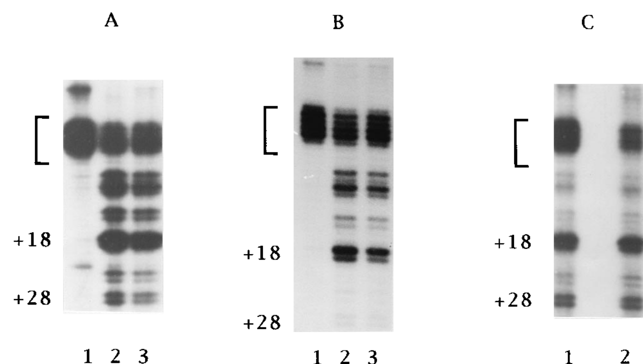

FIG. 1. Transcription bubble movement occurs prior to promoter clearance. Ten nanograms of supercoiled plasmid G9E4T was incubated with transcription extract for 30 min, and this incubation was followed by ordered additions of α-amanitin and nucleotides and permanganate probing. (A) Lane 1, α-amanitin and then 500 μM UTP for 2 min; lane 2, 500 μM UTP for 2 min; lane 3, 500 μM UTP for 2 min and then α-amanitin for 4 min. (B) Lane 1, α-amanitin and then 250 μM AMP-PNP and 125 μM CTP, UTP, and 3′-*O*-methyl-GTP [each] for 2 min; lane 2, 250 μM AMP-PNP and 125 μM CTP, UTP, and 3′-*O*-methyl-GTP (each) for 2 min; lane 3, 250 μM AMP-PNP and 125 μM CTP, UTP, and 3′-*O*-methyl-GTP (each) for 2 min and then α-amanitin for an additional 4 min. (C) Lane 1, 250 μM AMP-PNP and 125 μM GTP, CTP, and UTP (each) for 2 min and then α-amanitin for 4 min; lane 2, 250 μM AMP-PNP and 125 μM GTP, CTP, and UTP (each) for 2 min. The open complex signals represented by potassium permanganate hypersites at the transcription start site region are bracketed, and the sites near +18 and +28 are indicated.

combination of two observations (repeated below in Fig. 2). First, the addition of NTPs led to the disappearance of the open complex (Fig. 2, lane 3 versus 2; see also the negative control in lane 1), as can be expected when the polymerase escapes from the promoter in the process of elongation. Second, a subsequent addition of α-amanitin caused new open complexes to be trapped and thus led to the reappearance of an open complex signal (Fig. 2, lane 4 versus 3); this result was as expected (17) for the reinitiation event that accompanies the clearance step. We used this assay for the α-amanitin-induced appearance of bands over the transcription start site to detect promoter clearance.

The experiment thus involved first adding UTP, which both opens the DNA and allows the open complexes to begin initiation. Then, α-amanitin was added to assay for the formation of additional open complexes over the start site. The results show that α-amanitin does not cause a significant increase in start site open complexes (Fig. 1A; compare the bracketed regions in lane 3 and 2). This result indicates that clearance, which should lead to the formation of new open complexes, does not occur when UTP is used to form the initial transcribing complexes. Possibly, the initiated polymerases have not moved far enough downstream to allow a new polymerase to have access to the promoter, which will be tested next.

We added additional elongation substrates to allow the polymerase to move farther downstream. These substrates included UTP, CTP, and the ATP analog AMP-PNP; this last compound allows elongation but has a nonhydrolyzable beta-gamma bond, which avoids the potential for ATP stimulation of clearance. In addition, we included the chain terminator 3′-*O*-methyl-GTP. The methylated guanine was expected to prevent the RNA from progressing beyond the first G, which appeared downstream from the start site region (underline) in UUUUUUACACUG at position +9. The permanganate pattern under this condition is shown in Fig. 1B, lane 2. The lane shows that these conditions also led to the appearance of hypersensitive sites in the initial transcribed region downstream from the start site (compare Fig. 1B, lane 2, with control lane 1 in which RNA synthesis is absent). That is, transcription bubbles moved downstream in response to this combination of nucleotides.

Figure 1B also shows that these conditions were not associated with promoter clearance. That is, the addition of α-amanitin to these initial transcribing complexes did not lead to more open complex signals at the transcription start site; this result is indicated by the lack of increase in the start site opening (bracketed region) in lane 3 of Fig. 1B compared with lane 2. This lack of clearance under conditions in which bubbles move beyond the start site region confirmed that bubble movement can occur in the absence of promoter clearance. We also noted that this bubble movement occurred in the absence of beta-gamma-hydrolyzable ATP, although we cannot strictly rule out the possibility that higher concentrations of elongation substrates were used as a substitute hydrolysis source. Because the helicase activity of TFIIH is strictly ATP dependent (47), it appears not to be required to move the single-stranded transcription bubble.

We allowed the polymerase to move even farther downstream by substituting GTP for 3′-*O*-methyl-GTP (Fig. 1C). Lane 2 of Fig. 1C confirms that these conditions caused some transcription bubbles to appear in positions farther downstream. The permanganate pattern at this point consisted primarily of bands at regions near +28 and +18 and at the start site. We have not probed positions beyond +28 and thus cannot say if some polymerases moved even farther. The pattern in Fig. 1C contrasts with the patterns seen when RNA synthesis was more restricted (Fig. 1A and B). In Fig. 1A and B, the strong bands are spread relatively evenly between the start site and position +18. The additional movement of bubbles to at least position +28, seen under the conditions which obtained in Fig. 1C, could conceivably be associated with promoter clearance. This hypothesis can be tested by the addition of α-amanitin, which, as shown in Fig. 2 and previously in a different experimental context (60), can collect new open complexes that might form over a cleared promoter.

The result obtained was that which would be expected if some polymerases had moved far enough to clear the promoter. α-Amanitin now induced a significant increase in the start site opening (lane 1 versus 2 of Fig. 1C), as demonstrated by the increased intensity of bands that correspond to uninitiated open complexes (bracketed). It also froze the positions of the downstream transcription bubbles, as would be expected for an inhibitor of elongation. Thus, the downstream polymerases are frozen in place by α-amanitin under conditions in which new polymerases can enter and melt the promoter start site region. We infer that significant promoter clearance has occurred in this experiment. This outcome contrasts with the

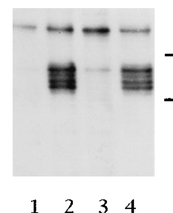

FIG. 2. Demonstration of promoter clearance. Closed complexes were assembled for 30 min prior to the indicated additions. Lane 1, no nucleotides added; lane 2, 500 μM ATP for 2 min; lane 3, all four NTPs at 500 μM for 2 min; lane 4, as in lane 3, with α-amanitin being added subsequently for 8 min to collect new open complexes over the cleared promoter. The bracket indicates the start site region.

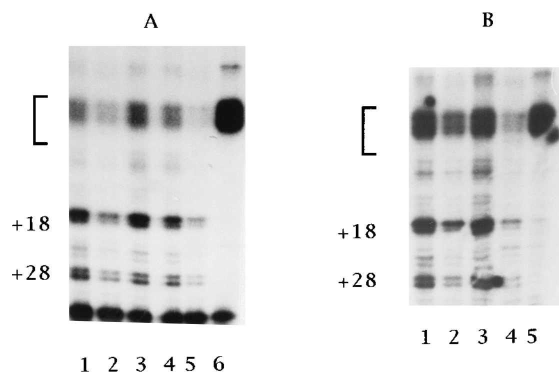

FIG. 3. Role of ATP and phosphorylation in polymerase escape. (A) Closed complexes were assembled on the supercoiled template for 30 min with or without H8, and then different combinations of nucleotides were added before potassium permanganate probing. Lane 1, 250 μM AMP-PNP and 125 μM GTP, CTP, and UTP (each) for 4 min; lane 2, the same nucleotide combination as that in lane 1 for 2 min and then 500 μM dATP for an additional 2 min; lane 3, 2 mM H8 during the preincubation and then nucleotides as in lane 2; lane 4, 2 mM H8 during the preincubation and then 125 μM ATP, GTP, CTP, and UTP (each) for 2 min; lane 5, 125 μM ATP, GTP, CTP, and UTP (each) for 2 min; lane 6, α-amanitin and then 125 μM ATP, GTP, CTP, and UTP (each) for 2 min. (B) The DNA template was linearized before being added to the preincubation mixture containing activator GAL-AH. Lane 1, 250 μM AMP-PNP and 125 μM GTP, CTP, and UTP (each) for 5 min; lane 2, the same nucleotide combination as that in lane 1 for 2 min and then 500 μM dATP for 3 min; lane 3, as in lane 2, except 2 mM H8 during the preincubation; lane 4, 125 μM ATP, GTP, CTP, and UTP (each) for 3 min; lane 5, as in lane 4, except α-amanitin during the preincubation. The open complex signals represented by potassium permanganate hypersites at the transcription start site region are bracketed. The permanganate hypersites at the +18 and +28 regions are indicated.

lack of clearance seen when polymerases were held closer to the promoter by the use of more restrictive combinations of nucleotides (Fig. 1A and B).

The existence of strong pauses associated with reactivity at positions +18 and +28 raised the question of why polymerases accumulate within this region. One possibility is that polymerases naturally stall prior to clearance, with the leading edge of their bubbles near +28. However, because the experiment did not result in the polymerases stopping at discrete points, it is not possible to define the exact position of clearance, which would require further study of an engineered series of templates. The clearance that occurred in this experiment could have been due to the polymerases associated with the +28 hypersites or with polymerases that moved farther. Recall that the experiment included four elongation substrates but lacked a source of hydrolyzable ATP. Thus, it is possible that hydrolyzable ATP is required for polymerase to escape from stalled positions within this initial transcribed region. In the next section, we will discuss the addition of a source of hydrolyzable ATP to test this possibility.

ATP facilitates polymerase escape from the initial transcribed region. The experiments discussed above demonstrated that initiation and DNA bubble movement can precede promoter clearance, which occurs after the bubble has moved some distance downstream. They also indicated that in the presence of four elongation substrates (125 μM UTP, CTP, and GTP [each] and 250 μM AMP-PNP), there are transcription bubbles associated with the initial transcribed region (repeated in lanes 1 of Fig. 3A and B). These bubbles were strongly diminished in identical experiments in which 125 μM ATP was used instead of its nonhydrolyzable (beta-gamma bond) analog AMP-PNP (lane 1 versus 5 of Fig. 3A and lane 1 versus 4 of Fig. 3B). This result suggests that ATP hydrolysis may be required to prevent the stalling of polymerases within this initial transcribed region. In order to rule out any effect of ATP as a superior elongation substrate, in this comparison, dATP was substituted as a source of beta-gamma-hydrolyzable ATP. dATP cannot be used in elongation, and therefore, any of its effects should be due solely to its role as a hydrolyzable cofactor. As DNA supercoiling has also been postulated to affect promoter clearance, the experiment was done with templates that were added as either supercoiled or linearized forms.

Lanes 1 of Fig. 3A (supercoiled DNA) and B (linear DNA) show the starting point for this set of experiments, which were aimed at testing a role for ATP in releasing a stalled polymerase. In these lanes (Fig. 3), elongation substrates were added to preformed open complexes for 4 min. Under these conditions, bubbles appeared over the start site, at positions +18 and +28 within the initial transcribed region, and probably beyond. This distribution is the beginning distribution of complexes for the testing of any effect of dATP. In lanes 2 of Fig. 3A and B, the incubations were identical for the first 2 min, but dATP was added during 2-min (Fig. 3A) and 3-min (Fig. 3B), chase periods (we observed less efficient chasing on linear DNA; hence, the longer chase and higher residual signal in Fig. 3B resulted). The result shows that for both linear and supercoiled DNA, dATP chased a significant fraction of the transcription bubbles (Fig. 3A and B; compare lanes 2 with lanes 1). Because dATP is not an elongation substrate, this chase cannot result from dATP being used by the polymerase for downstream elongation. Instead, the dATP appears to overcome a block to elongation, allowing the polymerase to be released and elongate away from the initial transcribed region.

This dATP-dependent loss of transcription bubbles near positions +18 and +28 can be blocked by the RNA elongation inhibitor α-amanitin (data not shown). This fact confirms that this process requires elongation, not simply the addition of dATP. Overall, the data suggest a pathway in which in the absence of hydrolyzable ATP, polymerases temporarily stall in the initial transcribed region. Clearance does not occur efficiently, because most polymerases have not moved far enough downstream to pass the position that leaves the promoter clear enough to bind a new polymerase. In the presence of hydrolyzable ATP, the release of the stalled polymerases is facilitated and they travel to positions which are far enough downstream to clear the polymerase from the upstream promoter.

The CTD kinase inhibitor H8 counteracts the stimulatory effect of ATP. This use of ATP in polymerase escape from the initial transcribed region could be related to the use of ATP in phosphorylation of the polymerase CTD. This possibility is suggested because phosphorylation is not required to form an open transcription complex (18) or the first mRNA bond (22), but it does occur sometime prior to the formation of an elongation complex (30, 39). ATP-dependent CTD phosphorylation has been shown to be inhibited by compound H8 in fractionated transcription systems (see, for example, references 18, 32, and 45). H8 did not inhibit ATP-dependent open complex formation or first-bond formation in the cases studied (18, 22). We investigated whether H8 inhibits the transition from open complex to elongation complex. Because the effect of H8 on polymerase phosphorylation has only been firmly established in fractionated systems, we first studied its inhibition of polymerase phosphorylation in the unfractionated system used in this study.

In this experiment, preinitiation complexes were assembled as described above in either the presence or absence of H8. Proteins were labeled with [γ-^{32}P]ATP, and then polymerase was precipitated with monoclonal antibody 8WG16 (54, 55). The precipitated proteins were subjected to SDS-PAGE, and

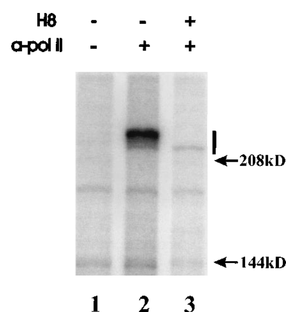

FIG. 4. Inhibition of polymerase phosphorylation by H8 in a HeLa nuclear extract. Proteins were labeled with [γ-^{32}P]ATP under preinitiation complex conditions in a HeLa nuclear extract. The polymerase was immunoprecipitated with monoclonal antibody. Proteins were then separated by SDS–5% PAGE, and the autoradiograph is shown. The positions of size markers and a Western blot marker using purified polymerase (indicated by a vertical bar that covers the heterogeneous forms) are shown at the right. The presence or absence of antibody (a-pol II) and CTD kinase inhibitor H8 are shown at the top.

the pattern was compared with those of size markers and a marker of pure polymerase subjected to Western analysis (Fig. 4). Lane 2 of Fig. 4 shows a strong band on the autoradiograph in a position that corresponds to that of polymerase. This result indicated that the polymerase incorporated phosphate from [γ-^{32}P]ATP, which is the only source of radioactivity. Lane 3 of Fig. 4 shows that the inclusion of H8 in the transcription reaction prevented the phosphorylation of the polymerase, which is known to occur primarily on the CTD (2). We conclude that H8 inhibits polymerase phosphorylation in this unfractionated system. The inhibition appears to be at least as strong, and perhaps stronger, than that observed previously in fractionated systems.

We also investigated the effect of H8 on promoter clearance. The experiment began with an observation of the permanganate pattern seen when transcription is allowed to occur freely in the presence of ATP, GTP, CTP, and UTP. A light pattern of bands was present (lane 5 of Fig. 3A and lane 4 of Fig. 3B), representing the small fraction of polymerases that traverse the initial transcribed region when permanganate probing is done during free transcription. When the experiment was repeated in the presence of H8, the abundance of all of these bubbles was substantially increased (Fig. 3A; compare lanes 4 and 5). This outcome indicates that H8 causes polymerases to collect in the initial transcribed region (H8 itself does not cause permanganate sensitivity in the absence of transcription [data not shown]).

The result demonstrates a role for an H8-sensitive kinase in polymerase escape from the initial transcribed region but does not firmly connect this role to the role of ATP in stimulating polymerase escape (demonstrated above). The next comparison showed that these two phenomena are indeed connected. Recall that dATP can trigger the release of polymerases that had been caused to stall in the initial transcribed region, as evidenced by the loss of transcription bubbles, as just described. The next experiment showed that dATP is essentially ineffective in this regard if H8 is present. That is, the pattern of bubbles seen in the presence of H8 and dATP was similar to that seen without dATP (lane 1 and 3 in Fig. 3A and B). The comparison indicated that H8 counteracts the stimulatory effect of dATP on polymerase escape. Thus, the data demonstrate that hydrolyzable ATP stimulates polymerase escape through an H8-sensitive kinase pathway. Because dATP can be used for CTD phosphorylation and H8 prevents CTD phosphorylation and because other data suggest that CTD phosphorylation may occur during the transition to elongation, the result suggests that the use of hydrolyzable ATP and the process of CTD phosphorylation are coupled in stimulating polymerase escape. That is, there may be a single ATP requirement for this process in which the CTD is phosphorylated, stimulating the escape of the polymerase from its stalled positions within the initial transcribed region.

The experiments discussed above also confirmed previous observations made in different experimental systems. H8, which can inhibit CTD phosphorylation, does not inhibit any step up to first-bond formation (18, 22). In addition, the data showed that H8 does not inhibit the movement of transcription bubbles into the initial transcribed region (see the downstream bubbles in lanes 3 and 4 of Fig. 3A and lane 3 of Fig. 3B). Thus, phosphorylation is not required to form the open complex or to form an initial transcribing complex, but it is required for the final stages of conversion to a downstream elongation complex.

These results imply that H8 should inhibit transcription in this system by restricting the polymerase to the initial transcribed region. The implication is important to test, because H8 does not inhibit transcription in certain highly purified systems (18, 45) but can inhibit transcription in a more complex system (32). We found that the strength of inhibition by H8 can vary somewhat, depending on experimental conditions (18), and this experiment was designed to mimic the conditions used for the permanganate assays discussed above. This design involved the use of very low amounts of DNA in order to ensure that the reaction will be very strongly dependent on the activator and the use of short reaction times.

Figure 5 shows that H8 strongly inhibited transcription (compare lanes 1 and 3 with H8 to lanes 2 and 4 without). This result was obtained under the same conditions in which the permanganate assay showed an H8-dependent inhibition of polymerase escape. The same conditions also obtained in the experiment illustrated in Fig. 4, in which a strong inhibition of polymerase phosphorylation by H8 was shown, confirming an important role for the kinase reaction. The transcription result differed from observations made with highly fractionated systems, implying that such systems transcribe independent of the factor that mediates the sensitivity to H8.

DISCUSSION

A model for initiation at the activated E4 promoter. The data presented here may be combined with prior data from several laboratories to arrive at a more detailed model for

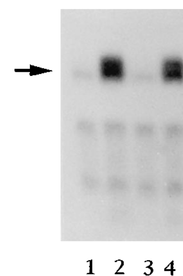

FIG. 5. Transcription in the HeLa nuclear extract is inhibited by CTD kinase inhibitor H8. DNA template, transcription activator GAL4-VP16, and HeLa nuclear extract were incubated in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 2 mM H8 for 30 min; then, NTPs at 125 μM each were added for 2 min. The transcript is indicated with an arrow. Repeated experiments with various preparations of nuclear extracts gave inhibitions ranging from 50 to the 90% shown here.

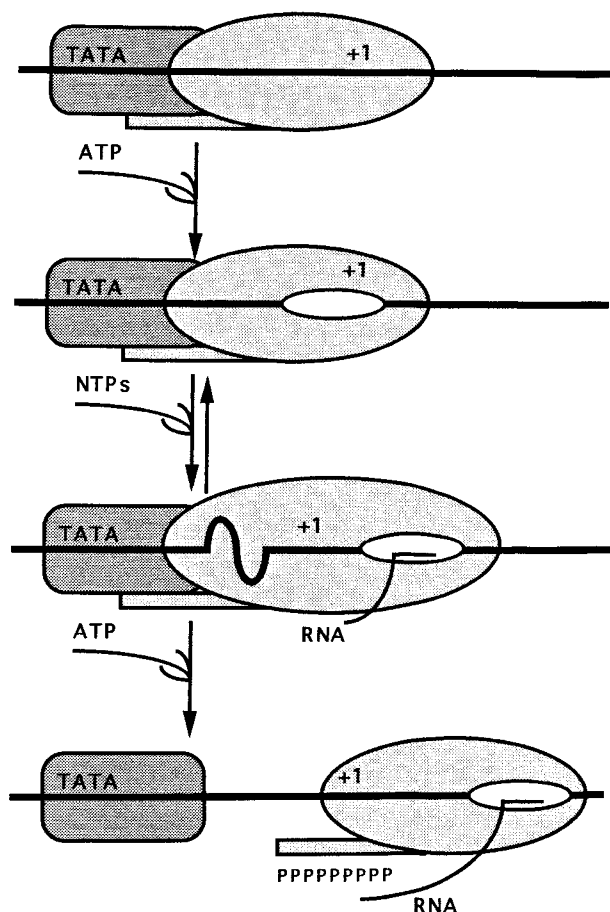

FIG. 6. A three-step pathway to transcription by RNA polymerase II preinitiation complexes. In the first step of this model, ATP is used to drive DNA strand opening to convert the closed complex to the open complex. In the second step, elongation substrates are used by open complexes to initiate RNA synthesis and drive the DNA melting bubble forward. At this step, some nascent RNA products might dissociate (abortive initiation), cycling the DNA bubble back to the transcription start site. This initial transcribing complex is held near the promoter by interactions that include those with the polymerase CTD. In the third step, ATP-dependent phosphorylation of the CTD breaks some of these interactions, allowing the polymerase to escape more easily and enter the elongation mode. The polymerase can then move easily and clear the promoter for the entry of a new polymerase. This transcription reinitiation may be facilitated by factors that remain on the promoter (17). The model derives from data on factors present in a crude transcription extract.

transcription initiation (Fig. 6). The first step in the model derives from previous data and shows the conversion of a closed transcription complex into an open complex. We showed previously that this step occurs with concomitant hydrolysis of the beta-gamma bond of ATP (19, 20, 60). The new data from this study are incorporated into the conception of the second and third steps.

The model indicates that after open complexes are formed, RNA synthesis begins. This synthesis is accompanied by the movement of the transcription bubble into the initial transcribed region. Two key elements in this previously uncharacterized second step are as follows: (i) this bubble movement occurs initially without the promoter being cleared, and (ii) it can occur without the need for phosphorylation of the polymerase CTD. The lack of clearance was demonstrated by showing that under these conditions, no new polymerases could associate with the template to form new open complexes. The lack of need for CTD phosphorylation was demonstrated by the lack of effect of CTD kinase inhibitor H8. Control experiments, done under conditions in which transcription proceeded farther downstream, showed that both clearance and H8 inhibition could be detected, supporting these interpretations.

The model also suggests that initiation of RNA synthesis is followed by or potentially coupled to (30, 39) phosphorylation of the polymerase CTD, which in turn facilitates the release of the polymerase from the initial transcribed region. The movement of transcription bubbles away from the initial transcribed region was stimulated by ATP, and this stimulation by ATP was blocked by H8. H8 was shown to block CTD kinase activity, and in view of prior results (see above), this activity is the most likely target for the inhibitor effect of H8. However, we cannot exclude the possibility that H8 is inhibiting another uncharacterized kinase and that this activity is contributing to its effect. In any case, the kinase activity helps to release the polymerase so that it can move to a region far enough downstream to have fully vacated the promoter. In a separate set of experiments, promoter clearance was shown directly via the formation of new open complexes at the promoter, which only occurred after the transcription bubbles had moved to positions farther downstream.

Overall, the data suggest a model in which promoter clearance occurs in three sequential substeps. First, an H8-sensitive activity, likely the CTD kinase, stimulates the escape of the polymerase from the contacts that hold it to factors bound at the promoter (for examples, see references 39, 53, and 58). Second, this escape allows the polymerase to move farther downstream. Third, the polymerase moves far enough downstream to sterically clear the promoter, allowing a new polymerase to bind there. The model also indicates that the binding of this new polymerase, and therefore reinitiation, is facilitated by certain factors that have previously been suggested to be left behind (17, 59, 62).

Prior reports suggest considerable diversity in how transcription systems use the CTD. Certain transcription systems, especially highly fractionated ones, appear not to require CTD phosphorylation (18, 45). The model in Fig. 6 represents transcription in an unfractionated system, in which CTD phosphorylation is important. In systems involving CTD phosphorylation, it appears to occur prior to the formation of an elongation complex (30, 39), which is in harmony with this model. The data support the notion that CTD phosphorylation occurs prior to promoter clearance and raise the possibility that such phosphorylation may primarily facilitate a clearance process that would occur anyway, but at a lower rate or efficiency.

One view is that experimental transcription systems are expected to differ in the extent that they impose a requirement for CTD phosphorylation (see the data in reference 32 for a comparison of two such systems). The differences may arise from coupling factors that mediate the interaction between general factors and the polymerase CTD. Such factors may be important in bringing the large multiprotein complex (holoenzyme) (24, 25) containing the polymerase to the promoter. With regard to initiation, the point is that such hypothetical coupling factors, or indeed anything that strengthens the CTD-general factor interaction, would have the potential to slow polymerase escape and promoter clearance. The stronger the coupling, the more the system may depend on CTD phosphorylation for maximal transcription rates. Existing data concerning supercoiling can be accommodated in this model by assuming that the energy of supercoiling can also be used to promote polymerase escape and thus promote transcription (see the references listed in the introduction). In some highly fractionated systems, coupling may be sufficiently weak so that either

CTD phosphorylation or DNA supercoiling may allow the polymerase to escape from the initial transcribed region and make transcript.

Factors and physiology. Many studies concerned with the effect of ATP on transcription have focused on general factor TFIIH, because it contains two ATP-dependent enzyme activities, a CTD kinase and a DNA helicase (see the introduction). As was just discussed, the current data are consistent with the view that the CTD kinase activity of TFIIH is important for the polymerase escape aspect of promoter clearance. The data do not support the view that TFIIH activities are important for the initiation events that move the transcription bubble into the initial transcribed region; this movement occurs in the presence of H8, ruling out an involvement of kinase, and occurs without ATP, which is needed for helicase action. We speculate that the demonstrated involvement of ATP and TFIIH in promoter clearance in a purified system (13) may be due to the kinase activity, but the differences between pure and impure systems make comparisons difficult.

It has been shown that the polymerase CTD physically interacts with certain general transcription factors in the preinitiation complex (53, 58). This observation has led to speculation that such interactions may hold the polymerase at the promoter and that phosphorylation may release the polymerase (reviewed in reference 8). As discussed above, our data support a version of this model. However, the model also implies that there is not a strict requirement for CTD phosphorylation, because there are a variety of influences, including hypothetical coupling factors and DNA supercoiling, that determine the rate and efficiency of polymerase escape. Given the rich variety of interactions that can occur at promoters of different types, it is conceivable that there will be interesting promoter-specific differences in the ease of achieving polymerase escape and promoter clearance, as has been observed with prokaryotic systems (9). Some of these interactions may be reflected in the various promoter-specific events that have been proposed to depend on stalled polymerases (see the introduction). Such regulatory events need not be restricted to acting as on-off switches but may involve modulating transcription levels by controlling the efficiency or the rate of polymerase escape.

Another aspect of polymerase escape and promoter clearance is that in the system studied here, reinitiation of transcription is faster than had been expected on the basis of the rate at which the first round of initiation occurs (17). Reinitiation can only occur after promoter clearance. Thus, when and how clearance occurs could control how readily reinitiation, which produces the bulk of RNA in an in vivo setting, occurs. Further studies of the detailed initiation mechanism proposed here should be useful in evaluating proposals concerning physiological regulation via polymerase release.

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