Phosphorylation of RNA Polymerase IIA Occurs Subsequent to Interaction with the Promoter and before the Initiation of Transcription*

Paul J. Laybourn$ and Michael E. Dahmus

From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616

(Received for publication, October 20, 1989)

The largest subunit of mammalian RNA polymerase II contains at its C terminus an unusual domain consisting of multiple tandem repeats of the seven-amino acid consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. This domain is unphosphorylated in RNA polymerase IIA and extensively phosphorylated in RNA polymerase IIO. To investigate the role of the C-terminal domain and the functional significance of its phosphorylation, changes in the level of phosphorylation were followed as a function of the position of RNA polymerase II in the transcription cycle. Complexes were formed with $^{32}$P-labeled RNA polymerase IIA and separated from the free polymerase by gel filtration. The phosphorylation state of the RNA polymerase II largest subunit was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Results indicate that RNA polymerase IIA interacts with the template-committed complex to form a stable preinitiation complex. RNA polymerase IIA associated with such complexes is converted to RNA polymerase IIO in the presence of ATP prior to the formation of the first phosphodiester bond. Furthermore, the observation that purified preinitiation complexes can catalyze the conversion of RNA polymerase IIA to IIO indicates that the protein kinase(s) responsible for phosphorylation of the C-terminal domain is a component of such complexes. The concentration of ATP required for the phosphorylation of RNA polymerase II associated with the preinitiation complex is two to three orders of magnitude lower than that required for the conversion of RNA polymerase IIA to IIO free in solution. These results support the idea that phosphorylation of the C-terminal domain of RNA polymerase subunit IIA occurs subsequent to the association of enzyme with the promoter and prior to the initiation of transcription.

The regulation of gene expression is determined to a major extent by the frequency with which RNA polymerase initiates the transcription of specific genes. An understanding of the mechanisms involved in gene regulation is, therefore, dependent on our understanding of the basic reactions involved in initiation. Although there has been considerable progress in our understanding of general factors required for the expression of class II genes (Van Dyke et al., 1988; Buratowski et al., 1989; Saltzman and Weinmann, 1989) and factors that influence the rate of initiation by RNA polymerase II (Ptashne, 1988; Mitchell and Tjian, 1989), relatively little is known about how these factors interact with RNA polymerase II to influence the rate of transcription.

The largest subunit of mammalian RNA polymerase II contains at its C terminus an unusual domain consisting of multiple repeats of the seven-amino acid consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (Corden et al., 1985). While this domain does not appear to be essential for the in vitro transcription of the Ad2-MLP, at least half of the repeats are required for in vivo function (Kim and Dahmus, 1989; Zehring et al., 1988; Payne et al., 1989; Nonet et al., 1987; Allison et al., 1988, Bartolomei et al., 1988). Mammalian cells contain two forms of RNA polymerase II, designated IIO and IIA, that differ with respect to the level of phosphorylation within this domain (Kim and Dahmus, 1986; Cadena and Dahmus, 1987). The C-terminal domain of RNA polymerase subunit IIA is unmodified, whereas the C-terminal domain of subunit IIO is extensively phosphorylated (Cadena and Dahmus, 1987).

Although the function of the C-terminal domain of subunit IIA is not known, recent results indicate that the level of phosphorylation of this domain changes during the course of transcription (Bartholomew et al., 1986; Laybourn and Dahmus, 1989; Payne et al., 1989). Based on these results, we have proposed that RNA polymerase IIA, containing an unphosphorylated C-terminal domain, interacts with DNA to form a preinitiation complex and that phosphorylation occurs during the transition from initiation to elongation (Dahmus et al., 1989; Laybourn and Dahmus, 1989; Payne et al., 1989). A similar model has been proposed by Sigler (1988). The cross-linking of nascent transcripts to RNA polymerase subunit IIO both in in vitro transcription systems (Bartholomew et al., 1986; Payne et al., 1989) and in isolated HeLa nuclei (Cadena and Dahmus, 1987), provides convincing evidence that the elongation of most class II genes is catalyzed by the phosphorylated C-terminal domain, interacts with DNA to form a preinitiation complex and that phosphorylation occurs during the transition from initiation to elongation (Dahmus et al., 1989; Laybourn and Dahmus, 1989; Payne et al., 1989).

Although the function of the C-terminal domain of subunit IIA is known, recent results indicate that the level of phosphorylation of this domain changes during the course of transcription (Bartholomew et al., 1986; Laybourn and Dahmus, 1989; Payne et al., 1989). Based on these results, we have proposed that RNA polymerase IIA, containing an unphosphorylated C-terminal domain, interacts with DNA to form a preinitiation complex and that phosphorylation occurs during the transition from initiation to elongation (Dahmus et al., 1989; Laybourn and Dahmus, 1989; Payne et al., 1989). A similar model has been proposed by Sigler (1988). The cross-linking of nascent transcripts to RNA polymerase subunit IIO both in in vitro transcription systems (Bartholomew et al., 1986; Payne et al., 1989) and in isolated HeLa nuclei (Cadena and Dahmus, 1987), provides convincing evidence that the elongation of most class II genes is catalyzed by the phosphorylated C-terminal domain, interacts with DNA to form a preinitiation complex and that phosphorylation occurs during the transition from initiation to elongation (Dahmus et al., 1989; Laybourn and Dahmus, 1989; Payne et al., 1989). A similar model has been proposed by Sigler (1988). The cross-linking of nascent transcripts to RNA polymerase subunit IIO both in in vitro transcription systems (Bartholomew et al., 1986; Payne et al., 1989) and in isolated HeLa nuclei (Cadena and Dahmus, 1987), provides convincing evidence that the elongation of most class II genes is catalyzed by the phosphorylated C-terminal domain, interacts with DNA to form a preinitiation complex and that phosphorylation occurs during the transition from initiation to elongation (Dahmus et al., 1989; Laybourn and Dahmus, 1989; Payne et al., 1989). A similar model has been proposed by Sigler (1988). The cross-linking of nascent transcripts to RNA polymerase subunit IIO both in in vitro transcription systems (Bartholomew et al., 1986; Payne et al., 1989) and in isolated HeLa nuclei (Cadena and Dahmus, 1987), provides convincing evidence that the elongation of most class II genes is catalyzed by the phosphorylated C-terminal domain, interacts with DNA to form a preinitiation complex and that phosphorylation occurs during the transition from initiation to elongation (Dahmus et al., 1989; Laybourn and Dahmus, 1989; Payne et al., 1989).

The abbreviations used are: Ad2-MLP, adenovirus-2 major late promoter; AMP-PNP, adenosine 5′,(3′,y-amino) triphosphate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid.

*This research was supported in part by Grant GM 33200 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.

$Supported in part with a grant from the California Biotechnology Research and Training Program. Present address: Dept. of Biology, University of California, San Diego, La Jolla, CA 92093.
Phosphorylation of RNA Polymerase IIA

IIO is shown to occur prior to the formation of the first phosphodiester bond.

EXPERIMENTAL PROCEDURES

Materials

Ultrapure nucleotides were purchased from Pharmacia LKB Biotechnology Inc. Radiolabeled nucleotides [2-32P]CTP and [5-32P]ATP were obtained from Amersham, and [3H]UTP was obtained from Du Pont-New England Nuclear. AMP-5NP was purchased from Boehringer Mannheim. Heparin-Sepharose CL-4B was prepared as described by the method of Teissere et al. (1977), and heparin-Sepharose CL-6B was obtained from Pharmacia. Purified RNA polymerase II was prepared as described by Kim and Dahmus (1988). Preinitiation complexes were formed by preincubation of 4 ml of transcription factor, 14 ml of DE0.15, 8 ml of DE0.25, and RNA polymerase II containing 50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.5 mM DTT, and 15% glycerol. Buffer D is the same as buffer B except that it contained 10% glycine.

Preparation of Transcription Extract and DNA Template—The S-100 transcription extract was prepared from HeLa cells by the method of Weil et al. (1979) as modified by Dahmus and Kederling (1983). S-100 extract was dialyzed against buffer containing 100 mM KC1 and chromatographed on heparin-Sepharose CL-4B (column volume was equal to 1.5 times the S-100 volume) according to the procedure of Davidson et al. (1983). The column was washed with two column volumes of buffer A containing 0.1 M KC1, one column volume of buffer A containing 0.24 M KC1, and the transcriptional activity was eluted with two column volumes of buffer A containing 0.6 M KC1. Stimulatory transcription factor (TFIIA) was purified from the flow-through peak by chromatography on a DEAE-Sepharose column as described by Moncell et al. (1986) and dialyzed against buffer B containing 50 mM KC1 (protein concentration approximately 5 mg/ml). Fractions from the heparin-Sepharose 0.6 M KC1 eluted peak (HS0.6) were assayed for transcriptional activity. Active fractions were pooled and dialyzed against buffer D containing 50 mM KC1, and 15–20 mg of protein were applied to a DEAE-SPW column pre-equilibrated with the same buffer. After washing with two column volumes buffer D containing 50 mM KC1, proteins were successively step-eluted with 0.15 M, 0.29 M, 0.25 M, and 0.6 M KC1 in buffer D according to the procedure of Moncell et al. (1986) as modified by Kim and Dahmus (1989). Three to four fractions from each peak, containing the greatest absorbance at 260 nm, were pooled and dialyzed against buffer B containing 50 mM KC1. The DE 0.25 M KC1 eluate contains TFIIE/F and has an overall protein concentration of 0.6–1.0 mg/ml. The DE 0.25 M KC1 eluate contains TFIID and TFIE/F and has an overall protein concentration of 0.6–1.0 mg/ml.

The adenovirus 27 Pml DNA fragment containing the major late promoter (positions –260 to +560), designated the 560 template, was prepared as previously described in Payne et al. (1989). A second template, designated the 405 template (positions –260 to +405), was produced by digestion of the 560 template with HindII.

Transcription Reactions—RNA polymerases I, II, and III were purified as described by Kim and Dahmus (1989). Preinitiation complexes were formed by preincubation of 4 ml of stimulatory transcription factor, 14 ml of DE0.15, 8 ml of DE0.25, and RNA polymerase II, IIA, or III (8 × 10^11 units) in buffer B containing 50 mM KC1 in a final volume of 50 ml for 15 min at 37 °C followed by the addition of 1 ml of DNA template (37.5 ng) and incubation for an additional 15 min at 25 °C. The molar ratio of RNA polymerase II to DNA in these experiments is about 1:3. Transcription was initiated by the addition of nucleotides and salts to a final concentration of 12 µM [α-32P]CTP (4.7 Ci/mmol), 600 µM ATP, UTP, and GTP, and 6 mM MgCl2 to a final volume of 40 µl. In reactions containing AMP-PNP, the final concentration of nucleotides was 12 µM [α-32P]CTP (4.7 Ci/mmol), ATP as indicated in the figure legends, and 30 µM AMP-PNP, UTP, and GTP. Complete transcription reactions were incubated at 30 °C for 45 min. RNA was purified and analyzed on 5% polyacrylamide-urea gels as described by Dahmus and Kederling (1985). The amount of specific transcript was quantitated as described previously (Laybourn and Dahmus, 1989).

Preparation of 32P-Labeled RNA Polymerase II—RNA polymerase II was purified from calf thymus by the procedure of Blatti (1977) as modified by Kim and Dahmus (1988, 1989). Modifications include the presence of 10 mM EDTA, 10 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride in all buffers, batch binding and step elution from DE52 and P11, and the substitution of heparin-Sepharose CL-4B in place of agarose A-1.5m as the final column. RNA polymerase IIA was purified from RNA polymerases IIO and IIB by chromatography on alkyl-Seperox (Pharmacia). An aliquot of heparin-Sepharose-purified RNA polymerase II (4–8 mg in a volume of 3–6 ml) was loaded onto the alkyl-Seperox column in buffer C containing 1.4 M (NH4)2SO4 and eluted with a 15-ml gradient of 1.4 M to 0 M (NH4)2SO4 in Buffer C. Casein kinase II was purified as previously described (Payne et al., 1989).

Purified RNA polymerase IIA (1 unit) was incubated in a 140-µl reaction containing 2 µM [γ-32P]ATP (approximately 2000 Ci/mmol), 40 mM Tris-HCl, pH 7.9, 10 mM MgCl2, 64 mM KCl, 0.4 mM DTT, and 5 units of casein kinase II at 37 °C for 10 min. The reaction mixture was then loaded onto a 50–µl heparin-Sepharose CL-6B column pre-equilibrated in buffer B containing 100 mM KC1. The column was washed extensively with the same buffer, and RNA polymerase IIA eluted with buffer containing 0.6 M KC1 and 0.2% Tween 80. The RNA polymerase IIA peak was pooled and dialyzed against buffer B. The nonselective and promoter-dependent transcriptional activity of 32P-labeled RNA polymerase IIA was determined as described by Kim and Dahmus (1988, 1989).

Fractionation of Free and Bound RNA Polymerase II by Gel Filtration—Protein fractions recovered from RNA polymerase II were gel-filtered on a 1-ml Sepharose CL-4B column equilibrated in Buffer B containing 37.5 mM KC1 and 6 mM MgCl2 as previously described (Carey et al., 1986). Fractions (50 µl) were collected, denatured, and run on 5% polyacrylamide-SDS gels according to the method of Laemmli (1970). Gels were silver-stained as described by Wray et al. (1981), dried, and exposed to x-ray film.

RESULTS

Fractionation of Preinitiation Complexes and Free RNA Polymerase II by Gel Filtration—An identification of the precise step in the transcription reaction in which phosphorylation of the C-terminal domain occurs is an essential prerequisite to defining the involvement of this domain in transcription. The overall strategy is to initiate transcription with purified RNA polymerase IIA in the presence of a fractionated transcription extract and to determine the level of phosphorylation of subunit IIA as RNA polymerase progresses through the various steps of transcription in order to assess the level of phosphorylation of RNA polymerase II in transcription complexes, it is essential to first separate these complexes from free enzyme.

Purified RNA polymerase IIA was incubated in the presence of a reconstituted HeLa cell transcription extract and a DNA template containing the Ad2-MLP to form preinitiation complexes. Complexes were then separated from free RNA polymerase II by gel filtration as described under "Experimental Procedures." RNA polymerase II associated with template is excluded and elutes in the void volume, whereas free RNA polymerase is included in the column. To determine whether or not complexes contained in the excluded fractions are indeed functionally active preinitiation complexes, the following experiment was done. Preinitiation complexes were formed in the absence of ATP by incubation of RNA polymerase IIA and a reconstituted transcription extract in the presence of DNA template. The DNA template was truncated to produce a 560-nucleotide runoff transcript. Preinitiation complexes were purified by gel filtration and incubated in the presence of increasing concentrations of a competing second template, (405 template) truncated to give a runoff transcript of 405 nucleotides. Nucleotides were then added, and, after an additional 5-min incubation, Sarkosyl was added to limit
transcription to a single round (Hawley and Roeder, 1987). The complete reaction was incubated for an additional 25 min, and the transcripts formed were analyzed as described under "Experimental Procedures." Results presented in Fig. 1 (lanes 1-3) show that purified preinitiation complexes formed with the 560 template produce only a 560-nucleotide transcript even in the presence of excess 405 template. Similarly, transcription from preinitiation complexes formed on the 405 template is restricted to that template even in the presence of excess 405 template. Similarly, transcription from preinitiation complexes formed on the 405 template is restricted to that template even in the presence of excess 405 template. Similarly, transcription from preinitiation complexes formed on the 405 template is restricted to that template even in the presence of excess 405 template.

The complete reaction was incubated for an additional 25 min, and the transcripts formed were analyzed as described under "Experimental Procedures." Results presented in Fig. 1 (lanes 1-3) show that purified preinitiation complexes formed with the 560 template produce only a 560-nucleotide transcript even in the presence of excess 405 template. Similarly, transcription from preinitiation complexes formed with purified RNA polymerase IIA in the absence of ATP and fractionated by gel filtration are stable and able to initiate and form completed transcripts upon the addition of nucleotides. Furthermore, the fact that end to end transcription of the competing template is not observed indicates that excluded fractions do not contain significant amounts of free RNA polymerase II.

Analysis of the Phosphorylation State of RNA Polymerase II in Transcription Complexes—The decreased electrophoretic mobility in SDS-PAGE of RNA polymerase subunit IIo, relative to subunit IIa, results from extensive phosphorylation within the C-terminal domain (Cadena and Dahmus, 1987; Cisek and Corden, 1989). Changes in the phosphorylation state of the largest subunit of RNA polymerase II can therefore be monitored by following the shift in electrophoretic mobility. In order to increase the sensitivity of such assays and to facilitate the analysis of factors that catalyze the phosphorylation of RNA polymerase II, Payne et al. (1989) developed an assay based on the utilization of 32P-labeled RNA polymerase IIA as substrate. Casein kinase II phosphorylates subunit IIa at a single site that does not result in a mobility shift in SDS-PAGE (Dahmus, 1981; Payne et al., 1989). 32P-Labeled RNA polymerase IIA, labeled by phosphorylation with casein kinase II as described under "Experimental Procedures," was incubated in the presence of a reconstituted transcription extract, with or without template DNA and ATP, and fractionated from transcription factors and free RNA polymerase II by gel filtration as described above. The distribution of RNA polymerase II in the column fractions and the state of phosphorylation of subunit IIa was determined by SDS-PAGE. In the absence of DNA, RNA polymerase IIa is found exclusively in the included fractions (Fig. 2A, lanes 5-8). The presence of DNA results in a fraction of RNA polymerase IIA eluting in the excluded fractions (Fig. 2B, lanes 2-3). The conditions for preinitiation complex formation and fractionation are identical with those employed in Fig. 1 in which excluded fractions were shown to contain template committed transcription complexes. Consequently, these results suggest that RNA polymerase IIA forms a stable preinitiation complex on the Ad2-MLP in the absence of ATP.

The fractionation of free and template-bound RNA polymerase II becomes more apparent in reactions that contain ATP. The inclusion of ATP, in the absence of DNA, results in the partial conversion of RNA polymerase IIA to IIA+ as indicated by the reduced electrophoretic mobility of subunit IIa in included fractions (Fig. 2C, lanes 6-8). This is in

![FIG. 1. Transcriptional activity of preinitiation complexes purified by gel filtration.](image)

Preinitiation complexes were formed on either the 560 or 405 template in a standard 40-μl transcription reaction lacking nucleotides as described under "Experimental Procedures." The transcription reaction was fractionated by gel filtration. The two excluded peak fractions (fractions 9 and 10) were pooled, and 30-μl aliquots were removed for assay of transcriptional activity. Competitor 405 or 560 template was added, and reactions were incubated for 15 min at 25°C. Nucleotides were added, and the incubation was continued for an additional 5 min. Sarkosyl was added to a final concentration of 0.08%, and the amount of transcript formed at the end of the 25-min incubation was determined as described under "Experimental Procedures." Lanes 1-3 contain transcripts from gel filtration-purified complexes formed on the 560 template and incubated with 0, 18.8, and 37.5 ng of competitor 405 template, respectively, prior to the addition of nucleotides. Lanes 4-6 contain transcripts from gel filtration-purified complexes formed on the 405 template and incubated with 0, 18.8, and 37.5 ng of competitor 560 template, respectively.

![FIG. 2. Fractionation of preinitiation complexes and free RNA polymerase II by gel filtration. 32P-labeled RNA polymerase IIA was incubated in the presence of a reconstituted transcription extract, with or without DNA and ATP, and fractionated by gel filtration as described under "Experimental Procedures." Panels A, B, C, and D contain column fractions from reactions containing no DNA or ATP (A), DNA only (B), ATP only (C), and both DNA and ATP (D). From each column, fractions 8-12, 14, 16, and 18 were run on a 5% polyacrylamide-SDS gel in lanes 1-8, respectively.](image)
agreement with previous results that demonstrate that transcription extracts contain a protein kinase active in the phosphorylation of the C-terminal domain of subunit IIa (Payne et al., 1989). In the presence of DNA and ATP, RNA polymerase IIO is recovered in the excluded fractions (Fig. 2D, lanes 2–4). These results, in conjunction with the results presented in Fig. 1, suggest that RNA polymerase IIa associates with the promoter and, in the presence of ATP, is converted to RNA polymerase IIO. Indeed, the formation of RNA polymerase IIO, under these reaction conditions, appears to be dependent on the formation of a preinitiation complex (compare lanes 3 and 4 with lanes 6–8 in Fig. 2D). The fact that RNA polymerase IIO does not appear to be formed in the absence of DNA under these conditions (Fig. 4C, lanes 6–8) suggests that phosphorylation of the C-terminal domain occurs after association of RNA polymerase with the preinitiation complex as opposed to RNA polymerase IIO selectively binding to the promoter.

In order to determine the phosphorylation state of RNA polymerase subunit IIa throughout the course of transcription, preinitiation complexes were formed as described above and subsequently incubated in the presence of various combinations of nucleotides. Transcription reactions included either no nucleotides, ATP alone, ATP and CTP, or ATP, CTP, UTP, and 3'-O-methyl-GTP thereby resulting in the formation of either a preinitiated complex, an activated complex, an initiated complex, or an elongating complex, respectively (Van Dyke et al., 1988; Buratowski et al., 1989; Saltzman and Weinmann, 1989). Complexes were fractionated from free RNA polymerase II by gel filtration and analyzed as described above. In agreement with the previous experiment, incubation of RNA polymerase IIa with transcription factors and DNA resulted in the formation of preinitiation complexes containing RNA polymerase IIA (Fig. 3A, lanes 2–3). In the presence of ATP, RNA polymerase IIA is converted to RNA polymerase IIO (Fig. 3B, lanes 2–4). RNA polymerase II remains as IIO through initiation (Fig. 3C, lanes 2–3) and early elongation (Fig. 3D, lanes 2–3). These results are in agreement with photoaffinity labeling experiments that demonstrate that the elongation phase of transcription is catalyzed by RNA polymerase IIO (Bartholomew et al., 1986; Cadena and Dahmus, 1987; Payne et al., 1989).

The observation that the conversion of RNA polymerase IIa to IIO occurs in the presence of ATP alone, implies that phosphorylation of the C-terminal domain precedes the formation of the first phosphodiester bond. The possibility exists, however, that trace amounts of contaminating nucleotides may be present in concentrations sufficient for initiation to occur. To test this possibility, the transcriptional activity of preinitiation complexes, formed in the presence of either ATP or dATP and purified by gel filtration, was determined in the presence of 0.08% Sarkosyl. If transcripts were initiated during the ATP incubation step, the purified transcription complexes would be stable in the presence of Sarkosyl, and completed transcripts would be formed upon the addition of remaining nucleotides (Hawley and Roeder, 1987). In order to monitor the formation of RNA polymerase IIO and to identify column fractions containing preinitiation complex, reactions were initiated by the addition of 32P-labeled RNA polymerase IIa. The experimental protocol is shown diagrammatically in Fig. 4. Results presented in Fig. 4A confirm that excluded fractions contain exclusively RNA polymerase IIO irrespective of whether the incubation contained ATP (lanes 1–7) or dATP (lanes 8–14). This is in agreement with previous results that show dATP can substitute for ATP in the conversion of RNA polymerase IIa to IIO (Payne et al., 1989).

The two peak fractions containing preinitiation complexes were pooled and divided into two reactions. The first reaction was incubated with ATP, CTP, GTP, and UTP as described under "Experimental Procedures" for 5 min at which time Sarkosyl was added to a final concentration of 0.08% and the incubation continued for an additional 40 min (Fig. 4B, lanes 1 and 5). The second reaction was incubated for 5 min in the presence of Sarkosyl prior to the addition of nucleotides (Fig. 4B, lanes 2 and 6). As an additional control, the included fractions containing free RNA polymerase IIA were also assayed for transcriptional activity (Fig. 4B, lanes 3–4 and 7–8). Completed transcripts were formed only in reactions containing purified preinitiation complexes that were incubated with nucleotides prior to the addition of Sarkosyl (Fig. 4B, lanes 1 and 5). The fact that no transcripts were formed when Sarkosyl was added prior to the addition of nucleotides establishes that transcripts were not initiated during the preincubation with ATP or dATP (Fig. 4B, lanes 2 and 6). These results therefore support the idea that phosphorylation of the C-terminal domain precedes the formation of an initiated complex.

Preinitiation Complexes Contain Factors Essential for the Conversion of RNA Polymerase IIa to IIO—The results pre-
Phosphorylation of RNA Polymerase IIA

![Diagram of phosphorylation process](image)

**FIG. 4.** Characterization of preinitiation complexes formed in the presence of ATP or dATP. Preinitiation complexes (3X reaction or 120 μl) were formed in the presence of either 700 μM ATP or dATP and 6 mM MgCl₂ and purified on a 3-ml gel filtration column as described under "Experimental Procedures." Fractions of 150 μl were collected. Aliquots of 50 μl were analyzed by electrophoresis on 5% polyacrylamide-SDS gels (A). Fractions 8-12, 14, and 16 from each column were run in lanes 1-7 (complexes formed in the presence of ATP) and in lanes 8-14 (complexes formed in the presence of dATP). The two peak fractions of each column containing preinitiation complexes (AdZ-MLP, lanes 2-3 and 8-9) were pooled and assayed for transcriptional activity (B, lanes 1-2 and 5-6). Included fractions containing RNA polymerase (AdZ-MLP, lanes 6 and 13) were also assayed for transcriptional activity (B, lanes 3-4 and 7-8). Lanes 1-4 and 5-8 of panel B are from preincubation reactions that contained ATP and dATP, respectively. Reactions in lanes 1, 3, 5, and 7 were incubated with nucleotides prior to the addition of Sarkosyl, whereas reactions in lanes 2, 4, 6, and 8 were incubated with Sarkosyl prior to the addition of nucleotides.

Presented in Figs. 2 through 4 establish that in the absence of ATP, RNA polymerase IIA is associated with the preinitiation complex, whereas in the presence of ATP, RNA polymerase IIO is associated with such complexes. If indeed the conversion of RNA polymerase IIA to IIO occurs after RNA polymerase IIA has associated with the preinitiation complex, the protein kinase that catalyzes the phosphorylation of the C-terminal domain of subunit IIa may be an integral component of such complexes. In order to test this possibility, preinitiation complexes were formed in the absence of ATP, purified by gel filtration, and assayed for RNA polymerase IIA to IIO conversion activity by incubation in the presence of ATP. Preinitiation complexes were formed by incubation of ³²P-labeled RNA polymerase IIA with a reconstituted transcription extract and the AdZ-MLP as described above and purified by gel filtration. Column fractions were incubated in the presence of ATP, and the level of phosphorylation of subunit IIa/o was determined by SDS-PAGE (Fig. 5A). A major fraction of RNA polymerase IIA contained in excluded fractions was converted to RNA polymerase IIO upon incubation with ATP (Fig. 5, lanes 2-3). In an effort to determine if the recovery of protein kinase in fractions containing preinitiation complex is indeed dependent on preinitiation complex formation, transcription factors and DNA were incubated in the absence of RNA polymerase II and fractionated by gel filtration. Fractions were assayed for conversion activity, as described above, following the addition of ATP and ³²P-labeled RNA polymerase IIA (Fig. 5B). The observation that incubation of RNA polymerase IIA and ATP in the presence of excluded fractions does not result in a significant shift in the electrophoretic mobility of subunit IIa suggests that the association of protein kinase with the preinitiation complex is dependent on the presence of RNA polymerase II. The protein kinase responsible for the conversion of RNA polymerase IIA to IIO is also not recovered in the excluded fractions when RNA polymerase IIA is present but DNA is absent from the preincubation reaction (data not shown). These results are consistent with the idea that the protein kinase responsible for phosphorylation of the C-terminal domain is a com-
ponent of TFIIE/F (Payne et al., 1989). Since TFIIE/F appears to interact directly with RNA polymerase II, the association of protein kinase with the complex would be expected to be dependent on the prior association of RNA polymerase II. The failure to observe the partial conversion of RNA polymerase IIA to IIO in included fractions is likely due to the dilution of protein kinase following chromatography.

Results presented above suggest that phosphorylation of RNA polymerase IIA may be facilitated by its association with the promoter. As a partial test of this idea, the concentration of ATP required for the phosphorylation of RNA polymerase IIA, free in solution and assembled into a preinitiation complex, was determined. Preinitiation complexes containing 32P-labeled RNA polymerase IIA were formed and purified as described above. Complexes were incubated in the presence of increasing concentrations of ATP, and the extent of RNA polymerase IIA to IIO conversion was determined. Results presented in Fig. 6A show that concentrations of ATP as low as 30 μM are sufficient for the conversion of RNA polymerase IIA to IIO when RNA polymerase IIA is associated with the preinitiation complex. The effect of increasing concentrations of ATP on the conversion of free 32P-labeled RNA polymerase IIA in the presence of the same transcription extract is shown in Fig. 6C. The conversion reaction in the absence of DNA was done with an otherwise complete reaction that was not fractionated by gel filtration. It is apparent from Fig. 6C that concentrations of ATP as high as 1 mM result in only the partial conversion of free RNA polymerase IIA to IIO. The association of RNA polymerase IIA with the preinitiation complex, therefore, results in a two to three order of magnitude reduction in the ATP concentration required for conversion (compare Fig. 6A, lane 2 with Fig. 6C, lane 6). One possibility is that the increased efficiency of conversion of RNA polymerase IIA associated with transcription complexes results from the removal of an inhibitor or protein phosphatase during the purification of such complexes. This was tested by examining the effect of unfractionated transcription extract on the rate of conversion of RNA polymerase IIA in purified transcription complexes. The inclusion of extract did not have an appreciable effect on the rate of appearance of RNA polymerase IIO even though RNA polymerase IIA was detectable at low ATP concentrations (data not shown).

The effect of increasing concentrations of GTP on the conversion of RNA polymerase IIA to IIO, either associated with a transcription complex or free in solution, is shown in Fig. 6B and D, respectively. In agreement with previous results (Payne et al., 1989), GTP can be utilized by the protein kinase that catalyzes the conversion of RNA polymerase IIA to IIO. The apparent Kₐ for GTP is, however, on the order of 100-fold higher than that of ATP (compare Fig. 6B, lane 6 with Fig. 6A, lane 2). Similar to the results with ATP, only partial conversion of free RNA polymerase IIA to IIO is observed in the presence of GTP (Fig. 6D).

RNA Polymerases IIO, IIA, and IIB Have Similar ATP Requirements—Adenosine nucleoside triphosphate (ATP or dATP) containing a hydrolyzable β, γ-phosphoanhydride bond is required for the activation of RNA polymerase II (Conaway and Conaway, 1988; Sawadogo and Roeder, 1984; Reinberg and Roeder, 1987). The nature of this energy dependence is not known. The ATP requiring step immediately precedes initiation and consequently appears to occur at about the same time as the conversion of RNA polymerase IIA to IIO. The possibility therefore exists that the energy-dependent step is the phosphorylation of the C-terminal domain of subunit IIA. If the conversion of RNA polymerase IIA to IIO is the sole energy-requiring step, transcription catalyzed by RNA polymerase IIB, that lacks a C-terminal domain (Corden et al., 1985), should not be dependent on the presence of ATP.

Preinitiation complexes were formed as described above by incubation of purified RNA polymerase IIO, IIA, or IIB with transcription factors, DNA template containing the Ad5-MLP, and increasing concentrations of ATP or dATP as indicated in the legend to Fig. 7. Transcription was initiated by the addition of AMP-PNP, CTP, GTP, and UTP and the amount of 560-nucleotide transcript produced was quantitated. AMP-PNP can be utilized as a substrate for elongation, but cannot satisfy the energy-requiring step. The results shown in Fig. 7A, B, and C demonstrate that RNA polymerases IIO, IIA, and IIB, respectively, have similar ATP requirements. RNA polymerases IIO, IIA, and IIB each exhibited a sharp stimulation of transcriptional activity between 10 and 100 μM ATP in the presence of 30 μM AMP-PNP. This contrasts with the ATP requirement of less than 10 μM for the conversion of RNA polymerase IIA to IIO in the preinitiation complex. The inhibitory effect of high concentrations of ATP on the transcriptional activity of RNA polymerase.
Transcription was initiated by the addition of AMP-PNP, followed by a second incubation with DNA at 25 °C for increasing concentrations of ATP or dATP at 25 °C for 15 min. CTP, UTP, GTP, and MgCl₂ as described under "Experimental Procedures." Each reaction contained 8 pmol of transcript produced for 100% value corresponds to 0.77, 0.74, and 0.86 × 10⁻²³ panel of transcript produced for RNA polymerases IIO, IIA, and IIB, respectively. The gel inset in each panel are reactions containing 0, 0.095, 0.95, 9.5, 95, and 950 μM ATP in lanes 1-6, respectively.

FIG. 7. ATP requirement of RNA polymerase IIO, IIA, and IIB. Transcription reactions, initiated with RNA polymerase IIO (panel A), IIA (panel B), or IIB (panel C), were incubated with increasing concentrations of ATP or dATP at 25 °C for 15 min followed by a second incubation with DNA at 25 °C for 15 min.

The state of phosphorylation of RNA polymerase II is dependent on its position in the transcription cycle. Results presented here indicate that RNA polymerase IIA, containing an unphosphorylated C-terminal domain, interacts with the template-committed complex to form a preinitiation complex and that phosphorylation occurs immediately prior to the initiation of a transcript. Evidence in support of the idea that RNA polymerase IIA can form a stable preinitiation complex comes from experiments in which purified ³²P-labeled RNA polymerase IIA was used to initiate transcription, and the state of phosphorylation was determined as a function of the position of RNA polymerase II in the transcription cycle.

Preinitiation complexes formed in the absence of ATP, and purified by gel filtration, were shown to contain RNA polymerase IIA (Figs. 2B and 3A). The fact that such complexes were transcriptionally active, as indicated by the synthesis of completed transcripts upon the addition of nucleotides and the failure to obtain transcription from a competing template, demonstrates that these complexes correspond to functional preinitiation complexes. These results indicate that RNA polymerase IIA can form a stable complex with the Ad2-MLP in the presence of a reconstituted HeLa cell transcription extract and in the absence of ATP. Earlier results, including an analysis of the in vitro transcriptional activity of purified RNA polymerases IIO, IIA, and IIB (Kim and Dahmus, 1989) and the effect of a monoclonal antibody, that differentially reacts with RNA polymerases IIA and IIO, on in vitro transcription (Laybourn and Dahmus, 1989), also support the idea that RNA polymerase IIA interacts with the template-committed complex to form a preinitiation complex.

The inclusion of ATP in the transcription reaction resulted in the recovery of preinitiation complexes that contained almost exclusively RNA polymerase IIO (Figs. 2D, 3B, and 4A). RNA polymerase IIO not associated with the preinitiation complex was, however, only partially phosphorylated. The exclusive localization of RNA polymerase IIO in the activated complex could result from either the preferential phosphorylation of RNA polymerase IIA in the preinitiation complex, relative to the free enzyme, or the preferential association of RNA polymerase IIO with the template-committed complex. The following observations support the idea that phosphorylation occurs subsequent to the association of RNA polymerase IIA with the promoter. The limited phosphorylation of unbound RNA polymerase IIO that occurs under standard transcription reaction conditions does not result in the formation of subunit IIO as determined by mobility shift in SDS gels (Fig. 2D). Furthermore, results presented in Fig. 6 show that the phosphorylation of RNA polymerase IIO is stimulated by its association with the promoter.

The nucleotide sequence at the 5' end of the major late transcript is ACUCU. The formation of the first phosphodiester bond therefore requires the presence of both ATP and CTP. The observation that the phosphorylation of RNA polymerase IIO, bound in a preinitiation complex, requires only the presence of ATP or dATP indicates that phosphorylation precedes the formation of the first phosphodiester bond. Furthermore, the fact that transcription complexes formed in the presence of either ATP or dATP cannot synthesize completed transcripts in the presence of 0.08% Sarkosyl indicates that initiation did not take place during the ATP preincubation. The observation that preinitiation complexes formed in the presence of ATP contain almost exclusively RNA polymerase IIO suggests that the transcripts initiated upon addition of the remaining nucleotides are initiated by RNA polymerase IIO. Quantitation of the amount of transcript produced indicates that about 10% of the RNA polymerase present in the preinitiation complex fraction is able to synthesize a completed transcript in the presence of Sarkosyl. It is, therefore,
unlikelihood that transcription is being catalyzed by a minor fraction of RNA polymerase with a structure different from that of RNA polymerase IIO. The state of phosphorylation, as determined by mobility shift in SDS gels, does not change upon initiation of the transcript or in the early phases of elongation (Fig. 3). This is in agreement with photoaffinity labeling experiments carried out in reconstituted transcription systems (Bartholomew et al., 1986; Payne et al., 1989) and in isolated HeLa nuclei (Cadena and Dahmus, 1987) that demonstrate elongation is catalyzed by the phosphorylated form of RNA polymerase IIA.

Several reports have appeared recently concerning the purification of protein kinases that phosphorylate the C-terminal domain of RNA polymerase subunit IIa (Cisek and Corden, 1989; Guilfoyle, 1989; Lee and Greenleaf, 1989; Payne et al., 1989). It is not possible at this point, however, to determine the relationship between these kinases or to establish whether or not they are involved in the in vivo phosphorylation RNA polymerase II. The observation that RNA polymerase IIA, associated with purified preinitiation complexes, can be converted to RNA polymerase IIO by incubation of the complex with ATP, demonstrates that the protein kinase responsible for the phosphorylation of the C-terminal domain of subunit IIa can form a stable association with the preinitiation complex. Furthermore, the association of protein kinase with the preinitiation complex appears to be dependent on the presence of RNA polymerase II. This is in agreement with recent results which show that a protein kinase purified from HeLa cells and active in the conversion of RNA polymerase IIA to IIO co-fractionates with transcription factor IIE/F (Payne et al., 1989). TFIIE/F appears to interact directly with RNA polymerase II and has been reported to contain ATPase activity (Reinberg and Roeder, 1987; Sawadogo and Roeder, 1984; Zheng et al., 1987). The fact that preinitiation complexes, purified by gel filtration as described above, can initiate and produce completed transcripts upon the addition of nucleotides indicates that these complexes contain all of the essential transcription factors, including TFIIE/F. These results are also in agreement with the recent observation of Dynan and Arias (1989) that the addition of [γ-32P]ATP to preinitiation complexes formed on an immobilized template results in the phosphorylation of the largest subunit of RNA polymerase II.

The dramatic difference in the apparent $K_m$ for ATP in the phosphorylation of RNA polymerase IIA associated with a preinitiation complex as opposed to free in solution may have important implications. According to the proposed model, RNA polymerase IIO is released upon termination of the transcript and must be converted back to RNA polymerase II before reinitiation. Consequently, the efficient cycling of RNA polymerase II requires conditions in which free enzyme is maintained in the dephosphorylated state, whereas RNA polymerase II associated with the preinitiation complex is readily phosphorylated. The 100-fold lower $K_m$ for ATP when protein kinase is associated with the transcription complex, as opposed to free in solution, provides a mechanism for the preferential phosphorylation of promoter-associated RNA polymerase II. In this regard, it is interesting to note that the inhibition of host transcription that results from infection of HeLa cells with poliovirus is associated with the disappearance of RNA polymerase IIO (Rangei et al., 1987, 1988). One interpretation of these results, taking into account the report that TFIIH may be altered as a result of viral infection (Kliwer and Dasgupta, 1988), is that the inhibition of initiation results in the accumulation of free RNA polymerase II that is primarily in the dephosphorylated state. Direct evidence for the involvement of protein phosphatase in the regulation of gene expression in yeast has also been recently reported (Arndt et al., 1989).

These studies provide further support for the model in which RNA polymerase IIA is involved in the initial interaction with the promoter and the assembly of a preinitiation complex. The extensive phosphorylation of the C-terminal domain of subunit IIa is catalyzed by a protein kinase that appears to be an integral component of the preinitiation complex and occurs prior to the initiation of a transcript. The conformational changes induced by this phosphorylation may be associated with either the formation of an open promoter complex or the release of RNA polymerase II from the preinitiation complex. If indeed the phosphorylation of the C-terminal domain plays a critical role in the transition from a preinitiation complex to an initiated complex, it may be difficult to understand how RNA polymerase IIB, that lacks the C-terminal domain (Corden et al., 1985), could specifically initiate and elongate transcripts from the Ad2-MLP (Kim and Dahmus, 1989). This is not an unexpected result, however, if we assume that the C-terminal domain of subunit IIa is involved in directing and orienting RNA polymerase to the start site of transcription via extensive protein-protein interactions with transcription factors involved in the formation of a preinitiation complex. Protein-protein interactions apart from those involving the C-terminal domain may be sufficient to bind and orient RNA polymerase IIB on a strong promoter such as the Ad2-MLP. This is consistent with the observation that the C-terminal domain of subunit IIa is not required for transcription from the Ad2-MLP, but appears to be essential for in vitro transcription from the dihydrofolate reductase promoter (Thompson et al., 1989). The dihydrofolate reductase promoter does not contain a TATA box and is significantly weaker than the Ad2-MLP. Consequently, if phosphorylation of the C-terminal domain serves to disrupt protein-protein interactions formed during preinitiation complex formation, then the phosphorylation of RNA polymerase II would be essential for the efficient cycling of RNA polymerase II/A. The disruption of preinitiation complexes formed with RNA polymerase II/A would not be dependent on phosphorylation.

Further studies are necessary to define the nature of the protein kinase that catalyzes the conversion of RNA polymerase IIA to IIO and to establish whether or not such an activity is essential for the transcription of class II genes. Whether or not the phosphorylation of the C-terminal domain of subunit IIa is important in the regulation of gene expression has also yet to be determined.

Acknowledgments—We gratefully acknowledge Grace Dahmus and Woo-Yeon Kim for many helpful discussions. We also thank Jon Chesnut and John Payne for critical review of this manuscript and many useful suggestions.

REFERENCES

Allison, L. A., Wong, J. K.-C., Fitzpatrick, V. D., Moyle, J., and Inglese, C. J. (1988) Mol. Cell. Biol. 8, 321-329

Arndt, K. T., Stiles, C. A., and Fink, G. R. (1986) Cell 56, 527-537

Bartholomew, B., Dahmus, M. E., and Meares, C. F. (1986) J. Biol.

Chem. 261, 14226-14231

Bartolomei, M. S., Halden, N. F., Cullen, C. R., and Corden, J. L. (1988) Mol. Cell. Biol. 8, 300-309

Buratowski, S., Hahn, S., Gaurrente, L., and Sharp, P. A. (1989) Cell

39, 549-561

Cai, H., and Luse, D. S. (1987) J. Biol. Chem. 262, 298-304

Carey, M. F., Gerrard, S. P., and Cozzarelli, N. R. (1986) J. Biol.

Chem. 261, 4309-4317

Cisek, L. J., and Corden, J. L. (1989) Nature 339, 679-684

2 W. S. Dynan and J. A. Arias, personal communication.
Phosphorylation of RNA Polymerase IIA

Conaway, R. C., and Conaway, J. W. (1988) J. Biol. Chem. 263, 2962-2968

Corden, J. L., Cadena, D. L., Ahearn, J. M., and Dahmus, M. E. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7934-7938

Dahmus, M. E. (1981) J. Biol. Chem. 256, 3332-3339

Dahmus, M. E., and Kedinger, C. (1983) J. Biol. Chem. 258, 2303-2307

Dahmus, M. E., Bartholomew, B., Cadena, D. L., Dahmus, G. K., Kim, W.-Y., Laybourn, P. J., and Payne, J. (1989) in NATO-ASI Series, Activation of Hormone and Growth Factor Receptors (Alexis, M. N., and Sekeris, C. E., ed) pp. 105-117, Kluwer Academic Publishers, Norwell, MA

Davison, B. L., Egly, J.-M., Mulvihill, E. R., and Chambon, P. (1983) Nature 301, 650-656

Guilfoyle, T. J. (1989) Plant Cell 1, 3452-3461

Hawley, D. K., and Roeder, R. G. (1987) J. Biol. Chem. 262, 827-836

Hodo, H. G., and Blatti, S. P. (1977) Biochemistry 16, 2234-2243

Kim, W.-Y., and Dahmus, M. E. (1986) J. Biol. Chem. 261, 14219-14225

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

Kliewer, S., and Dugas, A. (1988) Mol Cell Biol. 8, 3174-3189

Laemmli, U. K. (1970) Nature 227, 680-685

Laybourn, P. J., and Dahmus, M. E. (1989) J. Biol. Chem. 264, 6693-6698

Lee, J. M., and Greenleaf, A. L. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3624-3628

Mitchell, P. J., and Tjian, R. (1989) Science 245, 371-378

Moncollin, V., Miyamoto, N. G., Zheng, X. M., and Egly, J. M. (1986) EMBO J. 5, 2577-2584

Nonet, M., Sweeney, D., and Young, R. A. (1987) Cell 50, 990-915

Payne, J. M., Laybourn, P. J., and Dahmus, M. E. (1989) J. Biol. Chem. 264, 19621-19629

Ptashne, M. (1988) Nature 335, 683-689

Rangel, L. M., Fernandez-Tomas, C., Dahmus, M. E., and Gariglio, P. (1987) J. Virol. 61, 1002-1006

Rangel, L. M., Fernandez-Tomas, C., Dahmus, M. E., and Gariglio, P. (1988) J. Biol. Chem. 263, 19267-19269

Saltzman, A. G., and Weinmann, R. (1989) FEBS Lett. 231, 81-87

Sawadogo, M., and Roeder, R. G. (1984) J. Biol. Chem. 259, 5321-5326

Sigler, P. B. (1988) Nature 333, 310-312

Thompson, N. E., Steinberg, T. H., Aronson, D. B., and Burgess, R. R. (1989) J. Biol. Chem. 264, 11335-11340

Van Dyke, M. W., Roeder, R. G., and Sawadogo, M. (1988) Science 241, 1335-1338

Weil P. A., Segall, J., Harris, B., Ng, S.-Y., and Roeder, R. G. (1979) J. Biol. Chem. 254, 6163-6173

Wray, W., Boulita, T., Wray, V. P., and Hamcock, R. (1981) Anal. Biochem. 119, 197-203

Zehring, W. A., Leeu, J. M., Weeks, J. R., Jokisch, R. S., and Greenleaf, A. L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3608-3702

Zheng, X.-M., Moncollin, V., Egly, J.-M., and Chambon, P. (1987) Cell 59, 301-308