The Sensitivity of RNA Polymerase II in Elongation Complexes to C-terminal Domain Phosphatase*

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The phosphorylation state of the carboxyl-terminal domain (CTD) of the largest RNA polymerase (RNAP) II subunit plays an important role in the regulation of transcript elongation. This report examines the sensitivity of RNAP II to dephosphorylation by CTD phosphatase (CTDP) and addresses factors that regulate its sensitivity. The CTDP sensitivity of RNAP IIO in paused elongation complexes on a d-tailed template does not significantly differ from that of free RNAP IIO. RNAP IIO contained in elongation complexes that initiate transcription from the adenovirus-2 major late promoter in the presence of a nuclear extract is relatively resistant to dephosphorylation. Complexes treated with 1% Sarkosyl remain elongation-competent but demonstrate a 5-fold increase in CTDP sensitivity. Furthermore, the sensitivity of RNAP IIO in both control and Sarkosyl-treated elongation complexes is dependent on their position relative to the start site of transcription. Elongation complexes 11–24 nucleotides downstream are more sensitive to dephosphorylation than complexes 50–150 nucleotides downstream. The incubation of Sarkosyl-treated elongation complexes with nuclear extract restores the original resistance to dephosphorylation. These results suggest that a conformational change occurs in RNAP II as it clear the promoter, which results in an increased resistance to dephosphorylation. Furthermore, the sensitivity to dephosphorylation can be modulated by a factor(s) present in the nuclear extract.

RNA polymerase (RNAP) II is a large multisubunit enzyme responsible for catalyzing the transcription of protein coding genes in eukaryotes. The largest subunit of RNAP II contains at its C terminus a unique and highly conserved domain composed of tandem repeats of the consensus sequence YSPTSPS (for a review, see Ref. 1). Genetic analysis of the C-terminal domain (CTD) has established that it is essential for viability (for a review, see Ref. 1). The initial phosphorylation of the CTD results in a disruption of the protein-protein interaction that initially brought RNAP IIA to the preinitiation complex remains an attractive but unproven hypothesis. Transcription elongation is catalyzed by RNAP IIO. Completion of the transcription cycle is dependent on the dephosphorylation of RNAP IIO, a reaction that may be coupled to transcript termination. CTD phosphatase (CTDP) has been characterized in yeast and mammalian cells (10–16). Although active in the dephosphorylation of free RNAP IIO, its activity with respect to dephosphorylating RNAP IIO in an elongation complex has not been studied in detail. A recent report by Cho et al. (15) demonstrates that, when transcription is initiated in a defined system, the ternary elongation complex can be dephosphorylated by CTD phosphatase.

Increasing evidence suggests that an interplay of positive and negative factors regulate transcript elongation. Although the role of the CTD in elongation remains unclear, the CTD appears to be the regulatory focus of many protein factors. Distinct structural changes occur in early elongating RNAP II between +25 and +40 (17, 18). Furthermore, in this same region, the elongation factor P-TEFb can phosphorylate the CTD, resulting in a stabilization of the elongation complex (19). This reaction is inhibited by the nucleotide analogue DRB. The failure of P-TEFb to act can lead to abortive transcription. In addition to P-TEFb, several negative factors coordinately regulate the transition from abortive to productive elongation. DSIF was initially characterized as a protein factor required to repress transcription and antagonizes the positive action of P-TEFb (21). The negative effect of DSIF depends on the state of CTD phosphorylation. Prior phosphorylation of the CTD by TFIIH or P-TEFb should result in transcription complexes that are resistant to the effects of DSIF (20, 22). Interestingly, both NELF and DSIF are required to repress transcript elongation, although neither functions to repress transcription by RNAP IIO. This indicates that if RNAP becomes dephosphorylated during the course of transcription, DSIF and NELF can repress transcription (21).

Recent results suggest that CTDP can play a direct role in the regulation of transcript elongation. The human immunodeficiency virus type 1 transcriptional activator, Tat, interacts with and inhibits the activity of CTD phosphatase (23). Conversely, P-TEFb is recruited by Tat (24–27). Accordingly, the presence of Tat leads to a high level of CTD phosphorylation,
resulting in a highly processive RNAP II and the efficient expression of the viral genome. These results indicate that the elongation efficiency of RNAP II is regulated at least in part by protein kinases and phosphatase(s) that establish the level of CTD phosphorylation. The objective of these studies is to gain insights into the factors that regulate the sensitivity of RNAP IIO in elongation complexes to dephosphorylation. The results presented suggest that RNAP IIO in early elongation complexes is more sensitive to dephosphorylation than is RNAP IIO in elongation complexes that have cleared the promoter. Resistance to dephosphorylation appears to involve both a conformational change in RNAP II and the association of a specific factor(s).

**EXPERIMENTAL PROCEDURES**

**Buffers**

Buffer A contained 50 mM Tris-HCl, pH 7.9, and 0.1 mM EDTA. Buffer B contained 50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 5 mM MgCl₂, 0.5 mM dithiothreitol, 20 mM KCl, 0.025% Tween 80, and 20% glycerol. Buffer C contained 50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 5 mM MgCl₂, 0.5 mM dithiothreitol, 20% glycerol, and KCl as indicated. Buffer D contained 25 mM Tris, pH 7.9, 5 mM MgCl₂, 0.5 mM dithiothreitol, 0.025% Tween 80, 20% glycerol, and KCl as indicated.

**Purification and Labeling of RNAPs IIA and IIO**

RNAP IIA was purified from calf thymus by the method of Hobsley and Blatti (28) with the modifications described by Kang and Dahmus (29). RNAP IIA was labeled with [32P]P as described in Chambers et al. (13). Casein kinase II was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). [32P]Labeled RNAP IIO for dC-tailed transcription and control CTDP reactions was prepared in vitro by the phosphorylation of [32P]labeled RNAP IIA as described by Marshall and Dahmus (23).

**Preparation of DNA Templates**

To produce the DNA templates, 0.5 µg of pUC HTXB (30) was subjected to 30 rounds of polymerase chain reaction with 2 µM concentrations of the following primers: forward 5′-TTCCCAAGTCACGACGT-TGTA-3′ and reverse 5′-CACAGGAAACACGATAGCC-3′. The reaction buffer was 20 mM Tris, pH 7.9, 50 mM KCl, 1.5 mM MgCl₂, 200 µM dATP, dCTP, dGTP, and dTTP in a 2-ml reaction diluted into 100 µl aliquots. This results in a template DNA 94% base pairs in length that will give a promoter-dependent run-off transcript of 622 nucleotides. The polymerase chain reaction was loaded onto a 1-ml MonoQ column (Amersham Pharmacia Biotech) and eluted with a 20-ml linear gradient of 0.1–1 mM NaCl in buffer A. The HTXB DNA fragment elutes at ~0.75 mM NaCl. Promoter-independent transcription on dC-tailed templates was performed using templates produced with a biotinylated reverse primer. Promoter-dependent transcription was carried out on templates biotinylated on the forward primer.

Promoter-independent DNA templates (dC-tailed) were generated from 5 µg of HTXB. The template was cut with SaccI (Life Technologies, Inc.) following the manufacturer’s instructions, after which the DNA was phenol/chloroform- and chloroform-extracted, and ethanol-precipitated. After resuspension in buffer A, a tailing reaction was assembled with 38 units of terminal deoxynucleotide transferase (Promega) and 1 mM dCTP (Amersham Pharmacia Biotech) following the manufacturer’s instructions in a reaction volume of 100 µl. After 15 min of incubation (sufficient to add 60–90 dCMPs to the 3′-OH generated by SaccI), the labeled DNA reaction was adjusted to 750 mM NaCl and bound to Dynabeads (Dynal) as described by the manufacturer. 4.2 µg of DNA was added per 100 µl of Dynabeads previously equilibrated in buffer A. The reaction was incubated at 22 °C for 60 min in buffer A containing 750 mM NaCl while turning on a rotator. Dynabeads were washed free of unbound DNA with buffer A and resuspended at a DNA concentration of 20 ng/µl in buffer A. About 95% of input DNA was bound to the beads.

**Preparation of Transcription Extracts**

Transcription extracts were purified as described by Laybourn and Dahmus (31) with the following changes. HiTrap heparin (Amersham Pharmacia Biotech) was substituted for heparin-Sepharose, and the primary transcription extract (DE0.25) and RNA polymerase II (DE0.6) containing fractions were eluted from the DEAE-5PW (Waters) with 0.25 and 0.6 mM KCl, respectively. TFIIA does not bind to the heparin column (HS0.24 fraction) and was added back separately from the other general transcription factors found in the DE0.25 fraction.

**In Vitro Transcription**

**dC-tailed Templates**—Standard dC-tailed transcription reactions were initiated by the addition of 0.25 pmol of [32P]labeled RNA polymerase IIO in ~2 µl of buffer D containing ~500 mM KCl to 100 ng of dC-tailed HTXB DNA immobilized on Dynabeads (as described above) in the presence of 0.5 µg/ml BSA in buffer C containing no MgCl₂. Final reaction conditions prior to the addition of nucleotides were equivalent to buffer C containing 56 mM KCl, 0.5 µg/ml BSA, and no MgCl₂ in a 20-µl reaction volume. After incubation at 30 °C for 30 min, ATP, UTP, and GTP were added to 0.6 mM, and CTP and MgCl₂ were added to 10 µM and 6 mM, respectively. The final reaction volume was 25 µl. Five µCi of [-α-32P]CTP (Amersham Pharmacia Biotech) was included in reactions carried out to determine the size distribution of RNA transcripts and to quantify their amount. RNAP IIO was allowed to elongate for 10 min and then washed free of nucleotides with buffer C containing 50 mM KCl. If digestion of nascent RNA was required, 1 unit of RNase H (Life Technologies, Inc.) was added, and reactions were incubated for 10 min at 37 °C. RNA products or RNAP II was visualized as described below.

**Ad2-MLP Pulse Elongation Complexes**—Preinitiation complexes were formed in a standard reaction by the addition of 1 µl of HisTrap heparin flow-through in buffer C with 100 mM KCl (contains TFIIA), 0.5 µl of DE0.25 in buffer C with 50 mM KCl (contains remaining general transcription factors), 0.25 pmol of [32P]RNAP IIA in ~1 µl of buffer D containing ~500 mM KCl, and 100 ng of template DNA conjugated with Dynabeads. The reaction was adjusted to 56 mM KCl in a final volume of 20 µl in buffer C prior to incubation for 30 min at 30 °C. After incubation, 250 mM ATP and 250 mM KCl were added for a total reaction volume of 25 µl. Five µCi of [-α-32P]CTP was added when radiolabeled RNA was to be analyzed. Complete reactions were incubated for 2 min at 30 °C. Transcription was stopped as specified in the figure legends either by the addition of 15 mM EDTA or by twice washing the transcription complexes to remove free nucleotides.

**Ad2-MLP Chase Elongation Complexes**—Beginning with washed pulse complexes in a 20-µl volume, chase elongation complexes were formed by the addition of ATP, UTP, and GTP to 0.6 mM and CTP to 10 µM in buffer C with 200 mM KCl and 0.5 mM Mg/mL BSA in a final volume of 25 µl. Complexes were allowed to elongate for 1 min at 30 °C before being washed in buffer C with 50 mM KCl. Radiolabeled RNAP II in the transcription complexes was visualized by SDS-PAGE carried out according to the method of Laemmli (32) with a 5% polyacrylamide resolving gel. Radiolabeled RNA transcripts were analyzed by urea-PAGE as described by Chesnut et al. (5) utilizing 12.5% polyacrylamide. Specific RNA transcripts were quantitated on a Fuji phosphor imager by comparing radioactive signal intensity of transcript bands to known amounts of [-α-32P]CTP standard.

**CTDP Assays**

The sensitivity of RNAP IIO in elongation complexes was determined by a modification of the assay described by Chambers et al. (13). Unless otherwise specified, RAP74 was included in all CTDP assays. Input radiolabeled RNAP IIO in transcription complexes on immobilized DNA was washed twice in buffer B. CTDP was purified as described previously (23). Assays were quantitated on a Fuji phosphor imager by comparing radioactive signal intensity of transcript bands to known amounts of [-α-32P]CTP standard.

**RESULTS**

The CTD Phosphatase Sensitivity of RNAP IIO in Elongation Complexes on a dC-tailed Template Is Comparable with That of Free RNAP IIO—To establish a base line for the sensitivity of RNAP IIO, purified [32P]labeled RNAP IIO was incubated with increasing concentrations of CTD phosphatase (Fig. 1A, lanes 1–5). The amount of subunits IIA and IIB were quantitated from phosphor imager scans and plotted against mU of CTD phos-
phatase (Fig. 1B, left panel). The 50% conversion of RNAP IIO to IIA occurs at 9.5 mU of CTD phosphatase. Transcription was then initiated on a dC-tailed template immobilized on paramagnetic beads, and the CTD phosphatase sensitivity of RNAP IIO in elongation complexes was established. Analysis of RNA transcripts indicates that at 10 min RNAP IIO is distributed from about 150 to 650 nucleotides downstream from the site of initiation (Fig. 1C). The CTD phosphatase sensitivity of RNAP IIO in these elongation complexes does not differ appreciably from that of free RNAP IIO (Fig. 1A, compare lanes 1–5 with lanes 11–15, and in Fig. 1B, compare left and right panels). Accordingly, the formation of an elongation complex per se does not result in protection of the CTD from dephosphorylation. Furthermore, the addition of free DNA and paramagnetic beads does not appreciably alter the sensitivity of RNAP IIO (Fig. 1, A and B, lanes 6–10 and center panel, respectively).

**FIG. 1.** CTD phosphatase sensitivity of free RNAP IIO and elongating complexes on dC-tailed templates. A, increasing amounts of CTD phosphatase were incubated with RNAP IIO, and the resultant products were analyzed as described under “Experimental Procedures.” Lane 0 is a 10-fmol RNAP II reference standard of RNAP IIO. All CTD phosphatase reactions contained 16 pmol of RAP74 and either 0, 0.4, 4, 40, or 400 mU of CTD phosphatase as indicated. To establish the sensitivity of free RNAP IIO, 60 fmol of 32P-labeled RNAP IIO was incubated with increasing amounts of CTD phosphatase as indicated and resolved by 5% SDS-PAGE (lanes 1–5). Lanes 6–10 are identical to lanes 1–5 except that reactions also contained 25 ng of dC-tailed template DNA bound to Dynabeads. Lanes 11–15 contain paused elongation complexes on a dC-tailed template obtained as described under “Experimental Procedures” and incubated with CTD phosphatase. Reactions in lanes 1–10 and 16–17 were terminated by the addition of Laemmli buffer. Reactions on a dC-tailed template (lanes 11–15) were washed once to remove RNAP II that dissociated from the template during the CTD phosphatase reaction and were then terminated by the addition of Laemmli buffer. B, quantitation of the amount of subunits IIO and IIA, as well as the intermediate region (int) as described under “Experimental Procedures.” The results are graphed as percentage of each of IIO, int, and IIA against mU of CTD phosphatase. Numbers on the x axis above the graphs refer to the reaction in A from which the data were obtained (lane number). C, dC-tailed template transcription reactions were assembled as described under “Experimental Procedures.” After the addition of the nucleotide mix containing 5 μCi of [α-32P]CTP, aliquots were removed and stopped at 0, 2, 5, 10, and 15 min, extracted, precipitated, and resolved on 12.5% urea-PAGE.
Initiate Transcription on the Ad2-MLP in the Presence of a Nuclear Extract—Promoter-dependent transcription was carried out utilizing a DNA fragment containing the Ad2-MLP immobilized on Dynabeads. Preinitiation complexes were formed with 32P-labeled RNAII in the presence of a partially fractionated nuclear extract free of endogenous RNAII. ATP, CTP, and UTP were added, and the reaction was incubated for 2 min to allow the production of short transcripts. Although the first G is at position 11, the predominant product is a transcript produced relative to the amount of RNAII bound under these conditions produces a transcript.

The washed RNAII complexes were incubated with increasing amounts of CTD, in the presence of 35 pmol of RAP74. At the end of the CTD reaction, the complexes were washed, resulting in two populations of RNAII. Both populations were resolved by SDS-PAGE, and the sensitivity of the bound population was determined by quantitation of subunits IIo and IIA (Fig. 2, lane 3). The bound fraction (Fig. 2) is IIa (lane 0), the major fraction of RNAII associated with the DNA has been converted to RNAII (lane 1). Quantitation of the amount of transcript produced relative to the amount of RNAII bound indicates that 12–50% of RNAII bound under these conditions produces a transcript.

The washed RNAII complexes were incubated with increasing amounts of CTD, in the presence of 35 pmol of RAP74. At the end of the CTD reaction, the complexes were washed, resulting in two populations of RNAII. Both populations were resolved by SDS-PAGE, and the sensitivity of the bound population was determined by quantitation of subunits IIo and IIA (Fig. 2, lane 3). The bound fraction (Fig. 2, even lanes 2–10) remains attached to the template DNA while the wash fraction (odd lanes 3–11) is released. Only RNAII IIA is found in the wash fraction even in the absence of CTD (Fig. 2A, lane 3). Although some dephosphorylation is seen, complete dephosphorylation is not obtained with the highest concentrations of CTD tested under these conditions (Fig. 2A, lane 8). As a control, the reaction containing 400 mU of CTD was run in duplicate in the absence (lanes 8 and 9) and presence (lanes 10 and 11) of exogenous 32P-RNAII. The finding that free RNAII is not protected from dephosphorylation, as indicated by the absence of a subunit IIo band in the wash fraction (lane 11), indicates that protection is not conferred by a transacting factor. These results suggest that RNAII in complexes formed in the presence of a nuclear extract is about 50-fold more resistant to dephosphorylation than complexes formed on tagged templates (Table I). However, the interpretation of these results is complicated by the fact that the substrate for dephosphorylation is a mixed population of RNAII, only some of which is in functional elongation complexes.

**Sarkosyl Treatment Removes Nontranscribing RNAII**—A characterization of the CTD phosphatase sensitivity of RNAII in elongation complexes is dependent on the analysis of a homogenous population of functional complexes. This is assured only if the molar amount of transcript produced equals the molar amount of RNAII in complex. In an effort to remove nonproductively bound RNAII, complexes were treated with Sarkosyl. Transcription was initiated on immobilized DNA in the presence of an RNAII-depleted nuclear extract supplemented with 32P-labeled RNAII. Furthermore, in reactions to determine the size distribution of RNA and to quantify the amount of transcript produced, nascent transcripts were labeled by a 2-min incubation in the presence of [α-32P]CTP, ATP, and UTP. Pulsed complexes were washed with buffer C or 1% Sarkosyl in buffer C. The reaction scheme is shown in Fig. 3A. One pair of samples was assayed immediately following the pulse (Fig. 3, A–C, lanes 1 and 2 and lanes 5 and 6), whereas a second pair of samples was assayed after a 1-min chase in the presence of ATP, CTP, GTP, and UTP (Fig. 3, A–C, lanes 3 and 4 and lanes 7 and 8). To serve as a reference standard in the quantitation of RNAII, an aliquot containing 10 fmol of RNAII was removed prior to the first wash step and run on SDS-PAGE (Fig. 3, A and C, lane 0).

The size distribution of transcripts produced under pulse and chase conditions is shown in Fig. 3B. Transcripts up to 24 nucleotides in length are observed in pulse conditions. Treatment of complexes with 1% Sarkosyl does not appreciably change either the amount or distribution of bound nascent transcripts (Fig. 3B, compare lanes 1 and 5). Although slight variations in the relative amount of specific transcripts between control and Sarkosyl-treated complexes are sometimes observed, no consistent pattern has emerged. Following a 1-min chase, transcripts range in size from about 50 to 150 nucleotides in length (Fig. 3B, lanes 3 and 7). Furthermore, neither the amount nor distribution of transcripts differs appreciably between control or Sarkosyl-treated complexes. The absence of transcripts in the wash fractions (lanes 2, 4, 6, and...
Fig. 2. CTD phosphatase sensitivity of early elongation complexes initiated from the Ad2-MLP. To provide a reference point for the sensitivity of RNAP IIO in elongation complexes, transcription was initiated in the presence of a nuclear extract on the Ad2-MLP. Early elongation complexes, prepared in the absence of GTP, were purified and assayed for sensitivity to CTDP. A, lane 0 is a 10-fold RNAP II reference standard. Lane 1 is a reaction stopped prior to incubation with CTDP. Lanes 2 and 3 are the bound and wash fractions, respectively, resulting from a 30-min control incubation in buffer B. Lanes 4–9 are identical to lanes 2 and 3 except for the presence of increasing amounts of CTDP as indicated and 35 pmol of RAP74. Lanes 10 and 11 are identical to lanes 8 and 9 except for the inclusion of exogenous radiolabeled RNAP IIO as a control for CTDP activity. Lanes 12 and 13 are purified reference RNAP IIO incubated in the absence and presence of CTDP as indicated. B, quantitation of the amount of subunits IIo and IIa, as well as the intermediate region (int) as described under “Experimental Procedures.” Numbers above each graph correspond to the lane in A from which each set of numbers was obtained.

8) indicate that elongation complexes are stable under the conditions of the experiment. Furthermore, essentially all complexes passed after the initial labeling period are efficiently chased into longer transcripts (Fig. 3B, compare lanes 1 and 3, and compare lanes 5 and 7).

The amount and state of phosphorylation of the largest RNAP II subunit associated with the various elongation complexes is shown in Fig. 3C. Comparison of the subunit IIo bands retained after the first wash reveals that substantially less RNAP remains attached to the template DNA after treatment with Sarkosyl (Fig. 3C, compare lanes 1 and 5). Although treatment of pulse complexes with Sarkosyl removes a major fraction of the bound RNAP II, it does not significantly alter the amount of bound transcript (Fig. 3B, compare lanes 1 and 5). Following the pulse step, paused elongation complexes were chased by the addition of NTPs and 200 mM KCl. Chase conditions result in a substantial loss of RNAP IIO from control complexes (Fig. 3C, compare lane 1 with lanes 3 and 4) and no significant change in Sarkosyl-treated complexes (Fig. 3C, compare lane 5 with lanes 7 and 8). Quantitation of bound subunit IIo and transcript produced reveals that, in contrast to the control pulse reactions, approximately equimolar amounts of transcript and RNAP II are present in chase complexes (Fig. 3D). The observation that the chase step in control reactions results in the dissociation of nontranscribing RNAP II can be attributed to nucleotide and KCl destabilization of RNAP in the nonfunctional preinitiation complexes (34, 35).

Quantitation of the molar amount of RNAP II and RNA transcript was carried out as described under “Experimental Procedures.” Values in Fig. 3D are averages of three independent data sets. Although under pulse conditions with control complexes there is a molar excess of RNAP II, chase control complexes and both pulse and chase complexes treated with 1% Sarkosyl contain equimolar amounts of RNAP II and nascent transcript. The fact that Sarkosyl-treated pulse complexes contain equimolar amounts of transcript and RNAP II indicates that Sarkosyl efficiently and selectively dissociates nontranscribing RNAP II.

These results demonstrate that stoichiometric amounts of RNAP and transcript exist in pulse and chase complexes that have been treated with 1% Sarkosyl and that control chase complexes contain equimolar amounts of RNAP II and transcript. These complexes are ideally suited to assess the sensitivity of elongation-competent RNAP II to dephosphorylation by CTDP.

CTD Phosphatase Sensitivity Is Influenced by both the Position of the Elongation Complex and Sarkosyl Treatment—The objectives of experiments described in this section are 2-fold: first, to examine the CTD phosphatase sensitivity of RNAP IIO as a function of its position downstream of the transcriptional start site, i.e. promoter proximal (pulse) and distal (chase) elongation complexes; second, to examine the effect of Sarkosyl treatment on CTDP sensitivity. Elongation complexes were prepared as described above and incubated with increasing concentrations of CTD phosphatase. The experimental protocol is shown schematically in Fig. 4A. Although transcription was initiated by the addition of 32P-labeled RNAP IIA, transcripts were not labeled. The sensitivity of pulse and chase elongation complexes under both control and Sarkosyl wash conditions was examined. The distribution of subunits IIo and IIa is shown in Fig. 4B, whereas the quantitation is shown in Fig. 4C. The amount of CTD phosphatase required for 50% conversion of RNAP IIO to IIA differs for each complex and is shown in Table I. Pulse control complexes are more sensitive than chase complexes (Fig. 4C, compare upper left and right panels). Similarly, Sarkosyl pulse complexes are more sensitive than chase complexes (Fig. 4C, compare lower left and right panels). Accordingly, under both sets of conditions, RNAP IIO in elonga-
FIG. 3. Effect of Sarkosyl on transcript elongation and the molar ratio of RNAP IIO and transcript in purified elongation complexes. The stoichiometry of RNA transcript to RNAP II was quantitatively and qualitatively analyzed in a promoter-dependent assay. Reactions were carried out with 32P-labeled RNAP IIA utilizing a HeLa cell transcription extract depleted of RNAP II. Transcription was initiated on the Ad2-ML promoter in the presence of either [α-32P]CTP (B) or unlabeled CTP (C) in the absence of GTP (pulse conditions) and stopped by the addition of EDTA to 15 mM. Certain reactions were incubated further for 1 min in the presence of NTPs and 200 mM KCl (chase conditions). A, a schematic representation of the experiment. Filled circles, control wash steps; unfilled circles with an S, Sarkosyl wash steps. Reactions were assembled and preincubated for 30 min followed by a 2-min pulse and in some cases a 1-min chase as described under “Experimental Procedures.” Reactions indicated with lane numbers 1–8 refer to the corresponding lanes in the transcription and protein gels in B and C, respectively. Lane 0 refers to C. B, 12.5% urea-PAGE of RNA transcripts produced under different reaction conditions (4 × standard reaction). Transcripts were labeled with [α-32P]CTP as described above. The left two lanes correspond to reactions carried out in the absence of RNAP IIA. The right lane, designated M, is a DNA size marker generated by digestion of pBR322 with MspI. C, the distribution of RNAPs IIO and IIA corresponding to the transcription reactions in B. Lanes 1–8 in B and C correspond to equivalent points in the transcription reaction. Lane 0 is a 10-fmol RNAP II reference standard. Lanes 1 and 2 are the bound and first wash fractions from a control pulse reaction. Only 5% of the total wash fraction was loaded in lanes 2 and 6. Lanes 3 and 4 are the corresponding chase fractions. Lanes 5–8 are the corresponding 1% Sarkosyl pulse and chase fractions. D, a graphical representation of the calculated fmol of RNAP II and transcript derived from the bound lanes in B and C. Each pair of bars is derived from equivalent portions of the urea-PAGE and SDS-polyacrylamide gels and quantitated as described under “Experimental Procedures.” The numbers are the average of three independent experiments, and the error bars represent one S.D. nt, nucleotides.
tion complexes at positions 50–150 nucleotides downstream from the start site is more resistant to dephosphorylation than is RNAP IIO at positions 11–24. Although it is difficult to quantify the magnitude of the difference in control complexes given the extreme resistance of RNAP IIO, the difference in sensitivity between the Sarkosyl-treated complexes is about 8-fold.

Given that only a fraction of RNAP IIO in control pulse complexes is in functional elongation complexes, it is also difficult to directly compare the sensitivity of these complexes to control downstream complexes or Sarkosyl-treated complexes at the same position. Despite this qualification, it is clear that Sarkosyl treatment dramatically increases the sensitivity of RNAP IIO contained in elongation complexes (Fig. 4C, compare upper and lower panels). The sensitivity of RNAP IIO in promoter-proximal complexes treated with 1% Sarkosyl approaches that of RNAP IIO in elongation complexes initiated on dC-tailed templates (Table I, also compare right panel of Fig. 1C with lower left panel of Fig. 4C).

**CTD Phosphatase Sensitivity of Elongation Complexes**

The finding that elongation complexes washed with 1% Sarkosyl exhibit an increased sensitivity to CTDP suggests that Sarkosyl either dissociates a factor(s) that contributes to resistance or induces a conformational change that results in increased sensitivity. To test this hypothesis, pulsed complexes were washed with 1% Sarkosyl and then incubated for 10 min with transcription extract or one of several different protein fractions. After incubation with transcription extract, elongation complexes were washed into buffer B and assayed in a stand-
ard CTDP reaction. The reaction scheme is shown in Fig. 5A. The presence of increasing amounts of transcription extract results in increased protection of the CTD to dephosphorylation by CTDP (Fig. 5, B and C, compare lanes 3–6). The addition of BSA at a 33-fold higher concentration did not affect phosphatase sensitivity (Fig. 5, B and C, lanes 7 and 8). Similarly, the addition of the HS0.24 fraction containing TFIIA, which was present in the original reaction, had no effect on CTDP sensitivity although it was present at a 4-fold higher concentration than in transcription reactions (Fig. 5, B and C, compare lanes 6 and 10). These results suggest that the sensitivity of RNAP IIO in elongation complexes is determined in part by a specific factor(s) present in the transcription extract.

RAP74 Stimulates the Dephosphorylation of RNAP IIO in Elongation Complexes—The RAP74 subunit of the general transcription factor TFIIJ stimulates the dephosphorylation of free RNAP IIO by CTDP (13). Since TFIIJ influences both the initiation and elongation phase of transcription (36–39), it was of interest to determine if TFIIJ also stimulates the dephosphorylation of RNAP IIO in elongation complexes. To assess the effect of RAP74 on dephosphorylation, the sensitivity of pulse Sarkosyl-treated elongation complexes was determined in the absence (Fig. 6A, lanes 1–4) or presence (lanes 5–8) of RAP74. The major fraction of subunit IIO was converted to subunit IIA in the presence of 40 mU of CTDP and RAP74, whereas in the absence of RAP74, very little dephosphorylation was observed even in the presence of 400 mU CTDP (Fig. 6A, compare lanes 4 and 7). RAP74 also stimulates the dephosphorylation of pulse control complexes (data not shown).

DISCUSSION

The first indication that RNAP IIO was involved in elongation came from experiments in which nascent transcripts were shown to cross-link to subunit IIO but not subunit IIA (3, 40). Furthermore, the finding that no transcripts were cross-linked to subunits with an electrophoretic mobility between that of subunit IIO and IIA indicates that the elongating enzyme is fully phosphorylated. The simplest interpretation of these results is that the CTD is fully phosphorylated by TFIIH at the time of initiation and that there is no turnover of CTD phosphate during transcript elongation. However, the finding that the synthesis of long transcripts in vitro is inhibited by the nucleotide analogue DRB suggested that a protein kinase might play an important role in processive transcript elongation (41, 42). These studies resulted in the identification of P-TEFb, which was subsequently shown to be a CTD kinase (19). These results are consistent with a model in which TFIIH catalyzes CTDP phosphorylation during the initiation phase of transcription, whereas P-TEFb catalyzes the phosphorylation of RNAP II in elongation complexes. It is not presently possible to distinguish between models in which RNAP II cleared the promoter before it was fully phosphorylated as opposed to being dephosphorylated during early stages of transcript elongation. For this reason, as well as those noted below, it is important to understand the parameters that govern the sensitivity of RNAP IIO in elongation complexes to dephosphorylation by CTDP phosphatase.

Results presented here indicate that the sensitivity of RNAP
IIO to dephosphorylation can be dramatically influenced by its assembly into an elongation complex. The observation that RNAP IIO that initiates transcription on a dC-tailed template has a sensitivity comparable with that of free RNAP IIO indicates the CTD is readily accessible to dephosphorylation in elongation complexes that are free of other factors. Early elongation complexes that have initiated transcription on the Ad2-MLP in the presence of a nuclear extract and have been treated with 1% Sarkosyl also have a sensitivity comparable with that of free RNAP IIO. However, as RNAP IIO clears the promoter it becomes more resistant to dephosphorylation. This is supported by the observation that the resistance to dephosphorylation of Sarkosyl-treated elongation complexes increases 8-fold as RNAP IIO moves from positions +11–24 to positions +50–150. Since both promoter proximal and distal complexes have been treated with 1% Sarkosyl, this change in sensitivity is most likely the result of a conformational change as opposed to the association of specific proteins.

RNAP IIO in elongation complexes on the Ad2-ML template that have been purified in the absence of Sarkosyl is highly resistant to dephosphorylation. It is difficult to compare directly the sensitivity of control and Sarkosyl-treated early elongation complexes, because in the absence of Sarkosyl, a significant fraction of RNAP IIO bound to the template is not in functional elongation complexes. Accordingly, the sensitivity determined is an average value of different complexes and is not necessarily an accurate reflection of the sensitivity of early elongation complexes. However, the chase conditions for the production of promoter distal elongation complexes result in the dissociation of nonproductively bound RNAP IIO. Accordingly, the sensitivity of RNAP IIO at positions +50–150 downstream from the start site can be accurately assessed. Control downstream complexes are at least 5-fold more resistant to dephosphorylation than complexes at the same position treated with 1% Sarkosyl. This result suggests that a factor(s) present in the nuclear extract can associate with the elongation complex and confers protection against dephosphorylation. Presumably, the dissociation of this factor(s) by 1% Sarkosyl results in the increase in sensitivity. Alternatively, treatment with Sarkosyl may induce a conformational change that alters the sensitivity of RNAP IIO. The finding that incubation of Sarkosyl-treated elongation complexes with the nuclear extract can selectively reestablish resistance to CTDP suggests that a factor(s) is present that associates with the elongation complex to modulate the sensitivity of RNAP IIO.

The stabilization of RNAP IIO against dephosphorylation as it clears the promoter is seen in both control and Sarkosyl-treated elongation complexes. The mechanism for this decrease in sensitivity with increasing transcript length is unknown. However, RNAP is known to go through several transitions during the early stages of transcript elongation. Samkurashvili and Luse (17, 18) have described a conformational change that alters the footprint of RNAP II relative to the nucleotide addition site. As the RNA lengthens, the footprint of RNAP II becomes more trailing up to nucleotide +25. Beyond +25, the footprint becomes more centered on the site of nucleotide addition. The pulse (+11–24 nucleotides) and chase (+50–150 nucleotides) reactions bracket this transition zone. Since the decrease in sensitivity of chase complexes occurs in both control and Sarkosyl-washed complexes, it is likely to be the result of a conformational change associated with the early stages of transcript elongation.

A variety of biochemical studies establish that the CTD plays an essential role in co-transcriptional processing of the primary transcript (for a review, see Refs. 43 and 44). Since the phosphorylated CTD appears to play a key role in RNA processing, dephosphorylation of the CTD during the elongation phase of transcription would probably lead to dissociation of the processing machinery. This uncoupling could in principle lead to the arrest of transcription. These studies establish the importance in maintaining a high level of CTD phosphorylation during elongation and add significance to studies on the regulation of CTD phosphatase activity. The observation that RAP74 stimulates the ability of CTDP to dephosphorylate
RNAP IIO in an elongation complex suggests that TFIIF might function as a negative regulator of transcript elongation. This is not unprecedented in that the ability of elongin to stimulate transcript elongation is inhibited by the presence of TFIIF (45).

Recent studies also establish that the level of CTD phosphorylation can be regulated during transcript elongation by a protein factor that regulates both CTD kinase and CTD phosphatase. The observations that the human immunodeficiency virus type 1 transcriptional activator, Tat, is a positive regulator of P-TEFb and a negative regulator of CDTP support the idea that a high level of CTD phosphorylation is essential for processive elongation (23, 24, 46, 47). Furthermore, Tat is known to increase the ratio of RNAP IIO/IIA and activate transcription of the viral genome by increasing the processivity of RNAP II (48–50).

The assembly of preinitiation and elongation complexes in defined systems results in complexes that are sensitive to dephosphorylation by CDTP (15). Although it is difficult to directly compare these results with those presented here, transcription carried out in the presence of purified transcription factors probably results in complexes that are more similar to Sarkosyl-treated complexes than those formed in a nuclear extract.

These results suggest that the sensitivity of RNAP IIO IIO in elongation complexes is influenced by a number of factors. First, there appears to be a conformational change that accompanies promoter clearance and results in the establishment of an elongation complex that is relatively resistant to dephosphorylation. Second, factors in the nuclear extract probably associate with the elongation complex, resulting in an increased stabilization against dephosphorylation. There are several potential mechanisms that could account for the factor-induced protection. These include (a) interference with the association of CDTP with the elongation complex, (b) direct protection by the association of protein with the phosphorylated CTD, and (c) direct inhibition of CDTP activity. CDTP interacts with a docking site on free RNAP IIO that is distinct from the CTD (14). The observation that CDTP does not dephosphorylate either recombinant CDTo or free RNAP II subunit IIO suggests that the association of CDTP with the docking site on the native enzyme is essential. Accordingly, the association of factors with the elongation complex could regulate the accessibility of this site. Second, both the elongator complex (51) and proteins involved in processing of the primary transcript have been shown to directly interact with the phosphorylated CTD (43, 44). The association of these proteins with the CTD could in principle block dephosphorylation although CDTP is able to associate with the elongation complex, resulting in an increase in the activity of CDTP.

The observation that the sensitivity of RNAP IIO in an elongation complex can be influenced by its position relative to the promoter and the association of a factor(s) in the nuclear extract supports the hypothesis that an interplay between positive and negative factors including CTD kinases and CDTP works to regulate the transcription efficiency of RNAP II. It is now important to identify and characterize the factor(s) that influence CTD phosphatase sensitivity and to establish their relationship to known elongation factors.

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The Sensitivity of RNA Polymerase II in Elongation Complexes to C-terminal Domain Phosphatase

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