RNA Polymerases IIA and IIB Have Distinct Roles during Transcription from the TATA-less Murine Dihydrofolate Reductase Promoter*

(Received for publication, May 28, 1993, and in revised form, July 24, 1993)

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The largest subunit of RNA polymerase II (RNAP II) contains a remarkable region of tandem heptapeptide repeats of the consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser at its carboxy terminal. This COOH-terminal domain (CTD) is unphosphorylated in RNAP IIA, extensively phosphorylated in RNAP IIO, and absent in RNAP IIB. The reversible phosphorylation of the CTD has been proposed to be integral to each cycle of transcription from the adenovirus-2 major late promoter. The adenovirus-2 major late promoter, however, may not be a good paradigm for the study of CTD function because in vitro transcription from this promoter is not dependent on the CTD. Previous studies suggest that transcription from the murine dihydrofolate reductase (DHFR) promoter requires the CTD. In an effort to investigate the role of the CTD and its phosphorylation, a RNAP II-dependent reconstituted transcription system specific for the DHFR promoter was established. In this reconstituted system, RNAP IIA, but not RNAP IIB, can transcribe from the DHFR promoter. Furthermore, RNAP IIB does not compete with RNAP IIA for preinitiation complex assembly. These results suggest that the CTD plays a critical role in the recruitment of RNAP II to the DHFR promoter.

The analysis of preinitiation complexes assembled on the DHFR promoter indicates that RNAP IIA readily assembles into functional preinitiation complexes in contrast to the inefficient assembly of RNAP IIO. However, transcript elongation is catalyzed by RNAP IIo as demonstrated by the photoactivated cross-linking of nascent DHFR transcripts to subunit IIo. These results indicate that transcription from the DHFR promoter involves the reversible phosphorylation of the CTD and support the idea that RNAPs IIA and IIO have essential but distinct functions.

RNA polymerase II (RNAP II) is a large, multisubunit enzyme which catalyzes the transcription of protein coding genes in eukaryotic cells. The largest subunit of RNAP II contains a highly conserved heptapeptide sequence, Tyr-Ser-Pro-Thr-Ser-Pro-Ser, at its COOH terminal which is randomly repeated 26, 44, and 52 times in yeast, Drosophila, and mammalian cells, respectively (Allison et al., 1985; Corden et al., 1985). This unusual domain is commonly referred to as the CTD for "COOH-terminal domain" and is found in a heavily phosphorylated form in vivo (Kim and Dahmus, 1986). The form of the enzyme containing the phosphorylated CTD is referred to as RNAP IIO with its largest subunit (IIo) having an apparent molecular weight of 240,000. The form of the enzyme containing the unphosphorylated CTD is referred to as RNAP IIA with its largest subunit (IIa) having an apparent molecular weight of 214,000. A third form of the enzyme, designated RNAP IIB, lacks the CTD and is produced by limited proteolysis during the purification of RNAP II.

Genetic studies in yeast, Drosophila, and mouse have shown that the CTD is essential in vivo (Nonet et al., 1987; Allison et al., 1988; Bartolomei et al., 1988; Zehring et al., 1988). A variety of studies have been carried out in an effort to define the involvement of the CTD in transcription. In yeast, the CTD has been implicated to play a role in transcriptional activation. Truncations of the CTD seriously affect the ability of some upstream activating sequences to mediate the induction of transcription both in vivo and in vitro (Seafe et al., 1990; Liao et al., 1991). Conversely, GAL4 deletions are partially suppressed by the presence of additional repeats within the CTD (Allison and Ingles, 1989). In higher eukaryotes, however, the major late transcription factor and Sp1 are capable of activating transcription from the viral adenovirus-2 major late promoter (Ad-2 MLP) and a chimeric GC box/HSP 70 Drosophila promoter, respectively, in the absence of the CTD (Zehring and Greenleaf, 1990; Buratowski and Sharp, 1990). In addition, the CTD is not required during in vitro transcription from some promoters such as the Ad-2 MLP and Drosophila actin 5C promoter (Kim and Dahmus, 1989; Zehring et al., 1988). Nevertheless, functional interactions, both direct and indirect, have been demonstrated between the CTD and the TATA-binding protein (Conaway et al., 1992; Koleske et al., 1992; Usheva et al., 1992), a negative regulator of transcription encoded by SIN I (Peterson et al., 1991), and the initiator protein HIP1 (Buer-}

*This research was supported in part by United States Public Health Service Predoctoral Grant GM07377 (to M. E. K.) and Grant GM33300 (to M. E. D.) from the National Institute of General Medical Sciences, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: RNAP II, RNA polymerase II; CTD, COOH-terminal domain; Ad-2 MLP, adenovirus-2 major late promoter; DHFR, dihydrofolate reductase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
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II0 by a CTD kinase(s) which is itself stably associated with the preinitiation complex (Payne et al., 1989; Laybourn and Dahmus, 1990; Arias et al., 1991). RNAP II0 then catalyzes elongation of the transcript (Bartholomew et al., 1986; Cadena and Dahmus, 1987; Payne et al., 1989). A CTD phosphatase, whose activity has recently been identified in HeLa extracts, presumably dephosphorylates RNAP II0 after its release from the template to regenerate RNAP II A (Chesnut et al., 1992). RNAP II A can then begin another round of transcription. The observation that CTD phosphorylation occurs at some time during the transition from initiation to elongation suggests that this phosphorylation event could be of regulatory significance.

The RNAP II A/II0 transcription model discussed above is based primarily on studies utilizing the Ad-2 MLP which does not require the CTD for in vitro transcription (Kim and Dahmus, 1989). Consequently, this promoter is not ideally suited for the analysis of CTD function. Previous studies by Thompson et al. (1989) using HeLa nuclear extracts in which the endogenous RNAP II A was inactivated by anti-CTD monoclonal antibodies suggest that the CTD is required for transcription from the murine dihydrofolate reductase (DHFR) promoter. The structure of the DHFR promoter is different from that of the Ad-2 MLP in several important aspects. The DHFR promoter does not contain a TATA box in contrast to the strong consensus TATA sequence present in the Ad-2 MLP. Furthermore, the CTD is required for transcription from the murine DHFR promoter using transcription templates (post-initiation complexes). Therefore, RNAP II A was purified as described in Dahmus and Kedinge (1983), which was also purified on a Gen-Pak FAX column using the same linear gradient as described above. The Ad-2 MLP template gives a 560-nucleotide run-off transcript.

Preparation of Transcription Extract—The S-100 transcription extract was prepared from 2 x 10^{6} HeLa cells by the method of Weil et al. (1979) as modified by Dahmus and Kedinge (1983). The S-100 extract was chromatographed on heparin-Sepharose CL-4B as described by Laybourn and Dahmus (1990), and the transcriptional activity was assayed in the 0.6 M KCl eluted peak (designated HS0.6 extract). The HS0.6 peak fractions were pooled and chromatographed on a DEAE-5PW column as described by Laybourn and Dahmus (1990) with the exception that all of the general transcription factors were step-eluted at 0.25 M KCl. RNAP II A and DE0.25 extracts were dialyzed against the standard transcription reaction mixture.

Purification of RNAP II—RNAP II A and RNAP II B were purified from calf thymus by the method of Hodo and Blatti (1977) with the modifications described in Laybourn and Dahmus (1990). RNAP II A was further purified by chromatography on alkyl-Sepharose using a 15-ml linear gradient of 1.5-0 M (NH_{4})_{2}SO_{4} in Buffer C at a flow rate of 0.2 ml/min. RNAP II B was purified further on Mono Q using a 15-ml linear gradient of 0.24-0.39 M (NH_{4})_{2}SO_{4} in Buffer C at a flow rate of 0.4 ml/min. The RNAP II B fraction used in Fig. 1 was purified on alkyl-Sepharose as described above prior to purification on Mono Q. Each preparation of RNAP II was dialyzed against Buffer D and assayed for promoter-independent transcriptional activity (Kim and Dahmus, 1988).

In Vitro Transcription Reactions—Reconstituted transcription reactions contained either 6 or 12 µl of DE0.25 (as indicated in the figure legends), 30-50 ng of Spl, 2.5-40 milliunits of RNAP II (as indicated), 24 µg Hpes-KOH, pH 7.9, 6 µg MgCl_{2}, 60 µl KCl, 0.12 µl EDTA, 0.3 µM DTT, 0.12 µM phenylmethylsulfonyl fluoride, 12% glycerol, 25 µM [α-32P]ATP (800 000 Ci/mmol) and [γ-32P]GTP (300 000 Ci/mmol) were purchased from American Corp. West germ agglutinin was purchased from Vector Laboratories, Inc. and Sepharose CL-4B was purchased from Sigma. Syn- thetic oligodeoxynucleotides were purchased from University of California Davis Protein Structure Laboratory. The Gen-Pak FAX (0.46 x 10 cm) and Protein-Pak Glass DEAE-5PW (10 cm, 0.8 cm) columns were purchased from Waters Chromatography. The alkyl-Superase HR 5/5 and Mono Q HR 5/5 columns were purchased from Pharmacia.

Buffers—Buffer A contained 20 mM Hpes-KOH, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 20% glycerol. Buffer B contained 25 mM Hpes-KOH, pH 7.5, 12.5 mM MgCl_{2}, 1.0 mM DTT, 10 mM ZnSO_{4}, 0.1% Nonidet P-40, and 20% glycerol. Buffer C contained 50 mM Tris-HCl, pH 7.9, 5 mM EDTA, 0.25 mM DTT, and 15% glycerol. Buffer D contained 50 mM Tris-HCl, pH 7.9, 10 mM MgCl_{2}, 75 mM KCl, 0.1% EDTA, 0.5 mM DTT, 0.2% Tween-80, and 20% glycerol. Buffer E contained 24 mM Hpes-KOH, pH 7.9, 6 mM MgCl_{2}, 60 mM KCl, 0.12 mM EDTA, 0.3 mM DTT, and 12% glycerol.

Preparation of DNA Templates—The plasmid pS5625 containing the murine dihydrofolate reductase gene from positions −356 to +275 (Farnham and Means, 1990) was digested sequentially with Smal and Hind III to produce a 651-base pair fragment. This fragment was purified from plasmid DNA on a Gen-Pak FAX column using a 15-ml linear gradient of 0.60-0.75 M NaCl in 25 mM Tris-HCl, pH 7.5, at a flow rate of 0.5 ml/min. The purified DHFR template gives a run-off transcript of 560 nucleotides. The Ad-2 MLP template (post-initiation complexes) was prepared as described in Dahmus and Kedinge (1983), which was also purified on a Gen-Pak FAX column using the same linear gradient as described above. The Ad-2 MLP template gives a 560-nucleotide run-off transcript.

Experimental Techniques—This paper presents data from experiments using the following techniques: gel filtration-purified preinitiation complexes were assembled in the absence of ribonucleotides in a volume of 20 µl for 45 min at 24 °C. Sarkosyl was then added to a final concentration of 0.08% and the reaction incubated for an additional 15 min at 24 °C. Reactions were stopped with 125 µl of stop solution (100 mM NaOAc, pH 5.2, 0.4% SDS, 0.2 mM Escherichia coli tRNA) and extracted with 150 µl of phenol followed by 150 µl of chloroform:isoamyl alcohol (24:1). The nucleic acids were precipitated from the aqueous phase by the addition of 10 µl of 3 M NaOAc and 600 µg of ethanol and washed with 70% ethanol. The nucleic acids were then purified by gel filtration through a Bio-Gel A15M column and analyzed by electrophoresis on a 4% polyacrylamide, 8 M urea gel which was dried and exposed to x-ray film. The transcript bands were quantitated on a Betascope 603 Biotranalyzer (Betagen) or a BAS1000 phosphorimager (Fuji) as indicated.

Formation and Fractionation of Preinitiation Complexes Containing 32P-Labeled RNAPs IIA and IIO—RNAP II A was labeled with [γ-32P]ATP (as indicated) and [α-32P]CTP (8 Ci/mm) and [γ-32P]GTP (300 C i/mm) were purchased from Amer- sham Corp. West germ agglutinin was purchased from Vector Laborato- ries, Inc. and Sepharose CL-4B was purchased from Sigma. In the transcription experiment shown in Fig. 6, a 147-base pair 32P-labeled DNA fragment was added to the stop solution to serve as an internal control for recovery of 32P-labeled RNA during the extraction steps. The nucleic acids were precipitated from the aqueous phase by the addition of 10 µl of 3 M NaOAc and 600 µg of ethanol. The nucleic acids were then purified by gel filtration through a Bio-Gel A15M column and analyzed as described (Chesnut et al., 1992). To make 32P-labeled RNAP IIO, 32P-labeled RNAP IIA was incubated with excess cold ATP and a partially purified fraction of CTD kinase (Chesnut et al., 1992). Both 32P-labeled RNAP IIA and IIO were purified as previously described (Chesnut et al., 1992), dialyzed into Buffer D and assayed for promoter-independent transcriptional activity (Kim and Dahmus, 1988).
Preinitiation complexes were assembled on the DHFR promoter with $^{32}$P-labeled RNAP IIA, IIO, or a mixture of RNAPs IIA and IIO (milliunits indicated in the figure legends) in a 3 x transcription reaction (total volume was 60 pl) by incubation for 45 min at 24 °C in the absence of ribonucleotides. The complexes were fractionated from unbound RNAP II by gel filtration on a 3.5-ml Sepharose CL4B column equilibrated with Buffer E. Three-drop fractions (150 µl) were collected. One 50-µl aliquot was analyzed directly by electrophoresis in a 2% polyacrylamide-SDS gel which was silver stained (Wray et al., 1981), dried, and exposed to x-ray film. In Fig. 2, a second aliquot was assayed for transcriptional activity as described in the previous section. A third aliquot was assayed for the presence of CTD kinase(s) by incubation with 500 µM ATP, GTP, or GDP. The reaction was incubated for 15 min at 24 °C and analyzed by electrophoresis on a 2% polyacrylamide-SDS gel which was subsequently stained, dried, and exposed to x-ray film.

Photoaffinity Labeling of RNAP II Subunits with $^{32}$P-Labeled RNA—The photoaffinity labeling reagent 4-thio-UTP was synthesized as described by Bartholomew et al. (1986) except for the following modifications. The reaction mixture (5 ml) was incubated for 60 min at 37 °C and loaded directly onto a DEAE-5PW column. The 4-thio-UTP was eluted with a 40-ml linear gradient of 0-0.9 M NH$_4$Ac, pH 8.0, at a flow rate of 0.5 ml/min. Care was taken to minimize the exposure of the reagent to light.

The photoaffinity labeling reactions contained the same final buffer conditions as the transcription reactions described above in a total volume of 25 µl. Preincubation reactions contained 12 µl of DE025, 30-50 ng of Sp1, and 26 milliunits of RNAP IIA or 10 µl of HS0.6 extract. Each reaction containing 80 ng of DHFR template was incubated for 45 min at 24 °C. Ribonucleotides were then added to a final concentration of 5 µM [α-$^{32}$P]CTP (400 Ci/mmol), 2.5 µM GTP, 1.0 µM 3'-O-methyl-GTP, 200 µM 4-thio-UTP, and 200 µM ATP. The complete transcription reactions were incubated for an additional 15 min at 24 °C. The reaction mixture was irradiated with near ultraviolet light as described by Bartholomew et al. (1986) and loaded onto a 1-ml Sepharose CL-4B column equilibrated with Buffer E. Column fractions (50 µl) were treated with DNase I (0.50 unit) for 30 min on ice and analyzed by electrophoresis on a 2% polyacrylamide-SDS gel by silver staining and autoradiography.

RESULTS

The DHFR Promoter Requires the CTD for in Vitro Transcription—In an effort to establish more directly the requirement of the CTD for transcription from the DHFR promoter, a RNAP II-dependent reconstituted transcription system was developed which was specific for the DHFR promoter. The ability of purified RNAP IIA and RNAP IIB to support transcription from the DHFR promoter was examined. The Ad-2 MLP, which also can be transcribed in this reconstituted system, was included in the same reaction to serve as an internal control.

Increasing amounts of either RNAP IIA or RNAP IIB were added to the reconstituted transcription reaction in the presence of the DHFR promoter and the Ad-2 MLP. RNAP IIA was able to transcribe from both promoters (Fig. 1A, lanes 3-6). In contrast, RNAP IIB was able to transcribe from the Ad-2 MLP but was unable to transcribe from the DHFR promoter (lanes 7-10). The amount of RNA transcript synthesized by RNAP IIA and RNAP IIB from each of the DNA templates was quantitated and is shown in Fig. 1B. This result gives clear and direct evidence that in vitro transcription from the DHFR promoter requires the CTD of RNAP II.

RNAP IIA Forms a Stable and Functional Preinitiation Complex on the DHFR Promoter—Using the reconstituted transcription reaction, preinitiation complexes were assembled on the DHFR promoter with $^{32}$P-labeled RNAP IIA in the absence of ATP and fractionated from free RNAP IIA by gel filtration. The distribution of RNAP IIA in column fractions was determined by SDS-PAGE and autoradiography. Preinitiation complexes containing $^{32}$P-labeled RNAP IIA chromatographed in the excluded volume (Fig. 2A, -ATP panel, lanes 2-5) while the uncomplexed RNAP IIA was recovered in the included volume (Fig. 2A, -ATP panel, lanes 6-9) (Laybourn and Dahmus, 1990; Chesnut et al., 1992). To assay for transcriptional activity, the ribonucleotides ATP, GTP, UTP, and [α-$^{32}$P]CTP were added to aliquots of the same fractions shown in the -ATP panel of Fig. 2A. Only the included fractions which contain preinitiation complexes supported transcription from the DHFR promoter (Fig. 2B, lanes 2-3). The included fractions which contain the major portion of RNAP IIA were not transcriptionally active (Fig. 2B, lanes 6-8). These results demonstrate that stable and functional preinitiation complexes containing RNAP IIA as-

Fig. 1. Comparison of the ability of RNAPs IIA and IIB to transcribe from the DHFR promoter and Ad-2 MLP. In vitro transcription reactions using a RNAP II-dependent reconstituted transcription system were performed in the presence of both the DHFR promoter and the Ad-2 MLP in the same reaction. $^{32}$P-Labeled RNA transcripts were purified and analyzed by electrophoresis on a 5% polyacrylamide-8 M urea gel and autoradiography as described under "Experimental Procedures." A, increasing amounts of RNAP IIA (lanes 3-6) or RNAP IIB (lanes 7-10), as indicated, were added to reconstituted transcription reactions containing 12 µl of DE025 extract. Lane 1 contains $^{32}$P-labeled MspI-digested pBR322 DNA. Lane 2 contains the transcription reaction in the absence of RNAP II. The positions of the 560- and 295-nucleotide transcripts from the Ad-2 MLP and DHFR promoter, respectively, are shown on the right. B, the amount of specific transcript from each promoter catalyzed by RNAP IIA (•) and RNAP IIB (○) was quantitated using a Betascope 603 Blot Analyzer and plotted as a function of the amount of RNAP II.
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Fig. 2. Fractionation of DHFR preinitiation complexes containing 32P-labeled RNAP IIA. Preinitiation complexes were assembled on the DHFR promoter in a reconstituted reaction containing 32P-labeled RNAP IIA (90 milliunits) and 36 µl of DE0.25 extract in a total volume of 60 µl and purified by gel filtration on Sepharose CL-4B as described under "Experimental Procedures." A, aliquots of column fractions were analyzed by electrophoresis on a 5% polyacrylamide-SDS gel and autoradiography (-ATP panel, upper left). To other aliquots, ATP, dATP, or GTP was added to a final concentration of 200 µM, and the reactions were incubated for 15 min at 24 °C before loading on a 5% polyacrylamide-SDS gel (+ATP, +dATP, and +GTP panels). The positions of subunits I0 and IIA are shown on the left. The lane numbers are shown on the top of each autoradiogram, while the specific column fractions from the Sepharose CL-4B column are shown at the bottom. B, aliquots of column fractions were assayed for transcriptional activity by the addition of ribonucleotides, the purified transcripts analyzed by electrophoresis on a 5% polyacrylamide, 8 x urea gel, and the gel exposed to x-ray film. The 295-nucleotide DHFR run-off transcript is indicated on the left.

Fig. 3. Experimental scheme for photoaffinity labeling of RNA polymerase. Based on the method of photoaffinity labeling described by Bartholomew et al. (1986), elongation of the DHFR transcript was terminated at various positions. The complexes were irradiated with near ultraviolet light, purified by gel filtration, and analyzed by electrophoresis on a 5% polyacrylamide-SDS gel and autoradiography.

The Unphosphorylated CTD Is Necessary for the Efficient Production of Preinitiation Complexes on the DHFR Promoter
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**Fig. 4. Photoaffinity labeling of transcription complexes.** Elongation complexes were photoaffinity labeled, purified by gel filtration, and analyzed by electrophoresis on a 5% polyacrylamide-SDS gel and autoradiography as described under “Experimental Procedures.” A, fractions from the Sepharose CL-4B column are shown from transcription reactions containing 10 μl of HS0.6 extract. *Lane numbers and column fractions* are as described in the legend to Fig. 2. The positions of RNAP II subunits I10, IIA, IIB, and IIC are shown on the left in the stained gel. *B*, purified RNAP IIA (26 milliunits) was added to a reconstituted transcription system containing 12 μl of DE0.25 extract, and the resulting transcription complexes were irradiated. Fractions from the Sepharose CL-4B column are shown in the complete panel. The same experiment was carried out in the absence of DHFR promoter as well as in the absence of RNAP IIA as shown in the next two panels. The input RNAP IIA is shown on the left in the stained gel. *M* denotes a marker lane containing 32P-labeled RNAP IIA.

**Fig. 5. Comparison of the ability of RNAFs IIA and I10 to assemble into preinitiation complexes on the DHFR promoter.** 32P-Labeled RNAP IIA (24 milliunits, *IIA panel*), 32P-labeled RNAP I10 (24 milliunits, *I10 panel*), and a mixture of 32P-labeled RNAFs IIA and I10 (12 milliunits each, *IIA/I10 panel*) were separately incubated in a reconstituted transcription reaction containing 18 μl of DE0.25 extract in a total reaction volume of 60 μl. The reaction was chromatographed on Sepharose CL-4B, and fractions were collected as described under “Experimental Procedures.” The upper panels are autoradiograms showing the distribution of 32P-labeled RNAP II in column fractions as analyzed by electrophoresis on a 5% polyacrylamide-SDS gel. The positions of subunits I10 and IIA are shown on the left. *Lane numbers and column fractions* are as described in the legend to Fig. 2. *II* and *I10* to the left of the column fractions in each of the three panels denote the reaction input before and after preincubation, respectively. The lower panels show the distribution of 32P-labeled RNAP IIA [square] and RNAP I10 [circle] in column fractions as quantitated by the BAS1000 phosphorimager. The *abscissa* corresponds to the Sepharose CL-4B column fractions; the *ordinate* corresponds to PSL (Photo Stimulated Luminescence) values.

—To establish whether the state of phosphorylation of the CTD affects the ability of RNAP II to assemble into preinitiation complexes on the DHFR promoter, complex formation was carried out in the presence of 32P-labeled RNAFs IIA and I10 separately and in combination. Preinitiation complexes were purified from free RNAP II by gel filtration, and the column fractions were analyzed by SDS-PAGE and autoradiography. Similar to the results presented in Fig. 2, RNAP IIA efficiently assembled into preinitiation complexes on the DHFR promoter as shown by the presence of 32P-labeled RNAP IIA in excluded fractions (Fig. 5, *IIA panel*, lanes 5–6, and graph below). In the reconstituted reaction containing 32P-labeled RNAP I10, only a minor fraction of RNAP I10 assembled into preinitiation complexes as shown by the small amount of 32P-labeled RNAP I10 found in excluded fractions (Fig. 5, *I10 panel*, lane 5). 32P-Labeled RNAP IIA, generated from labeled RNAP I10 by a CTD phosphatase present in the DE0.25 extract, was also detectable in excluded and included fractions (Fig. 5, *IIA panel*, lanes 1, 2, 5, and 11–13, see graph below). The large difference in the efficiency with which RNAP IIA and RNAP I10 assemble into preinitiation complexes is evident in the reconstituted reaction containing equimolar amounts of 32P-labeled RNAFs IIA and I10. A significantly larger fraction of 32P-labeled RNAP IIA assembled into preinitiation complexes as compared to 32P-labeled RNAP I10 (Fig. 5, *IIA/I10 panel*, lanes 5–6, see graph below). These results indicate that phosphorylation of the CTD...
hinders the assembly of RNAP II into preinitiation complexes on the DHFR promoter.

The CTD Is Essential for the Recruitment of RNAP II to the DHFR Promoter—As shown in Fig. 1, RNAP IIB is unable to transcribe from the DHFR promoter. To determine whether the absence of the CTD prevents the assembly of RNAP IIB into preinitiation complexes, transcription was carried out in the presence of increasing amounts of RNAP IIB in reactions which contained a limiting amount of RNAP IIA. If RNAP IIB assembled into nonfunctional preinitiation complexes on the DHFR promoter, then transcription by RNAP IIA should diminish in the presence of increasing amounts of RNAP IIB. Conversely, transcription from the Ad-2 MLP catalyzed by a limiting amount of RNAP IIA should increase in the presence of increasing amounts of RNAP IIB. In the reconstituted reaction used for these experiments, the addition of 10 milliunits of RNAP IIA resulted in the maximal amount of transcription from both the DHFR promoter and the Ad-2 MLP (Fig. 6, A and B, lane 3). The addition of 2.5 milliunits of RNAP IIA gave a level of transcription which was approximately 2- and 4-fold less than the maximal level of transcription obtained from the DHFR promoter and the Ad-2 MLP, respectively (Fig. 6, A and B, lanes 2 and 6). In agreement with the results presented in Fig. 1, the addition of 2.5 and 25 milliunits of RNAP IIB to reactions containing the DHFR promoter did not result in appreciable transcription from the DHFR promoter (Fig. 6A, lanes 4–5). The addition of 2.5 and 25 milliunits of RNAP IIB to reactions containing the Ad-2 MLP resulted in a level of transcription which was comparable with that obtained from RNAP IIA (Fig. 6B, compare lanes 2–3 with lanes 4–5). Specific transcription from the DHFR promoter catalyzed by RNAP IIA was not diminished in the presence of increasing amounts of RNAP IIB, even up to a 10-fold excess (Fig. 6A, lanes 6–10, and 6C). Additionally, increasing amounts of RNAP IIB did not compete with 32P-labeled RNAP IIA in preinitiation complex formation as analyzed by gel filtration, SDS-PAGE, and autoradiography (data not shown). These results indicate that RNAP IIB is incapable of assembling into preinitiation complexes on the DHFR promoter and suggest that the CTD plays a direct role in the recruitment of RNAP II to this promoter. In contrast, specific transcription from the Ad-2 MLP catalyzed by RNAP IIA was augmented by the presence of increasing amounts of RNAP IIB (Fig. 6B, lanes 6–10, and 6C).

DISCUSSION

The hypothesis that reversible phosphorylation of the CTD plays an integral role in the RNAP II transcription cycle has been proposed based on studies of a single promoter, the Ad-2 MLP. In this model, RNAP IIA initially binds to the promoter to form a preinitiation complex and is subsequently phosphorylated to RNAP IIO by a CTD kinase which may be a basal transcription factor (Payne et al., 1989; Laybourn and Dahmus, 1990; Feaver et al., 1991; Chesnut et al., 1992; Lu et al., 1992; Serizawa et al., 1992). RNAP IIO then catalyzes the elongation of the transcript (Bartholomew et al., 1986; Cadena and Dahmus, 1987; Payne et al., 1989).

The DHFR promoter is involved in the regulation of a cellular, housekeeping gene, and its promoter elements differ significantly from that of the viral Ad-2 MLP. A previous study by Thompson et al. (1989) suggests that transcription from the DHFR promoter requires the CTD of RNAP II in contrast to the Ad-2 MLP which had previously been shown to be efficiently transcribed by RNAP IIB (Kim and Dahmus, 1989). Thompson et al. (1989) employed a monoclonal antibody directed against the CTD to inhibit transcription from the DHFR promoter in a HeLa nuclear extract. The addition of RNAP IIA, but not RNAP IIB, restored transcriptional activity from the DHFR promoter in the inhibited nuclear extract. The studies presented here utilize a recombinant, dependent reconstituted transcription system to directly assess the ability of RNAP IIA and RNAP IIB to transcribe from the DHFR promoter. In agreement with the results of Thompson et al. (1989) these studies demonstrate that RNAP IIA, but not RNAP IIB, is capable of transcribing from the DHFR promoter in the reconstituted system. This characteristic of transcription dependence on the CTD, which is lacking in the Ad-2 MLP, makes the DHFR promoter an attractive system for studies on the role of the CTD and its reversible phosphorylation during transcription.

These studies establish that RNAP IIA efficiently assembles...
into functional preinitiation complexes on the DHFR promoter in the absence of ATP. This is supported by the observation that preinitiation complexes containing RNAP IIA can be purified by gel filtration and can synthesize a run-off transcript upon the addition of ribonucleotides. In contrast, RNAP IIO is inefficient in assembling into preinitiation complexes on the DHFR promoter. Therefore, an unphosphorylated CTD is required for the efficient interaction of RNAP II with the preinitiation complex.

The observation that increasing amounts of RNAP IIB do not inhibit transcription from the DHFR promoter in reactions containing a limiting amount of RNAP IIA indicates that RNAP IIB cannot compete with RNAP IIA for assembly into preinitiation complexes. Consequently, the inability of RNAP IIB to transcribe from the DHFR promoter must stem from its failure to assemble into preinitiation complexes. This result rules out the possibility that RNAP IIB is stalled at some point in initiation or early in transcript elongation and supports a direct role for the CTD in the recruitment of RNAP II to the DHFR promoter.

The CTD requirement observed in the association of RNAP II with DHFR preinitiation complexes is not observed in the case of the Ad-2 MLP. This is supported by the observation that transcription from the Ad-2 MLP in the presence of a limiting amount of RNAP IIA increases with the addition of increasing amounts of RNAP IIB. It is interesting, however, that when the CTD is present in RNAP II, as it is in vivo, it must be in the unphosphorylated form in order to assemble efficiently into preinitiation complexes on the Ad-2 MLP (Lu et al., 1991; Chesnut et al., 1992). Recent studies demonstrate that not all promoters use the same set of "general" basal transcription factors (Parvin et al., 1992). Thus, it is possible that each promoter assemblies its own distinct array of general and specific transcription factors involving different protein-protein and protein-DNA interactions. The preinitiation complex that assembles on the Ad-2 MLP with RNAP IIB may be stable due to strong interactions which can compensate for the lack of the CTD. On the other hand, the stability of the preinitiation complex that assembles on the DHFR promoter depends on the interaction(s) of the CTD with components of that complex.

Preinitiation complexes assembled on the DHFR promoter also contain a stably associated CTD kinase activity which is able to utilize ATP, GTP, or CTP as nucleotide substrates in the conversion of RNAP IIA to IIO. This IIA to IIO conversion is significant in that RNAP IIO cannot be found cross-linked to the nascent DHFR transcript, thus implicating RNAP IIO as the elongating form of the enzyme in DHFR transcription. It is presumed that RNAP IIO dissociates from the template upon termination and is dephosphorylated to RNAP IIA in order to begin another round of transcription. These results establish that phosphorylation of the CTD occurs during transcription from the DHFR promoter and suggest that this may be a common feature in the transcription of class II promoters.

Recent experiments to address the obligatory nature of CTD phosphorylation during the transcription cycle utilized the protein kinase inhibitor N-[2-(methylamino)ethyl]-5-sulfoisoxazol-4-amine dihydrochloride (H-8) to inhibit CTD kinase activity (Serizawa et al., 1993). In the presence of H-8 concentrations which inhibited CTD phosphorylation by more than 99%, basal transcription from the Ad-2 MLP was virtually unaffected. These results suggest that CTD phosphorylation is not a necessary step during the transition from initiation to elongation. However, the possibility cannot be excluded that phosphorylation of a small percentage of RNAP II was not inhibited in these reactions and that this was responsible for the transcription observed both in the absence and presence of H-8. A more definitive experiment would be to photocross-link the nascent transcript to the elongating enzyme in the presence of H-8. Additionally, the interpretation of these H-8 inhibitor experiments is overshadowed by the fact that in vitro transcription from the Ad-2 MLP does not require the CTD. It will be of interest to establish whether or not transcription from the DHFR promoter, which has a dependence on the CTD, is sensitive to H-8.

A CTD kinase(s) is a component of preinitiation complexes assembled on both the DHFR promoter and the Ad-2 MLP (Payne et al., 1989; Laybourn and Dahmus, 1990; Arias et al., 1991). Two distinct kinase activities, CTDK1 and CTDK2, have been partially purified and characterized from HeLa transcription extracts (Payne and Dahmus, 1993). Additionally, other laboratories have identified CTD kinases such as TFIIF (BTF2) (Lu et al., 1992) and the template-associated CTD kinase (Arias et al., 1991) from human, factor δ from rat (Serizawa et al., 1992), cdk2 kinase from mouse (Cisek and Corden, 1989), and factor b and CTK1 from yeast (Feaver et al., 1991; Lee and Greenleaf, 1991). TFIIF, δ, and factor b have also been identified as basal transcription factors. The multiplicity of CTD kinases identified in in vitro studies suggests that multiple CTD kinases may function in vivo. TATA-containing and TATA-less promoters such as the Ad-2 MLP and DHFR promoter, respectively, could in principle assemble preinitiation complexes which contain distinct CTD kinases. In a broader sense, different promoters may bind different types of CTD kinases. Further studies are necessary to establish whether a single CTD kinase, as opposed to multiple CTD kinases, is involved in the transcription of class II promoters.

What specific interactions might serve to direct a promoter-specific CTD kinase to a promoter? A recent study demonstrated that during S phase, the E2F transcription factor is in a complex with cyclin A, Rb-related p107 protein, and p33 cdk2 protein kinase (Devoto et al., 1992). Consequently, these studies propose that the role of E2F may be to localize the cdk2 kinase to the DNA in a sequence-specific manner such that the cdk2 kinase may phosphorylate an adjacent DNA-bound factor. Devoto et al. (1992) suggest that a good candidate for the target of the cdk2 kinase is the CTD of RNAP II. Evidence in support of this idea comes from studies which show that the E2F-cyclin A-p107-cdk2 kinase complex can be found on the DHFR promoter at the E2F binding site (Wade et al., 1992).

The observation that phosphorylation of the CTD of RNAP II occurs with each round of transcription from both the Ad-2 MLP and the DHFR promoter suggests that this phosphorylation event may play a role in the regulation of transcription. Once assembled into a preinitiation complex, it is possible that phosphorylation of the CTD is involved in a key regulatory step to permit the transition from initiation to elongation. Alternatively, phosphorylation may be temporally but not causally related to the initiation process. Irrespective of whether CTD phosphorylation plays a role in the initiation, elongation, or termination phase of transcription, the presence of multiple CTD kinases could enhance the regulatory significance of this modification. Clearly, further studies are necessary to establish the functional and/or regulatory significance of CTD phosphorylation.

Acknowledgments—We gratefully acknowledge the technical assistance of Grace Dahmus and also thank Ross Chambers, Jon Chesnut, Sang Soo Lee, and Alan Lehman for their helpful comments and suggestions in the review of this manuscript.

REFERENCES

Allison, L. A., and Ingle, C. J. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 7794–7798
Allison, L. A., Moyle, M., Shakes, M., and Ingle, C. J. (1989) Cell 56, 599–610
Arias, J. A., Peterson, S. R., and Dyman, W. S. (1991) J. Biol. Chem. 266, 8055–8061
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Bartholomew, B., Dahmus, M. E., and Meares, C. F. (1986) J. Biol. Chem. 261, 14226–14231
Baralolomei, M. S., Halden, N. F., Cullen, C. R., and Corden, J. L. (1988) Mol. Cell. Biol. 18, 390–398
Blake, M. C., and Azizkhan, J. C. (1989) Mol. Cell. Biol. 9, 4994–5002
Buermer, A. B., Thompson, N. E., Strasheim, L. A., Burgess, R. R., and Farnham, P. J. (1992) Mol. Cell. Biol. 12, 2250–2259
Burstedt, S., and Sharp, P. A. (1990) Mol. Cell. Biol. 10, 5562–5564
Cadena, D. L., and Dahmus, M. E. (1987) J. Biol. Chem. 262, 12468–12474
Chesnut, J. D., Stephens, J. H., and Dahmus, M. E. (1992) J. Biol. Chem. 267, 10500–10506
Cieplak, L. J., and Corden, J. L. (1989) Nature 339, 679–684
Conaway, R. C., Bradsher, J. N., and Conaway, J. W. (1992) J. Biol. Chem. 267, 8464–8467
Corden, J. L., Cadena, D. L., Abearn, J. M., and Dahmus, M. E. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7934–7938
Dahmus, M. E., and Redinger, C. (1983) J. Biol. Chem. 258, 2303–2307
Devoto, S. H., Mudryj, M., Pine, J., Hunter, T., and Nevins, J. R. (1992) Cell 68, 167–176
Dyman, W. S., Sazer, S., Tjian, R., and Schimke, R. T. (1986) Nature 319, 246–248
Farnham, P. J., and Means, A. L. (1990) Mol. Cell. Biol. 10, 1390–1398
Feaver, W. J., Giese, O., Li, Y., and Kornberg, R. D. (1991) Cell 67, 1223–1230
Hodo, H. G., and Blatti, S. P. (1977) Biochemistry 16, 2334–2343
Jackson, S. P., and Tjian, R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1781–1785
Kim, W.-Y., and Dahmus, M. E. (1986) J. Biol. Chem. 261, 14219–14226
Kim, W.-Y., and Dahmus, M. E. (1988) J. Biol. Chem. 263, 18880–18885
Kim, W.-Y., and Dahmus, M. E. (1989) J. Biol. Chem. 264, 3169–3176
Koleske, A. J., Burstedt, S., Nonet, M., and Young, R. A. (1992) Cell 69, 883–894
Laybourn, P. J., and Dahmus, M. E. (1989) J. Biol. Chem. 264, 6693–6698
Laybourn, P. J., and Dahmus, M. E. (1990) J. Biol. Chem. 265, 13165–13173
Lee, J. M., and Greenleaf, A. L. (1991) Gene Exp. 1, 149–167
Liao, S.-M., Taylor, I. C. A., Kingston, R. E., and Young, R. A. (1991) Genes & Dev. 5, 2431–2440
Lu, H., Flores, O., Weinmann, R., and Reinberg, D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 10004–10008
Lu, H., Zawel, L., Fisher, L., Egli, J.-M., and Reinberg, D. (1992) Nature 358, 641–645
Meaux, A. L., and Farnham, P. J. (1990) Mol. Cell. Biol. 10, 653–661
Nonet, M., Sweetser, D., and Young, R. A. (1987) Cell 50, 909–915
Parvin, J. D., Timmers, H. T. M., and Sharp, P. A. (1992) Cell 70, 1335–1349
Payne, J. M., and Dahmus, M. E. (1990) J. Biol. Chem. 265, 89–87
Payne, J. M., Laybourn, P. J., and Dahmus, M. E. (1989) J. Biol. Chem. 264, 19621–19629
Peterson, C. L., Kruger, W., and Herskowitz, I. (1991) Cell 64, 1325–1343
Scafe, C., Chao, D., Lopes, J., Hirsch, J. P., Henry, S., and Young, R. A. (1990) Nature 347, 491–494
Schmitt, E. E., Owen, R. A., and Merrill, G. F. (1990) J. Biol. Chem. 264, 17397–17400
Serizawa, H., Conaway, R. C., and Conaway, J. W. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7476–7480
Serizawa, H., Conaway, J. W., and Conaway, R. C. (1993) Nature 363, 371–374
Thompson, N. E., Steinberg, T. H., Aronson, D. B., and Burgess, R. R. (1989) J. Biol. Chem. 264, 11511–11520
Usheva, A., Maldonado, E., Geldring, A., Lu, H., Hoehm, C., Reinberg, D., and Aloni, Y. (1992) Cell 70, 871–881
Wade, M., Kowalik, T. F., Mudryj, M., Huang, E.-S., and Azizkhan, J. C. (1992) Mol. Cell. Biol. 12, 4364–4374
Weil, P. A., Segall, J., Harris, B., Ng, S.-Y., and Roeder, R. G. (1979) J. Biol. Chem. 254, 6163–6173
Wray, W., Boulkhes, T., Wray, V. P., and Hancock, R. (1981) Annu. Rev. Biochem. 50, 197–203
Zehring, W. A., and Greenleaf, A. L. (1990) J. Biol. Chem. 265, 8351–8353
Zehring, W. A., Lee, J. M., Weeks, J. R., Jekson, R. S., and Greenleaf, A. L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3698–3702