4958–4962 Nucleic Acids Research, 1994, Vol. 22, No. 23

# RNA polymerase II phosphorylation: uncoupling from GAL4-VP16 directed open complex formation and transcription in a reconstituted system

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Received August 17, 1994; Revised and Accepted October 17, 1994

## ABSTRACT

**An activated transcription system was constructed using substantially purified liver factors, Hela TFIID and GAL4-VP16. The system was used to study the relationship between RNA polymerase II large subunit phosphorylation and other ATP-dependent processes occurring during activated transcription. When C-terminal domain (CTD) kinase activity was inhibited, activator dependent open promoter complex formation proceeded normally. These open complexes could function to produce RNA in the absence of CTD phosphorylation, although the level of RNA produced was changed somewhat. The results demonstrate that RNA polymerase II CTD phosphorylation is not generally required for the formation of activator-dependent, functional open promoter complexes. Taken together with prior results the experiments suggest that a requirement for CTD phosphorylation may be situation-dependent and thus serve a regulatory function.**

## INTRODUCTION

Several ATP-dependent processes occur during initiation of transcription by RNA polymerase II and associated transcription factors (1–5). One of these is the phosphorylation of the polymerase itself (6,7). The primary site of phosphorylation is the C-terminal domain (CTD) of the largest polymerase subunit. The CTDs of a wide variety of organisms contain 26–52 repeats of a sequence related to YSPTSPS (reviewed by 8,9). The serines and threonines within this sequence can be phosphorylated by certain ATP-dependent protein kinases (6,10–20).

There has been considerable discussion of the role of polymerase phosphorylation in transcriptional processes (for reviews see 8,9). The CTD is essential for viability in cells from a variety of organisms. Studies in yeast indicate that the CTD is necessary to obtain a significant transcriptional response to the activator GAL-VP16 (21) and the CTD appears to modulate the responsiveness to certain activators (22–24). The CTD interacts with a number of transcription factors (25,26) and this is in some cases affected by its phosphorylation state (25). The polymerase becomes phosphorylated as a normal part of transcription initiation at a variety of promoters. This phosphorylation inhibits the ability of the polymerase to re-enter pre-initiation complexes (27,28). Experiments such as these have led to models (25,28–31) in which CTD phosphorylation is involved in the conversion of the pre-initiation complex to an active form that can transit to the elongation phase of RNA synthesis.

On the other hand, there is substantial evidence that the CTD and its phosphorylation are not generally essential for transcription. Some promoters can be transcribed *in vitro* using polymerase that lacks the CTD (26,32–35). Previous experiments using purified mammalian factors have shown that basal transcription can occur in the presence of the CTD-phosphorylation inhibitor H8 (36). In a yeast-derived basal system it was shown that transcription does not depend on CTD phosphorylation unless additional proteins are present (37). Both of these studies assayed basal (unactivated) transcription and used compound H8 to inhibit CTD phosphorylation.

Similar experiments have not been done for activated transcription, which constitutes the larger body of information concerning potential CTD function. In this study we will use the CTD kinase inhibitor H8 to address whether CTD phosphorylation is necessary for activated transcription. In this initial test, we will use the activator GAL4-VP16 which in yeast is known to require the CTD as part of its transcription activation function (21).

CTD phosphorylation is one of three enzymatic activities associated with activated transcription that use ATP; the other two are helicase action and open promoter complex formation (3,5; Jiang and Gralla, manuscript submitted). Both the CTD-kinase activity and the helicase activity are associated with the homologous set of multi-subunit transcription factors TFIIH, BTF-2 and delta (16,18,20,36,38). The ATP-dependent activity that opens the DNA strands has not yet been identified, but it has been suggested that it could also be part of the TFIIH complex (39). Here we also investigate the potential coupling between ATP-dependent phosphorylation and ATP-dependent open complex formation.

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## MATERIALS AND METHODS

Nucleotides were from Pharmacia LKB Biotechnology Inc. and the CTD-Kinase inhibitor H8 (N-(2-(methylamino)ethyl)-5-isoquinoline sulfonamide dihydrochloride), was from Seikagaku American Inc. Monoclonal antibody 8WG16 was supplied by Drs Conaways (Oklahoma Medical Research Foundation) as were the transcription factors liver TFIIA, alpha (B), epsilon (E), beta gamma (F), delta (H) and polymerase. Alpha and epsilon were recombinant proteins purified from *E.coli* and beta gamma, delta and polymerase were purified from rat liver, and used as described (40−42; and unpublished).

Hela TFIID was purified with some modifications (suggested by Dr Michael Carey) to increase purity. Briefly, Hela nuclear extract was applied to a P-11 column (43), washed with buffer D (20 mM HEPES pH 7.9, 20% glycerol, 0.1 mM EDTA, 1 mM DTT, and 1 mM PMSF) containing 0.6 M KCl, and TFIID was eluted with 0.85 M KCl D buffer. This fraction was dialyzed against 0.05 M KCl D buffer and was applied to a DEAE 52 column equilibrated with 0.05 M KCl D buffer. This was eluted with 0.2 M KCl D buffer and the breakthrough was pooled and directly loaded on a heparin−sepharose column which was equilibrated with 0.2 M KCI D buffer. After washing with 0.3 M KCl D buffer the TFIID was eluted with 0.5 M KCl D buffer. The amount to be used was determined by titration of transcription reactions.

The hybrid transcription system used Hela TFIID, cloned factors and highly purified liver factors, as described above. Reactions were carried out in 1/2 D buffer, containing 10 or 20 ng supercoiled G9E4T as template (as indicated), 8.25 mM $Mg^{2+}$, 200 ng pGem as carrier and 500 $\mu$M NTPs. In some experiments nucleotides were added for 30 min only after a 30 min pre-incubation of the other components (Figs 3 and 4). When used, H-8 was included in the 30 min pre-incubation. RNA was assayed by primer extension as described (5). Because the RNA produced is shorter than 100 nucleotides, potential effects of H8 on long chain elongation are not assayed.

The assay for phosphorylated polymerase was as described (20) with modifications for activated transcription. Components were incubated for 30 min in the presence or absence of H8, followed by addition of gamma P32 ATP (sp. act. > 4000ci/mmol) and dATP or ATP (concentration indicated) for 30 min. Antibody 8WG16 was used to identify the polymerase large subunit (20,26). The *in vitro* potassium permanganate assay (3,5) used the same 30 min pre-incubation, with or without H8, followed by 500 $\mu$M dATP for 2 min. A 4 min treatment with 6 mM potassium permanganate was used.

## RESULTS

The initial experiments were modeled after those showing that CTD-phosphorylation was not required for basal level mammalian transcription (36). In that case highly purified liver factors were used to transcribe the adenovirus major late promoter. The CTD kinase inhibitor H8 was used to show that inhibition of polymerase phosphorylation was not accompanied by inhibition of basal transcription. We wished to do related studies of activated transcription because the CTD has been shown to have a critical role in activation in some cases (see Introduction). To accomplish this we adapted the liver system to study activated transcription. We chose to use the GAL4-VP16 activated adeno E4 promoter which has the advantage of being very strongly activator dependent (44,45) and CTD dependent (21). In addition the open transcription complex at this promoter has been identified and characterized (5).

### A hybrid liver-Hela transcription system which supports Gal4-VP16 activated pol II transcription

The newly constructed system uses recombinant and highly purified basal liver factors (40) alpha (TFIIB), epsilon (TFIIE), beta-gamma (TFIIF), delta (TFIIH), TFIIA (46), and polymerase, along with factor TFIID isolated from Hela cells using three columns. In the presence of GAL4-VP16 a strong transcription signal is obtained from template G9E4T in this hybrid system (arrow pointing to lane 2 of Fig. 1). This is the same transcript observed previously using factors derived exclusively from Hela cells (3,5,44,45,47,48). Formation of the transcript requires that GAL4-VP16 be present (compare lanes 1 and 2 of Fig. 1). We conclude that the hybrid system functions to yield GAL4-VP16 dependent transcription.

### Compound H8 inhibits CTD phosphorylation in the liver Hela hybrid transcription system

Next, we determined the effect of the CTD kinase inhibitor H8 on this system. H8 is a potent inhibitor of a number of protein kinases (49) and inhibits polymerase phosphorylation in the liver-based transcription system (36,50) and has been used in a variety of transcription systems (37,51). The new experiment addresses whether the presence of GAL4-VP16 and the substitution of Hela TFIID for TBP interferes with the action of H8 in inhibiting liver polymerase phosphorylation.

The CTD kinase assay was established in the hybrid system and the effect of H8 on the polymerase phosphorylation was tested (according to 20). In the initial assay, the complete system, with or without H8, was incubated in the absence of nucleotide triphosphates (NTPs) for 30 min. In order to label proteins, gamma-P-32 ATP and 10 $\mu$M unlabeled dATP were added for another 30 min. SDS−PAGE analysis and autoradiography show labeling of several protein bands. The labeling of only one band was inhibited by H8 and this has the mobility of the polymerase large subunit (compare lanes 1 and 2 of Fig. 2A). The experiment was repeated with increasing amounts of unlabeled ATP with

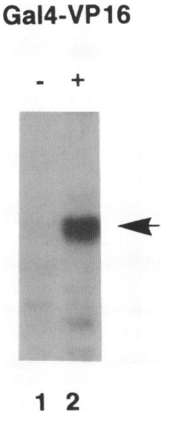


**Figure 1.** The hybrid system is capable of supporting Gal-VP 16 activated transcription. Transcription reactions with 20 ng G9E4T template were for 30 min in the absence (lane 1) or presence (lane 2) of Gal-VP16. The arrow shows the previously characterized transcript.

the result that the inhibition could not be reversed by up to 500 μM ATP (alternate lanes in Fig. 2B). The identity of the H8-inhibited band was confirmed to be the polymerase large subunit by immunoprecipitation; the band is precipitated with the polymerase CTD-specific antibody 8WG16 (lane 4 vs. lane 3 of Fig. 2C). We conclude that polymerase large subunit phosphorylation was severely inhibited by H8 under these experimental conditions.

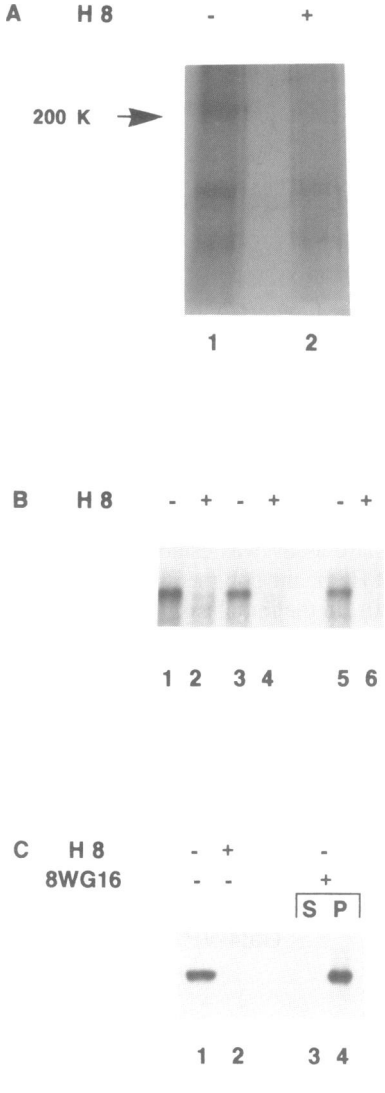


**Figure 2.** (**A**) H8 inhibits polymerase phosphorylation 10 ng G9E4T template was incubated in the complete hybrid system without H8 (lane 1) or with 1.2 mM H8 (lane 2) for 30 min, followed by addition of gamma P-32 ATP (4 μci) and 10 μM dATP for another 30 min. The reaction mixtures were subjected to 5% SDS–PAGE electrophoresis with parallel marker lanes followed by autoradiography . The arrow points to the position of 200Kd marker, and the band above it has the mobility expected of the large polymerase subunit. (**B**) Using 40 μci P-32 ATP, the protocol was repeated with 50 μM ATP (lanes 1 and 2), 100 μM ATP (lanes 3 and 4) and 500 μM ATP (lanes 5 and 6). Lanes 1, 3, 5 were without H8 and lanes 2, 4, 6 were with 1.2 mM H8. In this experiment 2.2% polyvinyl alcohol (PVA) was added in order to optimize the CTD kinase reaction (as suggested by Dr Serizawa). (**C**) The products of a reaction using 500 μM ATP (lane 1) were precipitated using the CTD specific antibody 8WG16. Lane 4 shows the pellet and lane 3 the supernatant. Lane 2 shows the effect of H8 on the original labeling reaction.

## Polymerase phosphorylation is not required for open complex formation

Both polymerase phosphorylation and DNA start site opening occur prior to elongation and both require ATP, raising the possibility that they are obligatorily coupled. The properties of H8 allow the testing of whether phosphorylation is necessary for formation of the open transcription complex. The open complex has been detected at this promoter by the use of the single strand selective reagent potassium permanganate. Enhanced reactivity appears over the transcription start-site under conditions associated with formation of a functional pre-initiation complex (3,5,47,48,52). In this experiment we used the same components and conditions used in the kinase assay. The goal was to learn whether open complexes still form in the presence of the CTD-kinase inhibitor H8.

The permanganate cleavage pattern, under conditions where no open complex forms because activator is absent, is shown in Figure 3, lanes 1 and 4. The pattern of bands represents the low reactivity of permanganate with double stranded DNA. When GAL4-VP16 is added, a new series of bands appears over the transcription startsite (lane 2 vs lane 1; see position of brackets), as identified previously (5). The formation of the open complex is confirmed by the similar but stronger reactivity using the activator GAL4-AH (lane 5 compared to lane 4 of Fig. 4); GAL4-AH was shown previously to yield a stronger open complex signal over the same region opened using GAL-VP16 (48).

Figure 3 also shows that addition of the inhibitor H8 does not prevent open complex formation. The bracketed signal is unchanged for the weaker open complex directed by GAL4-VP16 (compare lanes 2 and 3) and for the stronger open complex directed by GAL4-AH (compare lanes 5 and 6). We do not know the source of the extra downstream bands induced by the inclusion of H8 (lanes 3 and 6). They could represent interesting changes in the transcription complex associated with the state of the polymerase but may simply be artifacts since H8 does not change the transcription pattern (see below). Nonetheless the data show that inhibition of polymerase phosphorylation does not interfere with formation of the open transcription complex.

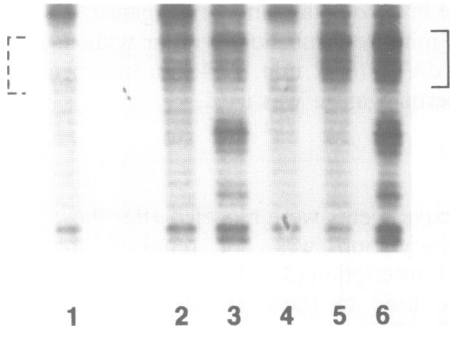


**Figure 3.** H8 does not inhibit Gal-VP16 dependent open promoter complex formation. Pre-incubation was as in Figure 2 followed by addition of 500 μM ATP for 2 min. Open promoter complexes were then probed with potassium permanganate. Lanes 1 and 4 lack activators; lanes 2 and 3 have Gal-VP16; lanes 5 and 6 have Gal-AH. H8 was present in lanes 3 and 6 at 1.2 mM from the very outset of incubation. The previously identified potassium permanganate hypersensitive sites that represent open complexes are bracketed.

### Polymerase phosphorylation is not necessary for transcription in this system

Next, we assessed the effect of the phosphorylation inhibitor H8 on GAL4-VP16 dependent transcription. The experiment used the protocol just described; this is a 30 min pre-incubation of all factors and template DNA, with or without H8, followed by a 30 min incubation in the presence of nucleoside triphosphates. Lane 1 of Figure 4 shows the substantial amount of transcript obtained in the absence of H8. The similar amount of transcript observed in lanes 2 and 3 shows that H8 does not inhibit transcription, which indicates that extensive polymerase phosphorylation is not necessary for transcription.

The comparison further indicates that under the experimental condition H8 actually stimulates transcription. Because Figure 3 showed that H8 has no effect on formation of open complexes the observed stimulation of transcription cannot arise from stimulation of initial complex formation; instead it is apparently a consequence of stimulating more rounds of transcription. This property is consistent with previous experiments demonstrating that CTD-phosphorylation inhibits polymerase from entering preinitiation complexes (27,28). Because H8 prevents phosphorylation it can increase the amount of polymerase that enters preinitiation complexes and thus can lead to more rounds of transcription. This explanation is further supported by an experiment (not shown) in which transcription is largely restricted to a single round; the stimulation disappears under these conditions. This stimulation should only occur with natural polymerase containing its CTD, confirming that it is this form of the polymerase that is yielding the transcription signal. This stimulation does not occur when the activator GAL-AH is used; instead, there is a modest inhibition of transcription (data not shown). Although the source of the stimulation cannot be stated with absolute certainty, the data demonstrate that CTD phosphorylation is not required for transcription in these systems that depend on the presence on hybrid GAL activators.

## DISCUSSION

In this study we have established a substantially purified activated transcription system combining liver factors (36) with Hela TFIID. Because initiation, CTD-phosphorylation, and open complex formation all require ATP we used the system to investigate whether the latter two processes are coupled. The results showed that in a system dependent on activator Gal4-VP16, ATP-dependent phosphorylation and ATP-dependent open complex formation proceed independently; that is, a normal complement of open complexes form under conditions where CTD phosphorylation is strongly inhibited, by the CTD kinase inhibitor H8.

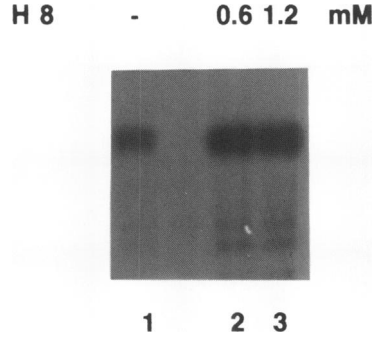


**Figure 4.** H8 does not inhibit Gal-VP16 activated transcription. Transcription was done as in Figure 2 without H8 (lane 1), or with the indicated concentrations of H8.

The results also addressed transcription from the activator-dependent test promoter and showed that RNA synthesis can also proceed in the absence of CTD phosphorylation, using the CTD kinase inhibitor H8. This result is consistent with two previous reports in partially purified basal transcription systems, both of which also relied on the properties of H8 (36,37). Factors have been isolated that induce inhibition of basal yeast transcription in confunction with the CTD phosphorylation inhibitor H8. Those proteins are likely to be present in crude Hela extracts, as we have observed that H8 can inhibit extract-directed transcription. However, these proteins, which are not necessary for transcription, are likely to be absent from our purified system, which is not inhibited by H8. These considerations suggest that both basal and activated transcription can proceed without CTD phosphorylation, but that factors exist that can impose a requirement for CTD phosphorylation. Thus, in the most highly purified transcription systems, both basal and activated, CTD phosphorylation is not required for transcription. Nonetheless, several lines of evidence indicate that the CTD itself is used in transcription. First, basal transcription by this liver polymerase is blocked by treatment with an anti-CTD antibody (36). Secondly, GAL4-VP16 dependent transcription in yeast requires an intact CTD (21). Thirdly, as discussed in the Results, the transcription stimulatory effect of kinase inhibitor H8 in this study is best understood as a natural consequence of the use of polymerase containing a CTD. Fourthly, factors can be isolated that impose a CTD phosphorylation requirement on basal yeast transcription (37).

Thus the existing data suggest that CTD phosphorylation is a normal part of the transcription pathway but not always an essential part. Because there is a considerable literature concerning the possible functional role of polymerase CTD phosphorylation, especially in activated transcription (see Introduction), these results need to be reconciled with previous studies. The CTD is known to be functionally important (see reviews 8,9). Moreover, the CTD has been shown to be critical in mediating the response to the very same activator studied here, GAL4-VP16 (21). The CTD binds certain transcription factors in its non-phosphorylated form, which could account for its role in assisting transcription (see for example 25,26). The observed lack of inhibition of transcription and open complex formation by H8 is in fact consistent with all of these prior observations. That is, the data do not imply that the CTD is unimportant, only that its phosphorylation is not essential for open complex formation or for transcription in highly purified systems.

Because our experiments demonstrate that it is possible to achieve activated transcription without CTD phosphorylation, situations *in vivo* where transcription can occur without it. Thus, CTD phosphorylation may function in activated transcription in a situation-dependent manner.

## ACKNOWLEDGEMENTS

We are very grateful to Ron and Joan Conaway for their generous gifts of material and advice. We also thank Drs Hiroaki Serizawa and Teijiro Aso for their help. This work was supported by a grant from the NIH (GM49048).

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