MOLECULAR AND CELLULAR BIOLOGY, May 1992, p. 2250–2259
0270-7306/92/052250-10$02.00/0

Vol. 12, No. 5

The HIP1 Initiator Element Plays a Role in Determining the In Vitro Requirement of the Dihydrofolate Reductase Gene Promoter for the C-Terminal Domain of RNA Polymerase II

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Received 16 September 1991/Accepted 26 February 1992

We examined the ability of purified RNA polymerase (RNAP) II lacking the carboxy-terminal heptapeptide repeat domain (CTD), called RNAP IIB, to transcribe a variety of promoters in HeLa extracts in which endogenous RNAP II activity was inhibited with anti-CTD monoclonal antibodies. Not all promoters were efficiently transcribed by RNAP IIB, and transcription did not correlate with the in vitro strength of the promoter or with the presence of a consensus TATA box. This was best illustrated by the GC-rich, non-TATA box promoters of the bidirectional dihydrofolate reductase (DHFR)-REP-encoding locus. Whereas the REP promoter was transcribed by RNAP IIB, the DHFR promoter remained inactive after addition of RNAP IIB to the antibody-inhibited reactions. However, both promoters were efficiently transcribed when purified RNAP with an intact CTD was added. We analyzed a series of promoter deletions to identify which *cis* elements determine the requirement for the CTD of RNAP II. All of the promoter deletions of both DHFR and REP retained the characteristics of their respective full-length promoters, suggesting that the information necessary to specify the requirement for the CTD is contained within approximately 65 bp near the initiation site. Furthermore, a synthetic minimal promoter of DHFR, consisting of a single binding site for Sp1 and a binding site for the HIP1 initiator cloned into a bacterial vector sequence, required RNAP II with an intact CTD for activity in vitro. Since the synthetic minimal promoter of DHFR and the smallest REP promoter deletion are both activated by Sp1, the differential response in this assay does not result from upstream activators. However, the sequences around the start sites of DHFR and REP are not similar and our data suggest that they bind different proteins. Therefore, we propose that specific initiator elements are important for determination of the requirement of some promoters for the CTD.

Nuclear eukaryotic RNA polymerases (RNAP) are complex, multisubunit enzymes that typically consist of two large subunits and many smaller subunits (see references 37 and 47 for reviews). The two largest subunits of RNAP I, II, and III from many organisms contain regions of homology to each other and to the β and β′ subunits of prokaryotic RNAP. The largest subunit of RNAP II from many different species contains an unusual repetitive carboxy-terminal domain (CTD) that is not found in the largest subunits of RNAP I or III or in bacterial RNAP (see reference 9 for a review). This domain consists of tandem repeats of a seven-amino-acid consensus sequence (Tyr Ser Pro Thr Ser Pro Ser).

Although the CTD has been shown to be essential for growth of *Saccharomyces cerevisiae* (2, 34), drosophila (50), and mouse (4) cells, the function of the CTD is still unclear. One proposed function is to interact with upstream activator proteins. Genetic evidence obtained from yeast cells has shown that RNAP II molecules with longer CTDs can compensate mutations that reduce the activation potential of transcription factor GAL4. Polymerases with short CTDs were no longer activated by GAL4 mutants with reduced activity (1). Another study has shown that several yeast promoters are differentially sensitive to CTD truncations (38). The differential sensitivity correlates with the presence of different upstream activation sequences. For example, transcription driven by the *HIS4* upstream activation sequence was unaffected by deletion of half of the heptapeptide repeats from the CTD, whereas transcription driven by the *INO1* upstream activation sequence was severely reduced when the same RNAP II CTD mutation was used. In vitro, progressive truncation of the yeast RNAP CTD causes progressive loss of activator-dependent transcription with acidic activators (27). The CTD has also been proposed to function by direct binding to DNA via intercalation of tyrosine residues (43). This could affect transcription by changing the DNA structure (e.g., helping to unwind the DNA strands to promote open complex formation) or by providing an anchor for the RNAP to the promoter region of the gene. A third proposal for the function of the CTD involves removal of transcriptional repressors from the DNA. Deletion of the *SIN1* gene, which may encode a nonhistone chromatin component (22), reduces the cold sensitivity of cells with CTD truncation mutations, whereas the full-length CTD is required for growth if *SIN1* is present (36). Although two of these possible functions imply an interaction between the CTD of RNAP II and other proteins, no direct physical interaction between the CTD and an activator or repressor protein has been observed or measured.

Multiple forms of RNAP II have been purified from mammalian cells (see references 37 and 47 for reviews). These forms contain similar smaller subunits but differ in the size of the largest subunit, depending on the state of the CTD. RNAP IIA contains a largest subunit of approximately 220 kDa which is believed to be the primary translation product (10). RNAP II0 contains an extensively phosphorylated largest subunit which migrates as approximately 240 kDa on sodium dodecyl sulfate-polyacrylamide gels. Studies of the adenovirus 2 (Ad2) major late promoter (MLP) have

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TABLE 1. Description of promoters used in these studies

Promoter[a]	Coordinates[b]	Plasmid	Enzyme(s)[c]	Reference
DHFR	−356/+275	pSS625	*Pvu*II	16
CAD	−332/+406	pC76B	*Eco*RI-*Hin*dIII	15
RAF-1	−650/+240	pRAF43	*Eco*RI/*Sac*II	—[d]
H2b	−175/+230	pH2b	*Hin*dIII/*Eco*RI	41
Ad2 MLP	−259/+270	pATS528	*Hae*II	14
REP	−195/+270	pSP65RT10+	*Sma*I-*Eco*RI	13
IRF1	−299/+225	pIRFCAT	*Pst*I	33
CMV MIEP	−806/+15	pCMV	*Eco*RI-*Bam*HI	14
c-MYC	−350/+513	pMP1P2	*Pvu*II	—[d]
β-ACTIN	−242/+515	pHbAPr1neo	*Sma*I	18
DHFR Δ downstream	−270/+20	pDMM285	*Pvu*II	31
DHFR Δ upstream	−65/+52	pDFX120	*Pvu*II	31
DHFR minimal	−56/+9	pGCDI	*Nde*I-*Hin*dIII	31
REP Δ downstream	−195/+14	pDHS205	*Pvu*II	—[d]
REP Δ upstream	−49/+265	pDDD310	*Pvu*II	—[d]
REP minimal	−49/+14	pDHD62	*Pvu*II	40

[a] DHFR (from the murine DHFR gene), CAD (from the Syrian hamster carbamoyl phosphate synthetase-aspartate transcarbamylase-dihydroorotase gene), RAF-1 (from the human c-*raf* gene), H2b (from the human histone H2b gene), Ad2 MLP (the sequences driving the Ad2 major late transcripts), REP (the upstream opposite-strand promoter from the murine DHFR locus that drives the *Rep-1* gene), IRF1 (from the murine interferon regulatory factor gene 1), CMV MIEP (sequences driving the major immediate-early transcripts from cytomegalovirus), c-MYC (from the human c-*myc* gene), β-ACTIN (from the human β-actin gene). The DHFR and REP promoter deletions are described in the text.

[b] Genomic sequences (+1 is the transcription initiation site) present on each template.

[c] Templates were prepared by digestion of the plasmid DNA with the indicated enzyme(s).

[d] See Materials and Methods for a description of this plasmid.

shown that it is the IIA form of the enzyme that assembles into preinitiation complexes at this promoter. Phosphorylation of the CTD to the II0 form then occurs before the first dinucleotide bond is made, and it is RNAP II0 that elongates the transcript. It has been proposed that a cycle of phosphorylation and dephosphorylation occurs during multiple rounds of transcription (23, 24, 35). Kinases that phosphorylate the CTD have been identified (3, 8, 25, 26, 35, 42, 48).

Although it is generally believed that conversion of RNAP IIA to II0 plays an important role in the transcription reaction, conclusions concerning the role of this conversion during transcription have been based on analysis of a promoter, the Ad2 MLP, that does not require the CTD for activity. Several groups have shown that the Ad2 MLP can be accurately and efficiently transcribed by RNAP IIB (7, 20, 46), which contains a 180-kDa largest subunit resulting from proteolytic removal of the CTD (37, 47). Different results may be obtained concerning the role of the CTD if transcription is analyzed using a system that is dependent upon the CTD for activity. Previous results have shown that the GC-rich, non-TATA box promoter driving the dihydrofolate reductase (DHFR) gene depends upon the CTD for transcriptional activity in HeLa nuclear extract (46). We expanded upon these results and found that several other promoters, in addition to that of DHFR, also require the CTD for transcriptional activity. Our data suggest that different initiator proteins determine the difference in transcriptional activity by RNAP IIB.

MATERIALS AND METHODS

Plasmids and DNA constructs. The promoter templates used for in vitro transcriptions are listed in Table 1, and additional notes are detailed here. After digestion of plasmid DNA with the indicated enzymes, templates were purified by polyacrylamide gel electrophoresis and then electroelution. An additional murine DHFR template, used interchangeably with the template described in Table 1, was prepared by digestion of pSS625 with *Hin*dIII and *Eco*RI and contains the same promoter sequences. Similarly, a second Ad2 MLP template was prepared from an *Eco*RI-*Pst*I digest of pATS528. The plasmid containing the human RAF-1 promoter, pRAF43, was created by insertion of an *Eco*RI-*Hin*dIII fragment of pUXCAT (5) into the same sites in pBSM13+. The plasmid containing the human c-MYC promoter, pMP1P2, consists of a *Pvu*II fragment of the human c-*myc* genomic sequence (−350 to +513) cloned into the *Sma*I site of pBSM13+. pDHS205, containing the REP downstream deletion, was created by insertion of an *Eco*RI-*Hin*cII fragment of pDBS321 into *Eco*RI-*Hin*cII-cut pBSM13+. pDBS321 contains a *Bst*NI-*Sma*I fragment (5′ overhangs filled in with the Klenow fragment of DNA polymerase [29]) of pSP65RT10+ cloned into *Hin*cII-cut pBSM13+. pDDD310 (REP upstream deletion) was cloned by insertion of a *Dde*I fragment (5′ overhangs filled in with the Klenow fragment of DNA polymerase) of pSP65RT10+ into *Hin*cII-cut pBSM13+.

Purification of MAb 8WG16. Monoclonal antibody (MAb) 8WG16 has already been characterized (44, 46). The MAb was purified from ascitic fluid and conjugated to CNBr-activated Sepharose 4B (Sigma, St. Louis, Mo.) as previously described (44). Antibody for inhibition of in vitro transcription reactions was purified as previously described (46) and then dialyzed against transcription buffer D (12) at 4°C by using a Microdialyzer System 500 (Pierce, Rockford, Ill.) in accordance with manufacturer instructions. Antibody concentration was determined either by reading the absorbance at 280 nm in 1-cm cuvettes by using an extinction coefficient ($E^{1\%}$) of 13.8 or with a Bio-Rad protein assay kit using bovine serum albumin as the standard.

Purification of RNAP II from calf thymus. Calf thymuses were obtained from Pel-Freez Biologicals (Rogers, Ark.). RNAP IIB was prepared by the method of Hodo and Blatti (19) and dialyzed against transcription buffer D (12) at 4°C by using a Microdialyzer System 500. RNAP IIA was prepared from 500 g of calf thymuses by a modification of the immunoaffinity chromatography procedure described by Thompson et al. (44). Thymuses were partially thawed, sliced

into pieces with a razor blade, and homogenized in 750 ml of buffer A (50 mM Tris-HCl [pH 7.9], 10 mM EDTA, 10 mM ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid [EGTA], 0.2 mM dithiothreitol, 5% [vol/vol] glycerol) in a 4-liter Waring blender (30% output for 1 min, then 70% output for 1 min). Another 750 ml of cold buffer A was added and blended at 70% output for 30 s. The extract was filtered through Miracloth (Calbiochem, San Diego, Calif.), and the volume was increased to 2,000 ml with cold buffer A. Solid ammonium sulfate was added (29.1 g/100 ml) and mixed at 4°C for about 1 h. The precipitate was collected by centrifugation (10,000 × *g*, 30 min, 4°C) and suspended in cold buffer B (50 mM Tris-HCl [pH 7.9], 0.1 mM EDTA, 15% [vol/vol] glycerol) until the ammonium sulfate concentration was 0.3 M (usually around 1,000 ml). Then 5.5 μl of a 10% (vol/vol) solution of polyethyleneimine (Polymin P; BASF, Charlotte, N.C.) was added per ml of extract and stirred for 10 min at 4°C. The precipitate was removed by centrifugation (10,000 × *g*, 15 min, 4°C); the supernatant was collected and diluted with cold buffer B until the ammonium sulfate concentration was 150 mM. Approximately 80 ml of DEAE–Sepharose CL-6B (equilibrated in buffer B containing 150 mM ammonium sulfate) was added and mixed gently for about 3 h at 4°C. The DEAE-Sepharose was washed in a batch with 500 ml of buffer B containing 150 mM ammonium sulfate, packed into a column, and eluted with buffer B containing 400 mM ammonium sulfate. UV-absorbing fractions were pooled and precipitated with ammonium sulfate (24 g/100 ml). After being stirred for 20 min at 4°C, the precipitate was collected by centrifugation (5,600 × *g*, 15 min, 4°C). The pellet was suspended in 25 ml of TE buffer (50 mM Tris-HCl [pH 7.9], 0.1 mM EDTA). The solution was clarified by centrifugation (5,600 × *g*, 15 min, 4°C) and gently mixed with 3 ml of 8WG16-Sepharose (equilibrated with TE containing 200 mM ammonium sulfate) for 1.5 to 2 h at 4°C. The resin was washed with 100 ml of TE containing 200 mM ammonium sulfate, 100 ml of TE containing 50 mM ammonium sulfate, and 100 ml of TE containing 200 mM ammonium sulfate. The RNAP IIA was eluted with three sequential elutions of 3 ml each of TE containing 500 mM ammonium sulfate and 30% (vol/vol) ethylene glycol (catalog no. 321455-8; Aldrich, Milwaukee, Wis.) as described previously (44). Purified RNAP IIA was dialyzed against TE containing 1 mM dithiothreitol and 20% (vol/vol) ethylene glycol for 2 h at 4°C and then against storage buffer (50 mM Tris-HCl [pH 7.9], 0.1 mM EDTA, 1 mM dithiothreitol, 150 mM ammonium sulfate, 50% [vol/vol] glycerol) for 2 h at 4°C. The RNAP IIA in storage buffer was severalfold less concentrated than the RNAP IIB prepared by the method of Hodo and Blatti (19) and was concentrated by using Centricon-30 microconcentrators (Amicon, Beverly, Mass.) as recommended by the manufacturer.

In vitro transcriptions. HeLa cells were grown in suspension at 37°C in Joklik-modified minimal essential medium (GIBCO, Gaithersburg, Md.) containing 5% (vol/vol) defined-supplemented calf serum (HyClone, Logan, Utah) or in α-minimal essential medium (GIBCO) containing 5% (vol/vol) fetal bovine serum (GIBCO). Nuclear extract from cells grown in the latter gave DHFR transcriptional activity two- to threefold higher than extracts from cells grown in the former. Cells were harvested at a density of 2×10^5 to 4×10^5/ml and frozen as described by Borelli (5a). Nuclear extract was made as previously described (12).

RNAP II activity was inhibited by incubating 60 μg of nuclear extract with 0 to 1,000 μg of MAb 8WG16 per ml at 4°C for 1 h in 12 μl of transcription buffer D (12). Reactions that received no exogenous RNAP II received 3 μl of buffer D, $MgCl_2$ (6 mM final concentration), and a promoter template fragment (5 nM final concentration). Reactions to be restimulated with RNAP IIB received 25 to 500 ng of purified polymerase in a volume of 3 μl of buffer D, $MgCl_2$, and a DNA template as described above. Restimulation with RNAP IIA was similar except that this polymerase was in storage buffer (described above). Therefore, control reactions for RNAP IIA restimulation experiments had 3 μl of storage buffer added instead of buffer D. After addition of RNAP II, all reactions followed the standard in vitro transcription protocol (25-μl total volume) previously described (17; see also Fig. 1). A radiolabeled RNA (116 nucleotides) was added to each reaction mixture with the stop buffer as a control for recovery during sample handling. RNA products from each promoter were identified on the basis of their migration next to DNA size markers on 8 M urea–5% polyacrylamide gels. Primer extension reactions did not include the internal standard RNA and were performed as previously described (31). Inhibited and restimulated reactions were always compared with uninhibited reactions done in the same experiment. Experiments with RNAP IIB always included a positive control (such as the Ad2 MLP) for IIB restimulation activity. Results were quantitated by measuring the radioactivity of the bands in the gel with a Betascope 2000 radioanalytical blot analyzer (Betagen, Waltham, Mass.).

Gel mobility shift assays. Binding of HIP1 to DNA was performed by incubating 5 ng of purified HIP1 with 5 ng of poly(dA-dT)–poly(dA-dT) and 1 ng of radiolabeled probe in 60 mM KCl–24 mM Tris HCl (pH 7.4)–6 mM $MgCl_2$–5% Ficoll (approximate molecular weight, 400,000)–0.12 mM EDTA–0.3 mM dithiothreitol in a total volume of 11 μl for 5 min at room temperature. Competitor DNA (7.5 to 30 ng [see the legend to Fig. 7]) was incubated with all reagents except the probe for 5 min at room temperature, the probe was added, and the incubation was continued for 5 min. The 81-bp probe, containing DHFR promoter sequences from −38 to +20, was prepared by digesting pDMM285 (31) with *Hin*dIII and *Fsp*I and filling in the 5′ overhang with the Klenow fragment of DNA polymerase and [α-^{32}P]dATP as previously described (29). The probe and competitor DNAs were purified by polyacrylamide gel electrophoresis and then electroelution of promoter-containing fragments. The 360-bp DHFR competitor (containing DHFR sequences from −200 to +153) was a *Hin*dIII-*Bgl*I fragment of pHH361 (16). The 444-bp REP competitor (containing REP sequences from −49 to +14) was the minimal promoter template described in Table 1. The 350-bp CAD competitor (containing CAD sequences from −113 to +126) was an *Eco*RI-*Hin*dIII fragment of pC350.1 (15). The 400-bp H2b competitor was the in vitro transcription template described above. The 310-bp RAF-1 competitor (containing RAF-1 sequences from −162 to +108) was prepared by digesting pRSP267 with *Eco*RI and *Pst*I. pRSP267 was created by inserting an *Sma*I-*Pvu*II fragment of pRAF43 (described above) into the *Sma*I site of pUC19. The reaction mixtures were electrophoresed for 60 to 120 min at 180 V on a 4% polyacrylamide gel which had been pre-electrophoresed for 90 min at 180 V. The gel buffer was 0.25× TBE (29).

RESULTS

DHFR requires RNAP IIA for efficient function in vitro. To investigate the abilities of different forms of RNAP II to transcribe various promoters, we used an assay, the RNAP

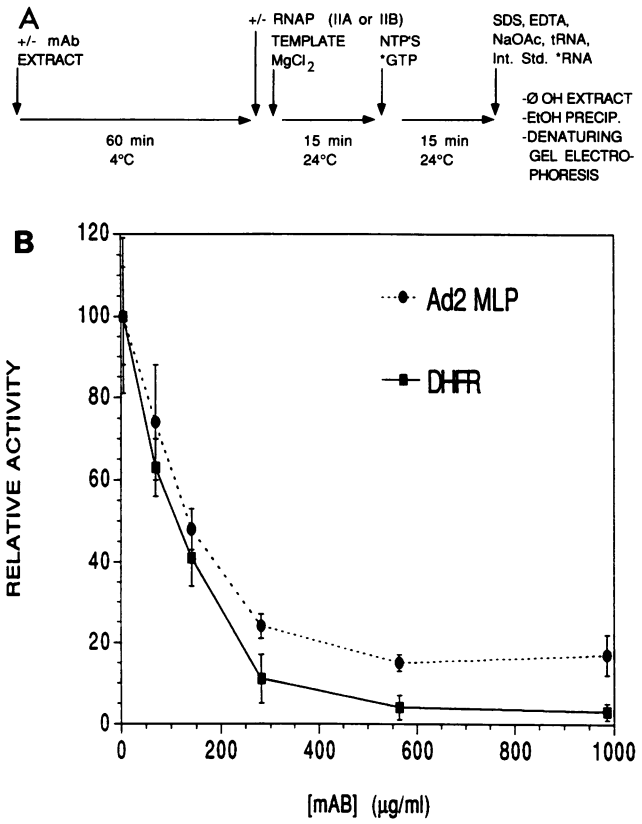

FIG. 1. (A) Schematic of the RNAP II inhibition-restimulation assay. RNAP II activity in HeLa nuclear extract was inhibited by 60 min of incubation with MAb 8WG16. Transcription was restimulated by addition of RNAP IIA or IIB, followed by a standard transcription reaction. Int. Std., internal standard; NTP'S, nucleoside triphosphates; SDS, sodium dodecyl sulfate; NaOAc, sodium acetate; EtOH PRECIP., ethanol precipitation. (B) Inhibition of in vitro transcription. Transcription from the Ad2 MLP and the DHFR promoter was inhibited with increasing quantities of MAb 8WG16. Transcriptional activity was plotted relative to the level of uninhibited transcription, which was approximately the same for the two promoters. Each point is the average from two experiments, and the error bars indicate the standard error of the mean.

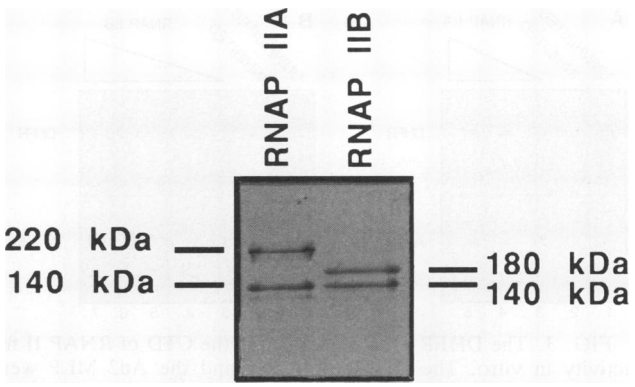

FIG. 2. RNAP IIA and IIB differ in the sizes of their largest subunits. Representative samples of the calf thymus RNAP IIA and IIB used in this study were analyzed by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. The sizes of the two largest subunits of each are indicated.

II inhibition-restimulation assay, that involves inhibition of transcription in HeLa nuclear extract with MAbs specific for the CTD of RNAP II (Fig. 1A) (46). The mechanism by which MAbs inhibit transcription (11, 46) is thought to involve steric hindrance of RNAP II. The MAb used by Thompson et al. (46) does not inhibit the elongation activity of wheat germ RNAP II, suggesting that the polymerase is not removed from solution by aggregation of polymerase-antibody complexes. In the experiments described here, the antibody was preincubated with extract for 1 h before template DNA and a buffer containing or lacking exogenous RNAP IIA or IIB were added. Preinitiation complexes were allowed to form for 15 min, and the nucleotides were added and incubation was continued for another 15 min. The reactions were stopped, and the RNA products were purified and analyzed by denaturing polyacrylamide gel electrophoresis. MAb 8WG16 (44, 46) has been shown to inhibit transcription from the Ad2 MLP in HeLa nuclear extract (45). Results in Fig. 1B demonstrate that transcriptions from two different RNAP II promoters, the DHFR promoter and the Ad2 MLP, were inhibited similarly by this MAb. Different preparations of extract differed in the level of response to a given MAb concentration, requiring a titration with each extract preparation. Similarly, different MAb preparations had different activities. However, the shapes of inhibition curves with different extracts or antibody preparations were qualitatively similar. For the experiments in this report, both promoters were inhibited to approximately the same level (10 to 20% of the level of uninhibited transcription) and were not maximally inhibited.

We next tested whether the DHFR promoter and the Ad2 MLP could be transcribed by different forms of RNAP II. Restimulation of transcription was assayed after addition of either RNAP IIA or RNAP IIB. Figure 2 demonstrates the size difference between the two largest subunits of representative samples of the RNAP IIA and IIB used in the assays. To demonstrate that the inhibition was specific for RNAP II, RNAP IIA was added to inhibited transcription reactions of the DHFR promoter and the Ad2 MLP (Fig. 3A). Both promoters responded similarly to RNAP IIA; transcription was restimulated approximately 2.5-fold above the level of inhibited transcription. Restimulation greater than 2.5- to 3.0-fold was not seen, even with RNAP IIA that had been microdialyzed into transcription buffer D (6). The reason for the difference in restimulation activity (2.5 versus 4.0) between RNAP IIA and IIB (see below) on the Ad2 MLP transcription is not known, but it may reflect differences in the methods of purification of the two forms of RNAP II. Primer extension analysis of the RNA products indicated that RNAP IIA correctly initiated at the promoters (6, 46). Also, addition of KSCN-treated RNAP II with an intact CTD to the MAb-inhibited reactions did not result in restimulation of DHFR promoter or Ad2 MLP activity (46). This control ensured that the added RNAP IIA did not restimulate transcription in the inhibited extracts by titrating MAb off endogenous HeLa cell RNAP II. When RNAP IIB was added to the inhibited reactions (Fig. 3B), transcription from the DHFR promoter was not restimulated above inhibited levels whereas transcription from the Ad2 MLP was restimulated approximately fourfold above the level of inhibited transcription. Using less MAb 8WG16, such that transcription was inhibited by only 50%, also did not allow restimulation of the DHFR promoter with RNAP IIB (6). Because RNAP IIB is stored in transcription buffer, the lack

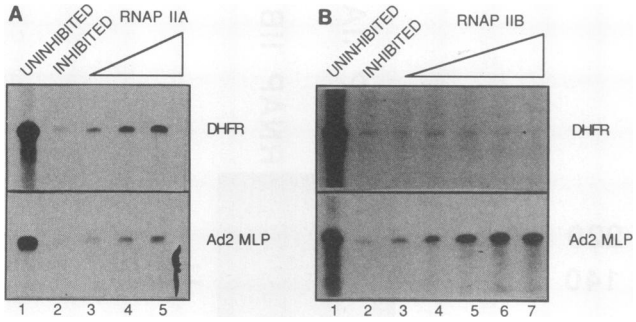

FIG. 3. The DHFR promoter requires the CTD of RNAP II for activity in vitro. The DHFR promoter and the Ad2 MLP were analyzed in the RNAP II inhibition-restimulation assay. (A) Transcription from the DHFR promoter and the Ad2 MLP was inhibited with 125 μg of MAb 8WG16 per ml (compare lanes 1 and 2), followed by addition of 100, 200, or 300 ng of purified calf thymus RNAP IIA (lanes 3, 4, and 5, respectively). Both promoters responded similarly and were restimulated up to 2.5-fold above the level of inhibited transcription. (B) Transcription from the DHFR promoter and the Ad2 MLP was inhibited with 250 μg of MAb 8WG16 per ml (compare lanes 1 and 2), followed by addition of 25, 62.5, 125, 250, or 500 ng of purified calf thymus RNAP IIB (lanes 3, 4, 5, 6, and 7, respectively). Transcription from the DHFR promoter was not restimulated, whereas transcription from the Ad2 MLP was restimulated up to fourfold above the level of inhibited transcription.

of transcription from the DHFR promoter with IIB cannot be due to the composition of the buffer. Furthermore, varying the time allowed for either complex formation or elongation from 0 to 45 min did not affect the results (6).

The different responses of the DHFR promoter and the Ad2 MLP in the RNAP II inhibition-restimulation assay could be explained by differences in the stability of preinitiation complexes that form on these two promoters. If stable complexes could form on the DHFR promoter in the presence of a MAb, then this promoter would be blocked for transcription from exogenous RNAP II. If such complexes did not form on the Ad2 MLP, then transcription by exogenous RNAP II would not be blocked. However, in experiments in which preinitiation complexes formed in nuclear extract on the DHFR promoter were challenged with a second DHFR promoter-containing template, transcription from both templates occurred (39), suggesting that extremely stable complexes do not form on the DHFR promoter. Our data from the RNAP II inhibition-restimulation assay confirmed the observations of Thompson et al. (46) and indicated that the DHFR promoter requires RNAP II with an intact CTD for efficient function in vitro.

The DHFR promoter is not unique in its requirement for the CTD. To determine whether other promoters also require an intact CTD for activity in vitro, we surveyed eight other RNAP II promoters of both cellular and viral origins in the RNAP II inhibition-restimulation assay (Table 2). Four of the eight promoters tested contained a consensus TATA box, whereas the remainder had no apparent TATA sequence spaced approximately 30 bp upstream of the transcription initiation site. As with the DHFR promoter, the CAD, RAF-1 and H2b promoters all required the CTD for activity in vitro. The other promoters, REP, IRF1, CMV MIEP, c-MYC, and β-ACTIN, all were transcribed by RNAP IIB. Transcription of the non-TATA box REP and IRF1 promoters demonstrated that a TATA box was not necessary for restimulation by RNAP IIB. A TATA box was also not sufficient for IIB restimulation, since the TATA box-containing H2b promoter was not transcribed by RNAP IIB. Furthermore, we found no correlation between RNAP IIB restimulation and the in vitro strengths (as measured by accumulation of full-length products in uninhibited transcription reactions) of the promoters. A correlation between restimulation with IIB and a binding site for a particular transcription factor could not be made because it is not known what transcription factors activate several of these promoters in HeLa nuclear extract. However, it is clear from these experiments that DHFR is not unique in its requirement for the CTD in vitro.

TABLE 2. DHFR is not unique in its requirement for the CTD of RNA polymerase II

Promoter	IIB restimulation[a]	IIA restimulation[a]	TATA box[b]	Strength[c]
DHFR	No	Yes	No	++
CAD	No	Yes	No	++
RAF-1	No	Yes	No	+
H2b	No	Yes	Yes	+
Ad2 MLP	Yes	Yes	Yes	++
REP	Yes	Yes	No	++
IRF1	Yes	Yes	No	+++
CMV MIEP	Yes	Yes	Yes	+++
c-MYC	Yes	Yes	Yes	+
β-ACTIN	Yes	ND	Yes	+

[a] The promoters listed were tested for responses to 250 ng of RNAP IIB and 300 ng of RNAP IIA in the RNAP II inhibition-restimulation assay. The β-ACTIN promoter was not assayed (ND) for restimulation by RNAP IIA.
[b] "Yes" indicates the presence and "No" indicates the lack of a consensus TATA box spaced approximately 30 bp upstream of the initiation site.
[c] The relative strength of the promoter in vitro. Each plus sign refers to approximately a 5- to 10-fold difference in the levels of full-length RNA transcript produced in an uninhibited reaction.

The bidirectional DHFR-REP locus. To define more precisely the sequences that specify the CTD requirement, we focused on two GC-rich, non-TATA box promoters that behaved differently in the RNAP II inhibition-restimulation assay, the DHFR promoter (which requires the CTD) and the REP promoter (which does not require the CTD). In the mouse genome, the promoters for the DHFR and REP genes are transcribed divergently from within the same locus, with the initiation sites located approximately 690 bp apart (28, 40). The DHFR gene encodes a key enzyme in the de novo synthesis of glycine, purines, and thymidylate. The REP promoter drives a gene with homology to bacterial genes involved in DNA mismatch repair (28). This locus is useful since transcription can be analyzed from the two promoters contained on one template DNA fragment that produces two different-size runoff products. We have assayed the responses of the DHFR and REP promoters contained on a single template fragment and on separate fragments and found that the REP promoter, but not the DHFR promoter, was able to be transcribed by RNAP IIB (reference 6 and Table 2, respectively). Both of these promoters lack a consensus TATA box and contain multiple binding sites for transcription factor Sp1. In vitro, the DHFR and REP promoters require similar optimum transcription conditions (17, 40). The low-temperature optimum for transcription (24°C) has been found to correlate with a temperature-sensitive DNA-binding activity of Sp1, and purified Sp1 can restore activity of the DHFR and REP promoters in an extract that has been heat treated at 40°C (6, 14). These two promoters also had similar activities in uninhibited transcription reactions (Table 2). We also found that the accumulation

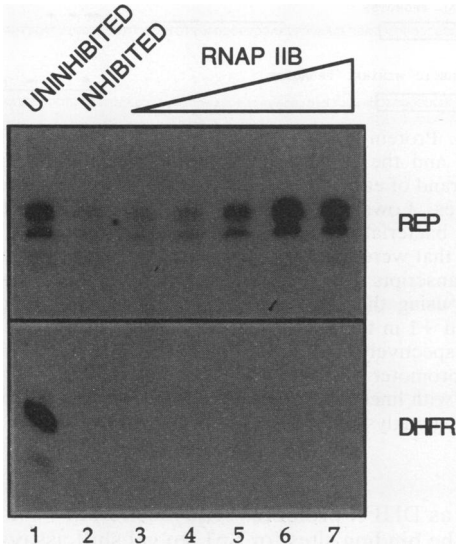

FIG. 4. Primer extension analysis of the responses of the DHFR and REP promoters to restimulation by RNAP IIB. Transcription from the DHFR and REP promoters was inhibited with MAb 8WG16 (compare lanes 1 and 2), and increasing amounts of RNAP IIB were added (lanes 3 to 7) as described in the legend to Fig. 3B. The RNA transcripts were analyzed by primer extension; the extended products from the REP and DHFR primers are indicated. The REP promoter was restimulated up to 4.5-fold above the level of inhibited transcription, whereas transcription from the DHFR promoter was not restimulated.

of full-length transcripts from the REP and DHFR promoters was the same when incubation of extract and promoter DNA was varied from 0 to 45 min (6). This finding suggests that the kinetics of complex formation of these two promoters are similar. The only functional difference we observed between these promoters is their differential responses in the RNAP II inhibition-restimulation assay. Thus, the bidirectional DHFR-REP locus is an ideal system for identifying what determines the requirement of a promoter for the CTD of RNAP II in vitro.

In experiments described thus far, promoter activity in the RNAP II inhibition-restimulation assay was measured by accumulation of a full-length RNA product in runoff transcription assays. We also analyzed the RNA products of the DHFR and REP promoters by primer extension to test whether RNAP IIB was initiated at the correct sites in the REP promoter. Primer extension analysis (Fig. 4) showed that, as in the runoff assays, the REP promoter was transcribed by RNAP IIB. The sizes of the primer extension products demonstrated that RNAP IIB accurately initiated at the REP promoter, utilizing the same start sites observed both in vitro and in vivo (40). However, primer extension analysis showed no restimulation of the DHFR promoter by RNAP IIB above inhibited levels (Fig. 4). Since the primer for the DHFR transcript binds only 60 nucleotides from the initiation site, this analysis would detect short products. Thus, it is unlikely that absence of transcription of the DHFR template by RNAP IIB is due to pausing and/or terminating of the polymerase during elongation (see also below).

All promoter deletions of DHFR and REP retain their differential requirements for the CTD. In dissecting the requirements for the CTD in vitro, we considered two possibilities: a DNA-binding negative regulator blocks the ability of RNAP IIB to transcribe the DHFR promoter or a DNA-binding positive regulator allows the REP promoter to be transcribed by IIB. Removal of a negative regulator, *SIN1*, has been reported to restore the ability of RNAP II with a shortened CTD to transcribe the *HO* gene in *S. cerevisiae* (36). Other studies suggest a positive interaction between the CTD and transactivating factors (1, 27, 38). We tested whether either of these mechanisms operated in our system by analyzing a parallel series of deletions of the DHFR and REP promoters in the RNAP II inhibition-restimulation assay (Fig. 5). Downstream deletions fused the same vector sequences to both promoters just downstream of their initiation sites, and upstream deletions deleted all Sp1-binding sites except the one closest to the initiation site of each promoter. All Sp1-binding sites could not be removed, since at least one is required for the murine DHFR promoter to function in vitro (16). A synthetic DHFR minimal promoter and a REP minimal promoter also were tested. The synthetic DHFR minimal promoter (31) consists of a single binding site for Sp1 and a binding site for initiator protein HIP1 cloned into the polylinker of pUC19. The REP minimal promoter (−49 to +14 relative to the initiation site) (40) includes a single Sp1 site and an additional protein-binding site which has been identified by DNase I footprinting analysis just upstream (−23 to −7) of the start sites (30). Responses of the DHFR and REP promoter deletion constructs to RNAP IIB in the RNAP II inhibition-restimulation assay are shown in Fig. 5C and D, respectively. Uninhibited reactions for the indicated deletion constructs are in lanes 1, 4, 7, and 10. Reactions in lanes 2, 3, 5, 6, 8, 9, 11, and 12 received 125 (Fig. 5C) or 250 (Fig. 5D) μg of MAb 8WG16 per ml. Reactions in lanes 3, 6, 9, and 12 received the MAb plus 250 ng of RNAP IIB. The DHFR promoter deletions shown were analyzed in runoff transcription experiments, whereas the products of the REP promoter deletions were assayed by primer extension analysis. None of the DHFR promoter deletions were transcribed by RNAP IIB (Fig. 5C), suggesting that there was no repressor-binding site in the DHFR promoter that blocked its ability to be transcribed by this form of polymerase. All of these promoter deletions were transcribed by RNAP IIA in control reactions (6). In contrast to the response of the DHFR promoter deletions, all REP promoter deletions were transcribed by RNAP IIB (Fig. 5D). These data indicated that the nonessential elements of both the DHFR and REP promoters could be deleted without changing their responses in the RNAP II inhibition-restimulation assay and that the minimal promoter constructs of DHFR and REP contain the information necessary to determine the response in this assay. Therefore, since the synthetic DHFR minimal promoter consists of only the Sp1- and HIP1-binding sites separated by polylinker DNA, the requirement for the CTD of RNAP II of this promoter is specified by one of these two elements.

DHFR and REP use different initiator proteins. The protein-binding sites in the DHFR and REP minimal promoters are shown schematically in Fig. 6. As mentioned above, both promoters are activated by transcription factor Sp1 and both also contain an additional protein-binding site at or near the transcription initiation site. To determine whether the proteins that bind these additional sites in the two promoters are different, gel shift competitions were performed (Fig. 7). A protein (HIP1) that binds the initiator element in the DHFR promoter has been purified from HeLa cells by DNA affinity chromatography (32). The probe is a fragment of the DHFR promoter (−38 to +20) that includes the HIP1-binding site

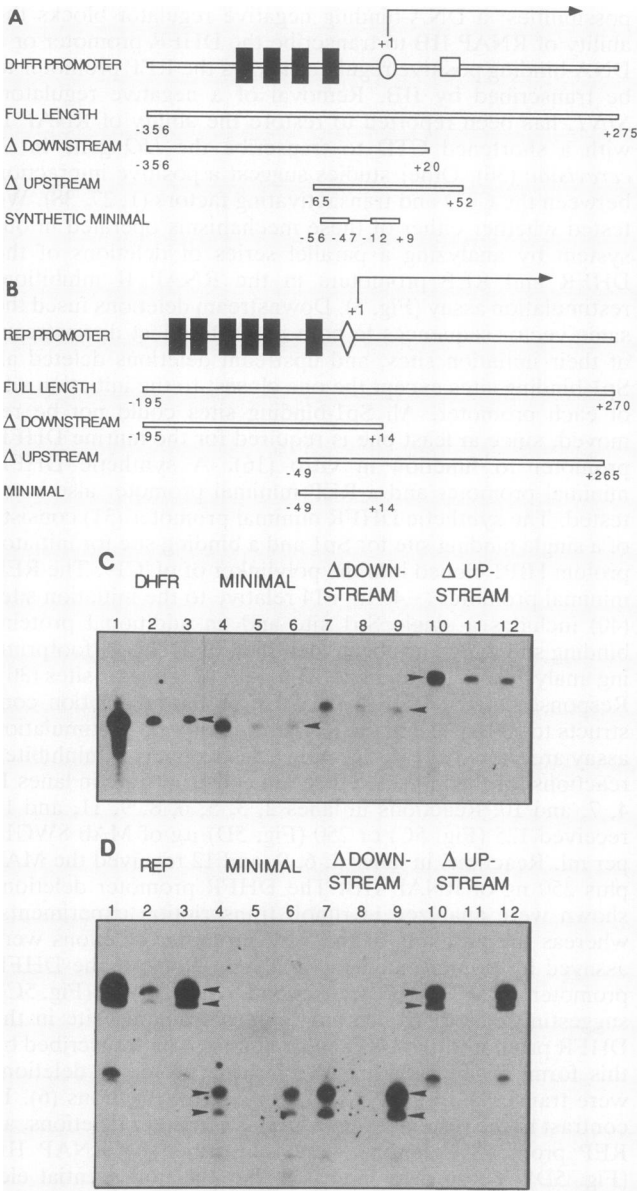

FIG. 5. All DHFR and REP promoter deletions retain the characteristics of their full-length promoters in the RNAP II inhibition-restimulation assay. Schematic diagrams of the DHFR (A) and REP (B) promoters indicate known protein-binding sites. Filled black rectangles are binding sites for transcription factor Sp1, the open oval is the HIP1 initiator element in the DHFR promoter, and the open square and open diamond are additional protein-binding sites identified by DNase I footprinting in the DHFR and REP promoters. The open rectangles depict the promoter sequences (coordinates are indicated below each construct) contained in the DHFR and REP deletion constructs that were analyzed in the RNAP II inhibition-restimulation assay. For the synthetic DHFR minimal promoter, the solid line represents the bacterial vector sequences that separate the Sp1- and HIP1-binding sites (DHFR sequences −56 to −47 and −12 to +9, respectively). Responses of the DHFR (C) and REP (D) promoter deletion constructs to RNAP IIB in the RNAP II inhibition-restimulation assay are shown. Uninhibited reactions for the indicated deletion constructs are in lanes 1, 4, 7, and 10. Reactions in lanes 2, 5, 8, and 11 received 125 (C) or 250 (D) μg of MAb 8WG16 per ml. Reactions in lanes 3, 6, 9, and 12 received the MAb plus 250 ng of RNAP IIB. The DHFR promoter deletions shown were analyzed in runoff transcription experiments, whereas the products of the REP promoter deletions were assayed by primer extension analysis.

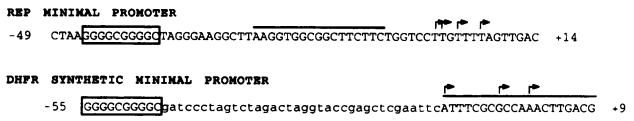

FIG. 6. Protein-binding sites in the synthetic DHFR minimal promoter and the REP minimal promoter. The sequence of the coding strand of each minimal promoter is listed with the promoter coordinates. Lowercase letters in the DHFR minimal promoter represent bacterial vector sequences. Arrows represent the initiation sites that were identified by comparing primer extension products of transcripts from the minimal promoters to DNA sequencing reactions using the same primers (6, 31). Previous studies have designated +1 in the DHFR and REP promoters as the furthest 3′ and 5′ (respectively) start sites utilized (6, 31). The Sp1-binding site in each promoter is boxed. The additional protein-binding sites indicated with lines above the sequence were identified by DNase I footprinting analysis (30, 31).

(defined as DHFR promoter sequences from −9 to −1 [32]) but not the binding sites for Sp1. In gel shift assays, purified HIP1 caused the appearance of a band of retarded mobility (lane 2) relative to the free probe (lane 1). Promoter fragments from DHFR and REP were then used as competitors for HIP1 binding. Addition of 7.5 ng (approximately 1.5-fold molar excess) of a DHFR promoter fragment containing the HIP1-binding site decreased the amount of the shifted band by approximately 95% (lane 3), and addition of 15 ng abolished the shift (lane 4). In contrast, there was no dose-dependent reduction of HIP1 binding to the probe in the presence of 7.5 and 15 ng of a fragment containing the REP minimal promoter (lanes 5 and 6). We also tested 30 ng of the REP promoter fragment and saw no competition for binding by HIP1 (6). These data indicate that HIP1 does not bind the REP minimal promoter, suggesting that distinct proteins bind the additional sites in the two minimal promoters.

We also determined whether HIP1 bound to the other promoters that required the CTD of RNAP II. The RAF-1 promoter contains sequences similar to the HIP1-binding

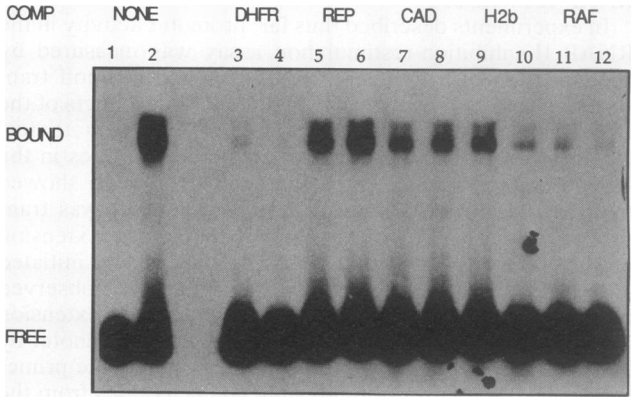

FIG. 7. The REP and DHFR promoters bind different initiator proteins. No protein (lane 1) or 5 ng of purified HIP1 protein (lane 2) was added to a radiolabeled DNA probe containing the DHFR initiator element, and the reaction mixtures were subsequently loaded onto prerunning nondenaturing polyacrylamide gels. Binding of HIP1 to the DHFR probe was competed for by 7.5 (lanes 3, 5, 7, 9, and 11) and 15 (lanes 4, 6, 8, 10, and 12) ng of fragments from the indicated promoters. The positions of the free and bound probes are indicated on the left. COMP, competitor.

site (an eight-of-nine match from +13 to +22, relative to the transcription initiation site at +1), and a fragment containing promoter sequences from −162 to +108 did compete for HIP1 binding (Fig. 7, lanes 11 and 12). The H2b promoter also has sequences similar to the HIP1 site (a five-of-nine match from −24 to −16), and a fragment containing promoter sequences from −175 to +230 competed slightly for HIP1 binding (lanes 9 and 10). Although these data suggest that HIP1 can bind in the RAF-1 promoter and perhaps the H2b promoter, it is not known whether HIP1 binding to these sites is of functional significance. A fragment of the CAD promoter containing sequences from −113 to +126 did not compete in a dose-dependent manner for HIP1 binding (lanes 7 and 8). This fragment contains a functional CAD promoter (15) and thus includes all of the *cis* elements necessary for in vitro transcription. Because the CAD promoter fragment did not compete for HIP1 binding, it is unlikely that HIP1 plays a role in initiation from this promoter. This finding suggests that HIP1 is not important for all promoters that require the CTD of RNAP II in vitro.

DISCUSSION

We used an RNAP II inhibition-restimulation assay to investigate the function of the CTD of the largest subunit of RNAP II. Our results confirm and extend the results of Thompson et al. (46) and demonstrate that several mammalian promoters require the CTD of RNAP II for efficient function. In the DHFR promoter, it appears that the HIP1 initiator element determines the requirement for the CTD of RNAP II. The requirement for the CTD for transcription of promoters such as DHFR and CAD, which are involved in nucleotide biosynthesis; histone H2b, which is necessary for nucleosome formation; and RAF-1, which is important in signal transduction, may partially explain the essential nature of this domain for cell viability. Importantly, these promoters will provide better model systems for studying the function of the CTD than promoters that do not depend upon the CTD for activity.

To understand which *cis* elements are responsible for the ability or lack of ability to be transcribed by RNAP IIB, we analyzed a series of deletion constructs of the DHFR and REP promoters, which do and do not require the CTD for activity, respectively. All deletions of both the DHFR and REP promoters retained the characteristics of their respective full-length promoters in the RNAP II inhibition-restimulation assay. Deletion of nonessential protein-binding sites from the DHFR promoter did not allow it to be transcribed by RNAP IIB, suggesting that the inability of the DHFR promoter to be transcribed by RNAP IIB is not due to the presence of a binding site for a repressor, similar to *SIN1* in *S. cerevisiae* (36). Our results do not rule out the possibility that the CTD is required to remove a specific repressor protein that binds to the DHFR initiator element and blocks the binding of HIP1. This model could be tested in a transcription system reconstituted from purified factors; however, no such system has been shown to transcribe GC-rich non-TATA box promoters such as the DHFR and REP promoters. We also cannot rule out the possibility that the DHFR initiator element is a binding site for the CTD. However, the CTD has been shown only to bind to DNA nonspecifically (36, 43).

Analysis of deletions that fuse the DHFR and REP promoters to similar plasmid DNAs allows us to rule out the possibility that the different responses in the RNAP II inhibition-restimulation assay are due to the transcribed sequences. This conclusion is supported by other studies that have demonstrated that RNAP II molecules having different forms of the largest subunit have similar activities in elongation assays on deproteinized templates (21, 27). Also, MAbs specific for the CTD do not inhibit the elongation by purified wheat germ RNAP IIA on denatured calf thymus DNA (46). We cannot, however, rule out the possibility that the first 10 to 20 bp that are transcribed determine the requirement for the CTD in this assay.

The synthetic DHFR minimal promoter and the REP minimal promoter behaved identically to their respective full-length promoters in the RNAP II inhibition-restimulation assay; the DHFR synthetic promoter, but not the REP minimal promoter, required the CTD of RNAP II for activity. Since the synthetic DHFR promoter consists of only two elements, the Sp1- and HIP1-binding sites, one of these elements must specify the requirement of this promoter for the CTD. Both the DHFR and REP minimal promoters are activated by transcription factor Sp1, indicating that the differential requirements for the CTD do not lie in the transcriptional activator. In support of this conclusion, it has been shown that the CTD is not required for Sp1 to activate transcription in vitro (49). It is therefore likely that the different responses in the RNAP II inhibition-restimulation assay are determined in both minimal promoters by the second protein-binding site: that for the HIP1 initiator protein in the DHFR promoter (31, 32) and that for an unknown protein(s) in the REP promoter. Because a single Sp1 cannot function to specify the start site of transcription, it is likely that the other protein binding in the REP minimal promoter, by analogy to the DHFR minimal promoter, is the initiator. Also, insertions between the Sp1-binding site and the second binding site in the REP minimal promoter do not alter the transcriptional start sites (6), indicating that the REP initiator is contained within the promoter sequences from −26 to +14. Further experiments to determine whether this protein-binding site functions as an initiator are in progress. Binding of purified HIP1 to the DHFR initiator element in a gel shift assay cannot be competed for by a fragment including the REP minimal promoter sequences, suggesting that the second protein that binds to the REP minimal promoter is not the same as the HIP1 protein. Since the DHFR and REP minimal promoters contain the same activator but different initiators, we propose that different initiator-binding proteins may determine whether some promoters require the CTD.

There are several possible mechanisms to account for the fact that transcription mediated by the HIP1 initiator element requires the CTD of RNAP II for promoter function, whereas transcription mediated by the REP initiator does not require the CTD. For example, the protein that binds near the REP initiation site may contain a domain which can functionally substitute for the CTD. Alternatively, the HIP1 protein may interact directly or indirectly with the CTD to form a preinitiation complex, whereas the protein that binds in the REP promoter may interact with another part of the polymerase to form a preinitiation complex. The HIP1 protein has been purified (32), and we are currently purifying the protein that binds to the REP promoter to begin to examine possible interactions between RNAP II and these proteins.

It is clear from other studies that additional mechanisms can determine a requirement for the CTD. Several studies have demonstrated that a specific upstream activator protein can influence the activity of RNAP II containing CTD truncations (1, 27, 38). It is possible that a promoter can be transcribed by RNAP IIB if it contains either an activator

(such as HIS4) or an initiator (such as the REP initiator) that obviates the requirements for the CTD. Multiple levels of regulation may be imposed in vivo such that the CTD is required both for relief of repression (as in the *HO* gene [36]) and for activation through upstream activators and initiators such as HIP1.

ACKNOWLEDGMENTS

We thank Anna L. Means for the generous gift of purified HIP1 protein and for communication of results prior to publication, Rick A. Young for communication of unpublished results, Tom W. Beck for the gift of the RAF-1 promoter construct, Jill E. Slansky and Lynda J. Schilling for sharing unpublished data and reagents, Stephanie McMahon and Holly Duncan for technical assistance, and David A. Brow, Bill M. Sugden, and members of the Farnham laboratory for valuable discussion and helpful comments on the manuscript.

This work was supported by Public Health Service grants CA07175 and CA23076 from the National Institutes of Health. A.B.B. was supported by Public Health Service training grants CA09135 and GM07215 from the National Institutes of Health.

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