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A TATA sequence-dependent transcriptional repressor activity associated with mammalian transcription factor IIA

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In the process of characterizing cellular proteins that modulate basal transcription by RNA polymerase II, we identified a novel repressor activity specific for promoters containing consensus TATA boxes. This activity strongly represses TATA-binding protein (TBP)-dependent transcription initiation from core promoter elements containing a consensus TATA sequence, but activates TBP-dependent transcription from core promoter elements lacking a consensus TATA sequence. Purification of this activity to near homogeneity from rat liver nuclear extracts led to the surprising discovery that it co-purifies closely with mammalian transcription factor IIA (TFIIA). The close association of TATA sequence-dependent transcriptional repressor activity with TFIIA adds a new and unexpected dimension to the already complex picture of this factor's function in transcription by RNA polymerase II.

Key words: core promoter/holo-TFIID/RNA polymerase II/TBP/TFIIA

Introduction

The initiation stage of messenger RNA synthesis is a major site for the regulation of gene expression. In eukaryotes, initiation is controlled by a complex set of interactions between RNA polymerase II, multiple transcription factors and the promoter-regulatory regions of genes. At least four distinct classes of transcription factors are proposed to regulate initiation. The general initiation factors transcription factor IID (TFIID) [either holo-TFIID or its DNA-binding subunit, the TATA-binding protein (TBP)], TFIIB, TFIIE, TFIIF and TFIIH are sufficient to direct a basal level of transcription by RNA polymerase II from the core regions of a large number of promoters (Sawadogo and Sentenac, 1990; Conaway and Conaway, 1993). An additional general factor, TFIIA, stimulates initiation from core promoters in some transcription systems. DNA-binding transcriptional activators, which fall into several broad classes including glutamine-rich activators such as Sp1, proline-rich activators such as CTF/NF1 and acidic activators such as GCN4, are not essential for basal transcription but control the rate of initiation by RNA polymerase II from the core promoter (Johnson and McKnight, 1989; Mitchell and Tjian, 1989; Struhl, 1989; Hahn, 1993).

In addition to the general initiation factors and transcriptional activators, a third class of transcription factors, collectively referred to as mediators, co-activators or positive co-factors, is believed to be essential for transcriptional activation and to mediate the action of activators on the basal transcriptional apparatus (Berger *et al.*, 1990; Kelleher *et al.*, 1990; Pugh and Tjian, 1990; White *et al.*, 1991). Some of these are among the subunits (TBP-associated factors, TAFs) of the TFIID complex (holo-TFIID) (Meisterernst *et al.*, 1990; Dynlacht *et al.*, 1991; Tanese *et al.*, 1991; White *et al.*, 1991, 1993; Pugh and Tjian, 1992; Zhu *et al.*, 1992, 1993; Brou *et al.*, 1993; Weinzierl *et al.*, 1993). Others are readily separable from holo-TFIID and appear to be distinct from the general transcription factors (Flanagan *et al.*, 1991; Meisterernst *et al.*, 1991; Zhu and Prywes, 1992).

Finally, a novel class of factors that repress basal transcription has been identified and proposed to play a key role in transcriptional regulation. This class includes the negative regulatory factors NC1, NC2, Dr1 and Dr2 which appear to block entry of RNA polymerase II and the general factors into the preinitiation complex by binding directly to TBP at the promoter and promoting dissociation of TFIIA or TFIIB from intermediary preinitiation complexes (Meisterernst and Roeder, 1991; Meisterernst *et al.*, 1991; Inostroza *et al.*, 1992). It has been proposed that these negative co-factors may be targets for transcriptional activators or co-activators, which promote their release from repressed preinitiation complexes thereby activating transcription. In this way, negative co-factors may function as molecular switches that maintain genes present in transcriptionally-active chromatin in a repressed but readily activatable state.

We have been engaged in efforts to understand how positive and negative co-factors regulate the formation of the RNA polymerase II preinitiation complex during transcriptional activation. In the course of these studies we identified a novel repressor activity capable of inhibiting basal transcription by RNA polymerase II in a TATA sequence-dependent manner. Purification of this activity to near homogeneity from rat liver nuclear extracts led to the surprising discovery that it co-purifies closely with mammalian TFIIA. Here we report these findings which bring to light a novel function associated with mammalian TFIIA.

Results

Identification of a repressor of core promoter function

An activity that represses TBP-dependent basal transcription by RNA polymerase II from the adenovirus 2 major late (AdML) promoter was identified in side fractions from the purification of TFIIE (Conaway *et al.*, 1991) from rat liver nuclear extracts. TBP is the DNA-binding subunit of the native TATA factor (holo-TFIID) and can replace holo-TFIID in basal but not activated transcription by RNA polymerase II in the presence of the general initiation factors

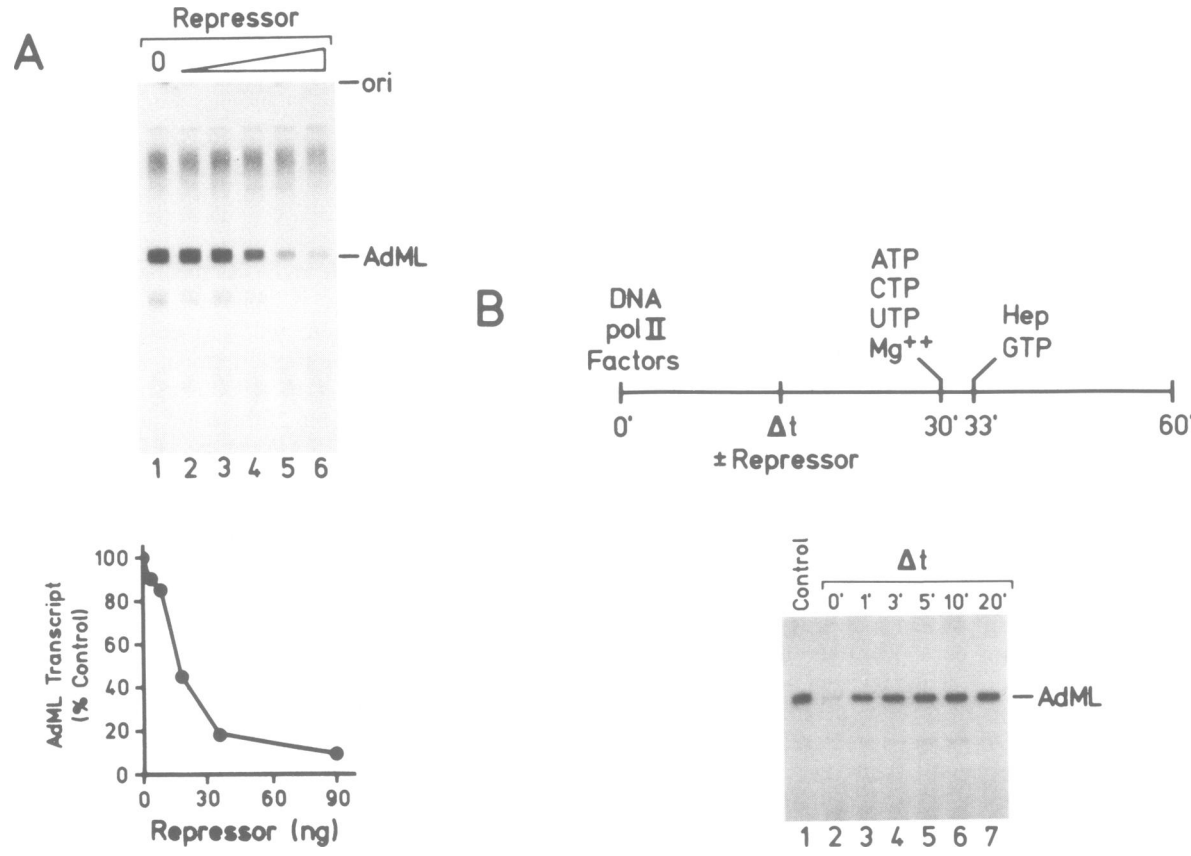

Fig. 1. A repressor of core promoter function. **(A)** Inhibition of basal transcription by RNA polymerase II from the core region of the adenovirus 2 major late promoter. Run-off transcription reactions were carried out as described in Materials and methods with 100 ng of *Nde*I-digested pDN-AdML DNA as template and 30 ng of recombinant yeast TBP as the TATA factor. Approximately 3.6 (lane 2), 9 (lane 3), 18 (lane 4), 36 (lane 5) and 90 ng (lane 6) of repressor (Fraction VIII) were added to reaction mixtures. The control reaction (lane 1) lacks repressor. Quantitation of the amount of run-off transcript synthesized in this experiment was determined by densitometry of the autoradiogram shown in the figure. AdML transcript refers to the relative synthesis, expressed as the percent of the control reaction, of the 254 nucleotide run-off transcript synthesized from the adenovirus 2 major late promoter in pDN-AdML. AdML indicates the position of the 254 nucleotide run-off transcript. **(B)** Repressor inhibits the initiation stage of basal transcription. Run-off transcription reactions were carried out as described in Materials and methods and as illustrated in the figure with 100 ng of *Nde*I-digested pDN-AdML DNA as template and 20 ng of recombinant yeast TBP as the TATA factor. Approximately 200 ng of repressor (Fraction VII) was added to reaction mixtures at the times indicated in the figure. The control reaction (lane 1) lacks repressor. AdML indicates the position of the 254 nucleotide run-off transcript synthesized from the adenovirus 2 major late promoter in pDN-AdML. pol II, RNA polymerase II; Hep, heparin.

TFIIB, TFIIE, TFIIF and TFIIH. As shown in Figure 1A, the addition of increasing amounts of a highly purified repressor fraction to reactions containing DNA template, TBP and saturating amounts of RNA polymerase II, TFIIB, TFIIF, TFIIE and TFIIH resulted in as much as a 10-fold reduction in synthesis of run-off transcripts initiated at the AdML promoter. This activity represses basal transcription since the template (pDN-AdML) used in these reactions includes only the AdML core promoter (−50 to +10 relative to the site of transcription initiation) but lacks AdML promoter sequences (−51 to −63) that mediate transcriptional activation by USF/MLTF (Carthew *et al.*, 1985; Miyamoto *et al.*, 1985; Sawadogo and Roeder, 1985; Moncollin *et al.*, 1986). In this and subsequent experiments, transcription was limited to a single round of initiation by adding heparin, which inhibits transcription initiation but not elongation (Egly *et al.*, 1984; Conaway and Conaway, 1990), shortly after the addition of ribonucleoside triphosphates.

Because of its distinct chromatographic properties this repressor activity appeared to be different to that of the previously identified repressors NC1, NC2, Dr1 and Dr2 (Meisterernst and Roeder, 1991; Meisterernst *et al.*, 1991; Inostroza *et al.*, 1992). As described in more detail below, further investigation led to the surprising discoveries that repressor activity (i) inhibits TBP-dependent transcription in a uniquely TATA sequence-dependent manner and (ii) is chromatographically inseparable from rat TFIIA.

Repressor blocks formation of the functional preinitiation complex

Basal transcription is a multi-stage process that begins with the formation of an RNA polymerase II preinitiation complex, followed by the initiation of RNA synthesis and elongation of nascent RNA transcripts. To determine whether repressor inhibits the assembly of the preinitiation complex or a later stage in transcription, order-of-addition experiments were performed. Preinitiation complexes were assembled at the core region of the AdML promoter by preincubation of *Nde*I-digested pDN-AdML DNA with RNA polymerase II, TBP and initiation factors TFIIB, TFIIF, TFIIE and TFIIH. Repressor was added to the reaction mixtures along with, or at various times after the addition of, RNA polymerase II and initiation factors. The results of these experiments

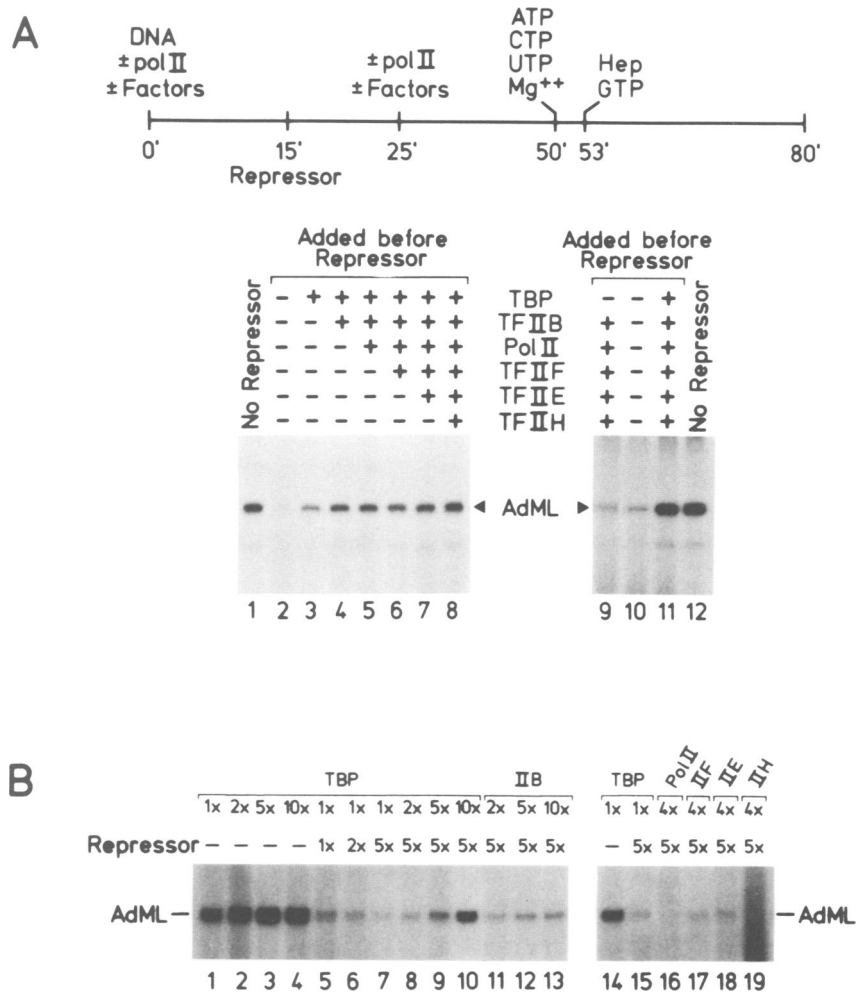

Fig. 2. Effect of repressor on the formation of the preinitiation complex. **(A)** Repressor interferes with an early step in the assembly of the preinitiation complex. Run-off transcription reactions were carried out as described in Materials and methods and as illustrated in the figure with 100 ng of *Nde*I-digested pDN-AdML DNA as template and 20 ng of recombinant yeast TBP as the TATA factor. Approximately 200 ng of repressor (Fraction VII) was added to reaction mixtures as indicated in the figure. Control reactions (lanes 1 and 12) lack repressor. AdML indicates the position of the 254 nucleotide run-off transcript synthesized from the adenovirus 2 major late promoter in pDN-AdML. pol II, RNA polymerase II; Hep, heparin. **(B)** Excess TBP restores basal transcription in the presence of repressor. Run-off transcription reactions were carried out as described in Materials and methods with 100 ng of *Nde*I-digested pDN-AdML DNA as template and 20 ng of recombinant yeast TBP as the TATA factor. RNA polymerase II and transcription factors were purified as described in Materials and methods. The amounts of transcription factor added to reaction mixtures were as follows: 1 × repressor (18 ng of Fraction VIII); 1 × TBP (20 ng of recombinant yeast TBP); 1 × IIB (10 ng of recombinant TFIIB); 1 × pol II (0.01 U of RNA polymerase II); 1 × IIF (15 ng of recombinant TFIIF); 1 × IIE (15 ng of recombinant TFIIE); 1 × TFIIH [25 ng of TFIIH(δ) Fraction VII]. AdML indicates the position of the 254 nucleotide run-off transcript synthesized from the adenovirus 2 major late promoter in pDN-AdML. pol II, RNA polymerase II; IIB, TFIIB; IIE, TFIIE; IIF, TFIIF; IIH, TFIIH(δ).

revealed that the repressor interferes with a step in the assembly of the preinitiation complex. Repressor strongly inhibited transcription when added to the reaction mixtures along with RNA polymerase II and initiation factors (Figure 1B, lanes 1 and 2). Transcription was not significantly affected, however, when repressor was added to the reaction mixtures 1 min or more after the addition of RNA polymerase II and initiation factors, but prior to the addition of ribonucleoside triphosphates (Figure 1B, lanes 3–7). In addition to RNA polymerase II and initiation factors, protection from repressor activity requires DNA (data not shown). Thus, the repressor exerts its inhibitory effect on preinitiation complex formation rather than on the initiation or elongation stages of transcription.

Repressor is an antagonist of TBP function

Biochemical studies have resolved preinitiation complex formation into several distinct steps, beginning with the binding of the TATA factor to the core promoter and culminating with the formation of the complete preinitiation complex containing RNA polymerase II, TFIIB, TFIIF, TFIIE and TFIIH (Conaway and Conaway, 1993; Zawel and Reinberg, 1993). In an effort to determine which step in preinitiation complex formation is blocked by repressor, the order-of-addition experiment illustrated in Figure 2A was performed. Various combinations of RNA polymerase II and initiation factors were first preincubated with the AdML core promoter. Repressor was then added to the reaction mixtures and, following an additional preincubation, the remaining transcription proteins were added. After a final preincubation, ribonucleoside triphosphates were added to the reaction mixtures to allow the synthesis of run-off transcripts.

As shown, preincubation of the AdML core promoter with RNA polymerase II, TFIIB, TFIIE, TFIIF and TFIIH, in the absence of TBP, did not relieve transcriptional repression (Figure 2A, lanes 9–12). On the other hand, repressor

activity was substantially reduced when repressor was added to the reaction mixtures after preincubation of TBP with the AdML core promoter (lanes 1–3). Preincubation of TBP in the absence of promoter DNA was not sufficient to relieve transcriptional repression (data not shown), and little or no further reduction in the ability of repressor to inhibit transcription was observed when RNA polymerase II, TFIIB, TFIIF, TFIIE and TFIIH were included in preincubations (lanes 4–8). Thus, repressor appears to interfere with the ability of TBP to interact productively with the AdML core promoter, but to have little effect on subsequent steps in the assembly of the preinitiation complex.

To explore this possibility further, we asked whether transcriptional repression could be overcome by the addition of excess TBP, TFIIB or any other component of the basal transcription apparatus. As shown in Figure 2B, the addition of excess TBP restored transcription to a level near that observed in the absence of repressor (compare lanes 1 and 7–10), even though additional TBP did not substantially increase transcription in the absence of repressor (lanes 1–4). In contrast, neither the addition of excess TFIIB (lanes 11–13), RNA polymerase II, TFIIF, TFIIE or TFIIH (lanes 14 and 16–19) nor the addition of excess AdML promoter DNA (data not shown) were able to relieve transcriptional repression. Taken together, the results presented in Figure 2 provide strong support for the idea that repressor antagonizes TBP function, most probably through a direct interaction with TBP.

Repressor activity is specific for promoters containing a consensus TATA element

Studies carried out in many laboratories have established that TBP recognizes and binds selectively to a short core promoter region limited to sequences in the immediate vicinity of the TATA box (Cavallini *et al.*, 1989; Hahn *et al.*, 1989b,c; Horikoshi *et al.*, 1989b; Hoey *et al.*, 1990; Kao *et al.*, 1990; Muhich *et al.*, 1990). TBP binds most strongly to and directs transcription most efficiently from core promoters containing TATA elements closely related to the consensus TATA sequence TATAAA (Wobbe and Struhl, 1990; Conaway *et al.*, 1991; Wiley *et al.*, 1992), but it also binds to (Wiley *et al.*, 1992) and is sufficient to direct transcription from promoters containing non-consensus TATA elements (T.Aso, unpublished results).

To characterize the repressor activity further, we compared its ability to inhibit transcription from a variety of core promoter elements containing either consensus or non-consensus TATA elements; the sequences of the TATA regions of each of the promoters tested are shown in Figure 3. Repressor inhibited transcription from both the AdML and mouse mammary tumor virus (MMTV) promoters, which have identical consensus TATA elements but which differ in sequences 5′ and 3′ of the TATA element. In addition, repressor inhibited transcription from the rat β-fibrinogen (β-Fib) and mouse interleukin-3 (IL-3) promoters, whose TATA regions differ from the consensus sequence only in the fifth or sixth positions of the TATA element. As we observed previously, the AdML, MMTV, β-Fib and IL-3 promoters all support comparable and relatively efficient TBP-dependent transcription *in vitro* (Conaway *et al.*, 1991). In contrast to the results obtained with these consensus TATA promoters, a significant stimulation of transcription was observed when highly purified repressor was added to reactions containing either an AdML promoter mutant

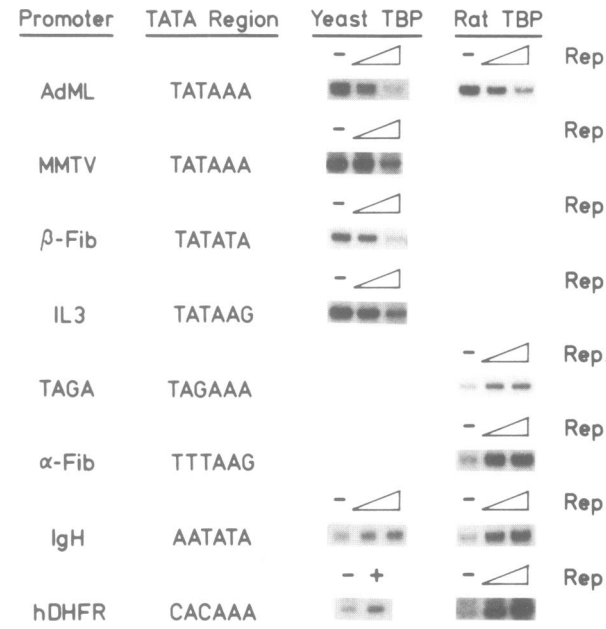

Fig. 3. Repressor inhibits basal transcription from core promoter elements containing the consensus TATA sequence (TATA), but activates transcription from non-TATA core promoter elements. Run-off transcription reactions were carried out as described in Materials and methods with 100 ng of the indicated DNA templates and 20 ng of recombinant yeast or rat TBP. All promoters except hDHFR were tested with 0, 20 and 200 ng of repressor (Fraction VII); hDHFR was tested with 0 and 200 ng of repressor (Fraction VII) in the presence of yeast TBP. AdML, *Nde*I-digested pDN-AdML DNA; MMTV, *Nde*I-digested pDN-MMTV DNA; β-Fib, *Eco*RI to *Nde*I fragment containing the rat β-Fib core promoter from pDN-β-Fib; IL3, *Eco*RI to *Nde*I fragment containing the mouse IL-3 core promoter from pDN-IL3; TAGA, *Eco*RI to *Nde*I fragment from pDN-TAGA; α-Fib, *Eco*RI to *Nde*I fragment containing the rat α-Fib core promoter from pDN-α-Fib; IgH, *Eco*RI to *Nde*I fragment containing the hIgH core promoter from pDN-IgH; hDHFR, *Eco*RI to *Nde*I fragment containing human dihydrofolate reductase core promoter sequences between ~−40 to +8 from pDN-hDHFR. The data shown in the figure do not accurately reflect the relative strengths of promoters used in these experiments, since the duration of autoradiographic exposures was different for different promoters.

(TAGA), which contains a single T → G substitution (TATA → TAGA) in its TATA element, or the rat α-fibrinogen (α-Fib), human immunoglobulin heavy chain (IgH) and human dihydrofolate reductase (hDHFR) promoters, which each have non-consensus TATA regions and are only weakly transcribed in the presence of TBP. Thus, while repressor inhibits TBP function at efficiently transcribed core promoter elements containing near-consensus TATA sequences, it activates TBP-dependent transcription from a variety of less efficiently transcribed non-TATA core promoters. It is important to note that the relative efficiencies of transcription from the different promoters cannot be compared directly from the data shown, since the data are derived from different autoradiographic exposures. Even in the presence of high levels of repressor fraction, promoters containing near-consensus TATA elements are transcribed more efficiently than either the hDHFR or rat α-Fib promoters, which lack consensus TATA elements (data not shown).

Repressor activity is associated with mammalian TFIIA

Repressor activity was initially identified in side fractions from the purification (Conaway *et al.*, 1991) of rat TFIIE.

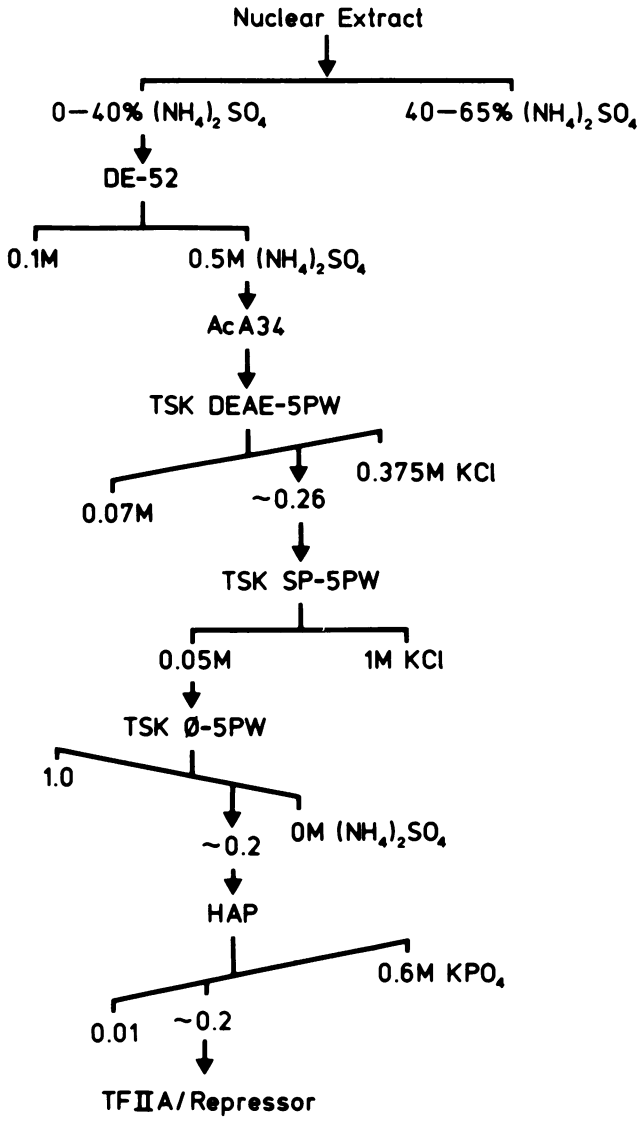

Fig. 4. Origin of fractions containing transcriptional repressor and stimulatory activities. ϕ, phenyl; HAP, Bio-Gel HPHT (hydroxylapatite).

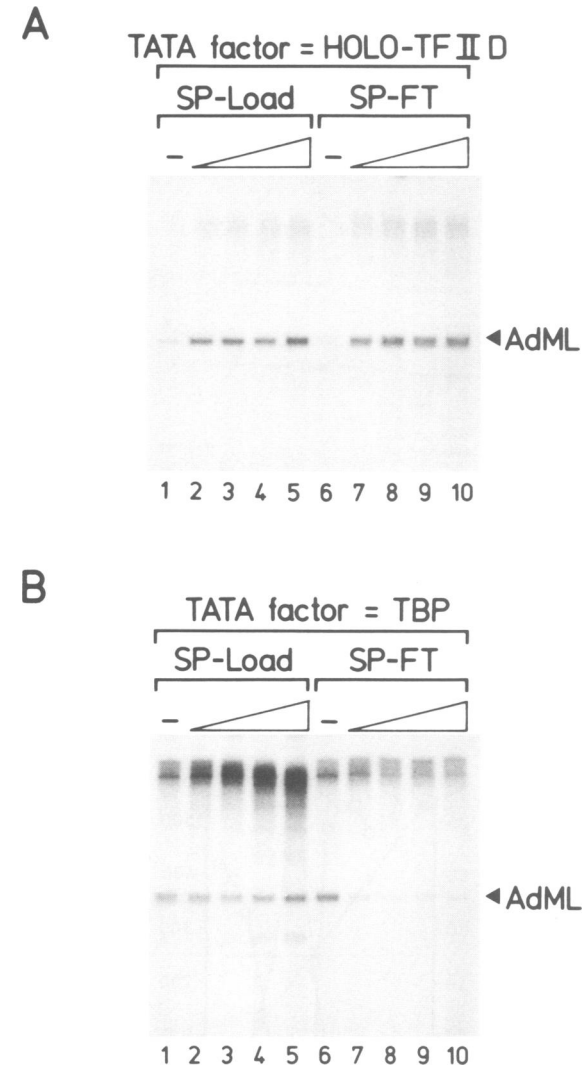

Fig. 5. Assay of transcriptional repressor and stimulatory activities in the TSK SP-5-PW load and flow-through fractions. Run-off transcription assays were performed as described in Materials and methods with 100 ng of *NdeI*-digested pDN-AdML DNA as template. 20 ng of recombinant yeast TBP was used for repressor assays and 80 ng of native rat holo-TFIID was used for activator assays.

In these experiments, repressor activity as well as an activity capable of stimulating holo-TFIID-dependent basal transcription by RNA polymerase II, were identified in the 0.05 M KCl flow-through fraction from TSK SP-5-PW HPLC. The origin and subsequent purification of fractions containing transcriptional repressor and stimulatory activities is summarized in Figure 4. Assay of fractions from the purification of TFIIE revealed that transcriptional stimulatory activity overlapped but was chromatographically distinguishable from TFIIE activity during both gel filtration on AcA 34, where it eluted with a native molecular mass of ~160 kDa, and anion exchange HPLC on TSK DEAE-5-PW, where it eluted at ~0.26 M KCl; repressor activity was not assayable in early fractions but was unmasked by cation exchange HPLC on TSK SP-5-PW. As shown in Figure 5, whereas the activator of holo-TFIID-dependent transcription could be assayed in both the TSK SP-5-PW load and flow-through fractions (A), repressor activity could be assayed only in TSK SP-5-PW flow-through fractions (B). Further investigation revealed that transcriptional repressor and stimulatory activities were chromatographically inseparable, and several lines of evidence argue that they are closely associated with rat TFIIA.

First, purification of the TSK SP-5-PW flow-through fraction by high-resolution chromatography on successive TSK phenyl-5-PW and Bio-Gel HPHT HPLC columns revealed that, like human TFIIA (Cortes *et al.*, 1992; Coulombe *et al.*, 1992), transcriptional repressor and stimulatory activities co-purified with three polypeptides of ~35, 20 and 14 kDa. As shown in Figure 6A, transcriptional repressor and stimulatory activities co-purified closely with three polypeptides of ~35, 20 and 14 kDa during TSK phenyl-5-PW chromatography; detectable quantities of these polypeptides were not present in fractions that lacked these activities. A small amount of an additional polypeptide of ~21.5 kDa also co-eluted with repressor and stimulatory activities. The major portion of this polypeptide, however, appeared to elute from the column in fractions 25 and 26 before repressor and stimulatory activities, and in fraction 32 and subsequent fractions after repressor and stimulatory activities. We note that the 20 kDa polypeptide stains variably

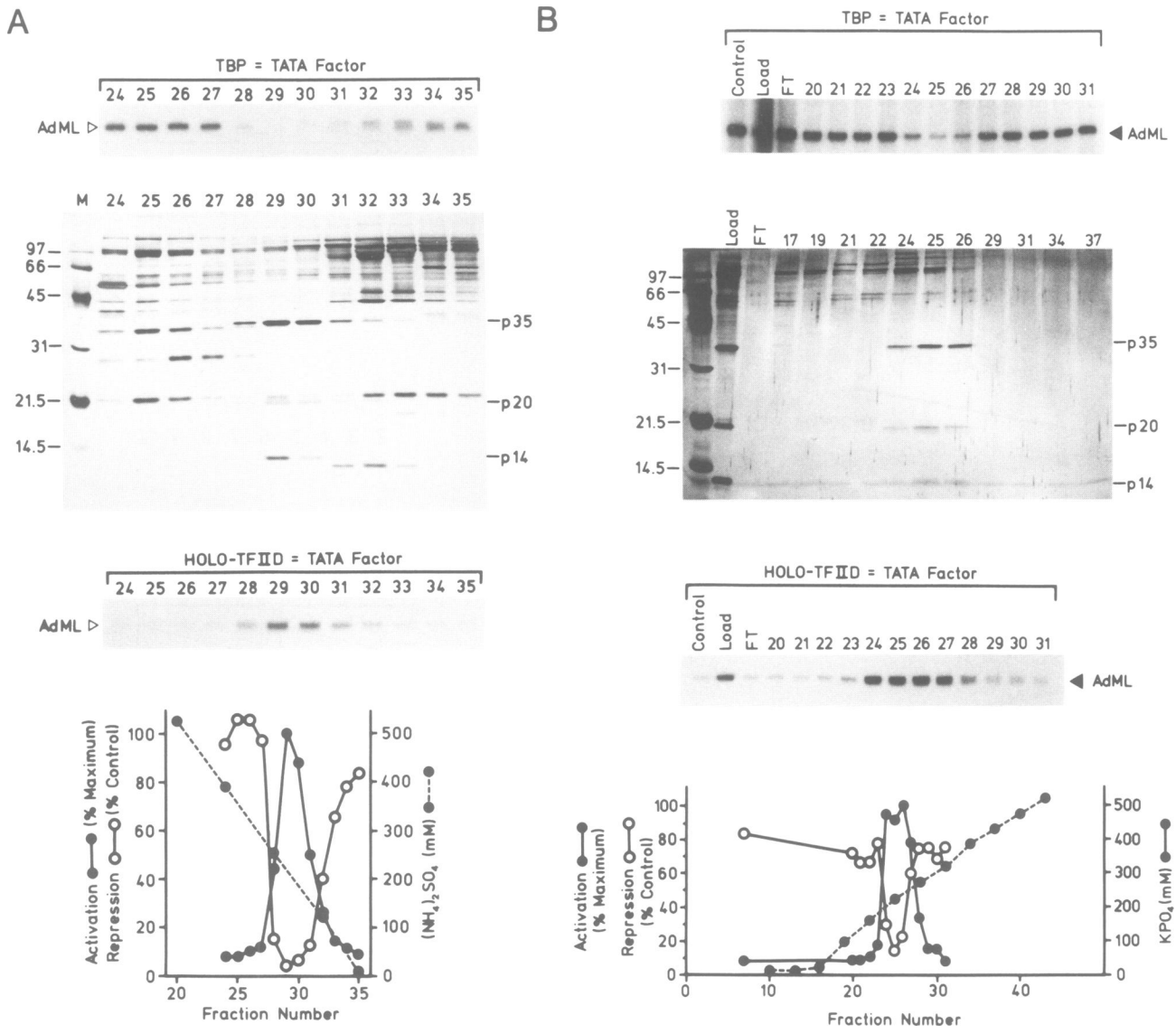

Fig. 6. Co-purification of transcriptional repressor and stimulatory activities with polypeptides of ~35, 20 and 14 kDa during TSK phenyl-5-PW (**A**) and Bio-Gel HPHT (**B**) HPLC. Run-off transcription assays were performed as described in Materials and methods with 100 ng of *Nde*I-digested pDN-AdML DNA as template. 20 ng of recombinant yeast TBP was used for repressor assays and 80 ng of native rat holo-TFIID was used for activator assays. Activation (●), expressed as % maximum, and Repression (○), expressed as % control, refer to the relative synthesis of the 254 nucleotide run-off transcript synthesized from the AdML promoter in pDN-AdML. The amount of AdML run-off transcript synthesized in these experiments was determined by densitometry of the appropriate exposures of the autoradiograms shown in the figures. Aliquots of column fractions were analyzed by 13% SDS–PAGE and protein was visualized by silver staining.

with silver (in the SDS gel of Figure 6A, fractions 28–30, the 20 kDa polypeptide silver stained poorly; in some experiments this polypeptide was more efficiently stained as, for example, in Figure 6B, 'Load', which contains a pool of fractions 29 and 30 from the TSK phenyl-5-PW column). Likewise, the ~20 kDa polypeptide of human TFIIA stains poorly with silver (Cortes *et al.*, 1992).

As shown in Figure 6B, the ~35, 20 and 14 kDa polypeptides also co-chromatographed with transcriptional repressor and stimulatory activities during Bio-Gel HPHT HPLC. The bulk of repressor and stimulatory activities, as well as of the ~35, 20 and 14 kDa polypeptides, eluted from the column in fractions 24–26; neither repressor activity, stimulatory activity nor the three polypeptides were detectable by fraction 29. The minor 21.5 kDa polypeptide, which appeared to co-elute with repressor and stimulatory activities from the TSK phenyl-5-PW column, was separated from them on Bio-Gel HPHT where it eluted at a lower phosphate concentration.

Besides this similarity in their polypeptide compositions, the chromatographic properties of transcriptional repressor and stimulatory activities are strikingly similar to those of human TFIIA. Neither repressor activity, stimulatory activity nor human TFIIA binds to cation exchange resins at 0.1 M KCl (Matsui *et al.*, 1980; Samuels *et al.*, 1982; Davison *et al.*, 1983; Egly *et al.*, 1984). Furthermore, repressor activity, stimulatory activity and human TFIIA (Cortes *et al.*, 1992) adsorb to and elute from TSK DEAE-5-PW and Bio-Gel HPHT under similar conditions. In addition, transcriptional repressor and stimulatory activities and human TFIIA (Usuda *et al.*, 1991; Cortes *et al.*, 1992) exhibit an apparent native molecular mass by gel filtration of ~160 kDa (data not shown).

Second, we observed that, like human TFIIA (Matsui

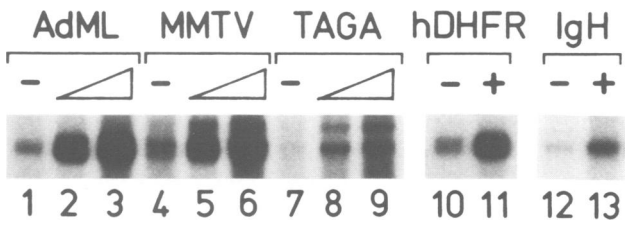

Fig. 7. Activation of basal transcription directed by holo-TFIID. Run-off transcription reactions were carried out as described in Materials and methods with 100 ng of the indicated DNA templates and 80 ng of rat holo-TFIID. All promoters except hDHFR and IgH were tested with 0, 20 and 200 ng of repressor (Fraction VII); hDHFR and IgH were tested with 0 and 9 ng of repressor (Fraction VIII). The templates, which are described in the legend to Figure 4, were as follows: AdML, 100 ng *Nde*I-cut pDN-AdML; MMTV, 100 ng *Nde*I-cut pDN-MMTV; TAGA, 100 ng *Nde*I-cut pDN-TAGA; IgH, 200 ng *Eco*RI to *Nde*I fragment from pDN-IgH; hDHFR, 200 ng *Eco*RI to *Nde*I fragment from pDN-hDHFR.

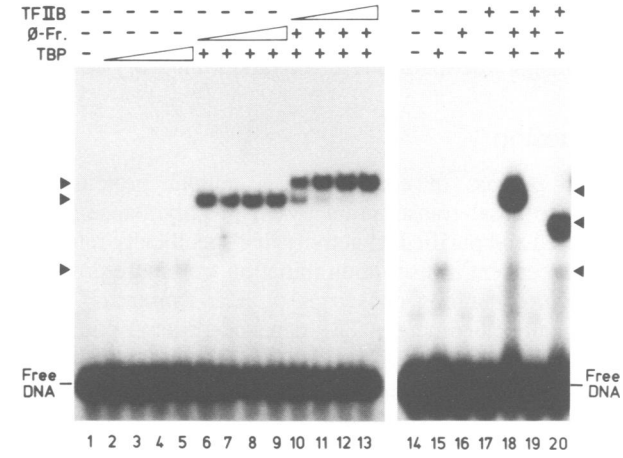

Fig. 8. An activity that interacts with the TBP–promoter complex. A 5′ ^{32}P-labeled double-stranded oligonucleotide containing the AdML core promoter sequences was incubated for 45 min at 28°C with the indicated amounts of yeast TBP (0, 10, 20, 40 and 80 ng in lanes 1–5, respectively; 10 ng in lanes 6–13; 20 ng in lanes 15, 18 and 20), repressor (Fraction VII) (5, 10, 20 and 40 ng in lanes 6–9, respectively; 5 ng in lanes 10–13, 16, 18 and 19) and TFIIB (10, 20, 40 and 80 ng in lanes 10–13, respectively; 40 ng in lanes 17, 19 and 20).

et al., 1980; Samuels *et al.*, 1982; Davison *et al.*, 1983; Usuda *et al.*, 1991; Pugh and Tjian, 1991; Cortes *et al.*, 1992; Zhou *et al.*, 1992), fractions containing transcriptional repressor and stimulatory activities from TSK phenyl-5-PW and Bio-Gel HPHT HPLC could stimulate holo-TFIID-dependent transcription by RNA polymerase II from a wide variety of promoters containing both consensus and non-consensus TATA elements. As shown in Figure 7, holo-TFIID-dependent transcription from the AdML and MMTV promoters, which have consensus TATA elements, as well as from the TAGA, hDHFR and IgH promoters, which have TATA elements that deviate substantially from the consensus sequence, could be stimulated as much as 20-fold by the addition of aliquots from TSK phenyl- 5-PW and Bio-Gel HPHT HPLC column fractions. Unlike transcriptional repression, therefore, stimulation of holo-TFIID-dependent transcription is not promoter specific.

Third, we observed that, like human TFIIA (Buratowski *et al.*, 1989; Cortes *et al.*, 1992; Moncollin *et al.*, 1992), highly purified fractions containing transcriptional repressor and stimulatory activities are capable of interacting with a TBP–AdML promoter complex in electrophoretic mobility shift assays. As shown in Figure 8, the addition of the TSK phenyl-5-PW fraction to a mixture of TBP and promoter DNA leads to the formation of a complex with a significantly reduced electrophoretic mobility than the complex of TBP and DNA alone; no protein–DNA complexes were detected when the repressor fraction was incubated with promoter DNA in the absence of TBP. We observe that TBP-binding activity co-chromatographs with transcriptional repressor and stimulatory activities during chromatography on TSK phenyl-5-PW and Bio-Gel HPHT (data not shown).

Although the results of many studies show that TBP is capable of binding the AdML promoter efficiently in the absence of other proteins (e.g. Buratowski *et al.*, 1988, 1989; Hahn *et al.*, 1989a; Horikoshi *et al.*, 1989a), the TBP-AdML promoter complex is often unstable during native gel electrophoresis (Buratowski *et al.*, 1989; Hahn *et al.*, 1989a). In agreement with previous results indicating that TFIIA not only interacts with TBP but also helps the TBP–promoter complex survive during native gel electrophoresis (Buratowski *et al.*, 1989; Hahn *et al.*, 1989a; Moncollin *et al.*, 1992), considerably more protein–DNA complex is detected in the presence than in the absence of fractions containing repressor and stimulatory activities. The addition of sufficient TSK phenyl-5-PW fraction to inhibit transcription from the AdML promoter by >80% does not cause measurable complex dissociation, suggesting that repressor may not inhibit TBP-dependent transcription by disrupting the TBP–promoter complex. We observe that less repressor fraction is required to saturate binding of TBP–AdML promoter complexes in electrophoretic mobility shift assays than to inhibit or stimulate the synthesis of accurately initiated transcripts in run-off transcription assays. Since (i) repression appears to involve a stoichiometric interaction between repressor and TBP (Figure 2B) and (ii) binding of TFIIA to TBP is known to be salt-sensitive (Usuda *et al.*, 1991; Cortes *et al.*, 1992; Coulombe *et al.*, 1992), it is possible that less repressor fraction is needed to saturate electrophoretic mobility shift assays because protein–DNA complexes are subjected to conditions of lower ionic strength during native gel electrophoresis than during transcription assays. Consistent with this possibility, Cortes *et al.* (1992) could recover sufficient TFIIA TBP-binding activity from SDS–polyacrylamide gels to supershift a TBP–AdML promoter complex, but not to stimulate transcription detectably.

It has been shown previously that TBP–TFIIA–DNA complexes are capable of interacting with the basal TFIIB to form complexes detectable by electrophoretic mobility shift assays (Buratowski *et al.*, 1989; Maldonado *et al.*, 1990; Moncollin *et al.*, 1992). We therefore asked whether TFIIB could interact with the complexes formed in the presence of TBP and fractions containing transcriptional repressor and stimulatory activities. As shown in Figure 8 (lanes 10–13), the addition of increasing concentrations of TFIIB led to the disappearance of the complex formed in the presence of TBP and the TSK phenyl-5-PW fraction alone and to the appearance of a new complex with reduced electrophoretic mobility. A TBP–TFIIB–promoter complex, formed in the absence of the TSK phenyl-5-PW fraction, has a significantly greater electrophoretic mobility than a similar complex formed in the presence of TBP, TFIIB and the TSK phenyl-5-PW fraction (compare lanes 6–13

with lanes 18 and 20), suggesting that TFIIB does not simply displace the TBP-binding activity present in fractions containing transcriptional repressor and stimulatory activities.

Discussion

In the process of characterizing cellular proteins that modulate basal transcription by RNA polymerase II, we identified and purified an activity that specifically represses TBP-dependent transcription initiation from core promoter elements containing consensus or near-consensus TATA boxes, but that stimulates TBP-dependent transcription from core promoter elements lacking consensus TATA boxes. Several lines of evidence argue that this novel transcriptional repressor activity is closely associated with mammalian TFIIA.

(i) Repressor activity and mammalian TFIIA have similar polypeptide compositions. As we have shown, repressor activity co-purifies with polypeptides of ~35, 20 and 14 kDa. Although the precise polypeptide composition of TFIIA has been controversial (Egly *et al.*, 1984; Samuels and Sharp, 1986; Usuda *et al.*, 1991), results of two recent studies suggest that the human factor is composed of polypeptides of ~35, 20 and 14 kDa (Cortes *et al.*, 1992; Coulombe *et al.*, 1992).

(ii) Repressor activity and mammalian TFIIA have similar chromatographic properties. Unlike the previously described repressors NC1, NC2, Dr1, and Dr2 (Meisterernst and Roeder, 1991; Meisterernst *et al.*, 1991; Inostroza *et al.*, 1992), neither repressor nor TFIIA binds to cation exchange resins at 0.1 M KCl. In addition, repressor and mammalian TFIIA adsorb to and are eluted from TSK DEAE-5-PW and Bio-Gel HPHT resins under similar conditions (Cortes *et al.*, 1992). Finally, repressor and TFIIA (Usuda *et al.*, 1991; Cortes *et al.*, 1992) exhibit similar native molecular masses by gel filtration.

(iii) Highly purified repressor preparations mediate activities previously ascribed to TFIIA. First, like TFIIA (Matsui *et al.*, 1980; Samuels *et al.*, 1982; Davison *et al.*, 1983; Egly *et al.*, 1984; Samuels and Sharp, 1986; Usuda *et al.*, 1991; Cortes *et al.*, 1992; Coulombe *et al.*, 1992; Waldschmidt and Seifart, 1992), repressor co-purifies with an activity that strongly stimulates holo-TFIID-directed transcription initiation by RNA polymerase II from promoters containing either consensus or non-consensus TATA elements. Moreover, our highly purified repressor preparations replace human TFIIA in a reconstituted transcription system from human cells (Y.Jiang and J.Gralla, personal communication). Second, like TFIIA (Buratowski *et al.*, 1989; Maldonado *et al.*, 1990; Moncollin *et al.*, 1992), repressor co-purifies with an activity that is capable of interacting with a TBP–promoter complex in electrophoretic mobility shift assays. Complexes formed in the presence of TBP and repressor fraction, like those formed in the presence of TBP and TFIIA, can be bound by TFIIB.

Taken together, these observations argue that mammalian TFIIA is closely associated with a previously unrecognized transcriptional repressor activity. Direct demonstration that repressor is an inherent activity of one or more of the TFIIA polypeptides awaits reconstitution of transcriptionally active TFIIA from isolated polypeptides. Until this has been accomplished, it will be impossible to rule out the possibility that repressor is closely associated with, but physically separable from, TFIIA.

Our finding that TFIIA is associated with an activity that modulates transcription directed by TBP in a TATA sequence-dependent manner was unexpected, since no such TFIIA-associated activity has been previously observed. Instead, the results of some previous studies indicate that TFIIA has little or no effect on TBP-dependent transcription (Cortes *et al.*, 1992; Sayre *et al.*, 1992), while the results of others indicate that TFIIA is capable of strongly stimulating TBP-dependent transcription (Hahn *et al.*, 1989a; Usuda *et al.*, 1991; Roy *et al.*, 1993). The reasons for the discrepancies between these studies and ours are unclear. Our observation that TFIIA-associated repressor activity was detectable only after chromatography on TSK SP-5-PW raises the possibility that preparations of TFIIA or other factors used in previous studies may have contained activity(s) that counteract repressor activity. In addition, we have observed that repressor activity can be overcome by the addition of excess TBP; therefore, if previous studies were carried out using a greater molar ratio of TBP to TFIIA than used in our experiments, repressor activity would not have been detected.

How might TFIIA repress transcription from core promoters containing consensus or near-consensus TATA elements, but activate transcription from promoters lacking consensus TATA elements? Consistent with previous studies indicating that TFIIA binds both free and DNA-bound TBP (Buratowski *et al.*, 1989; Maldonado *et al.*, 1990; Ranish and Hahn, 1991; Usuda *et al.*, 1991; Buratowski and Zhou, 1992; Cortes *et al.*, 1992; Coulombe *et al.*, 1992; Lee *et al.*, 1992), our finding that transcriptional inhibition can be overcome by the addition of excess TBP, but not by the addition of excess TFIIB, TFIIE, TFIIF or TFIIH, argues that TBP is the primary target of repressor action. In addition, our observation that the preincubation of TBP with template DNA renders transcription resistant to inhibition, suggests that TFIIA-associated repressor activity somehow interferes with the functional interaction of TBP with promoters containing consensus TATA elements. Substantial evidence argues, however, that TFIIA does not actually prevent binding of TBP to promoters. Results of previous DNAase I footprinting studies indicate that TFIIA has either no significant effect (Hahn *et al.*, 1989a) or a modest stabilizing effect (Buratowski *et al.*, 1989; Cortes *et al.*, 1992) on the interaction of TBP with the AdML promoter. In addition, as shown previously for human and yeast TFIIA (Buratowski *et al.*, 1989; Hahn *et al.*, 1989a; Maldonado *et al.*, 1990; Ranish and Hahn, 1991; Cortes *et al.*, 1992; Moncollin *et al.*, 1992; Ranish *et al.*, 1992), our highly purified TFIIA/repressor preparations do not dissociate TBP–AdML promoter complexes in electrophoretic mobility shift assays, but stabilize them. In the light of these results, we suggest that TFIIA could mediate both transcriptional repression and stimulation by interacting with TBP and inducing it to assume, or trapping it into, a conformation with either an altered promoter specificity or an altered ability to nucleate the assembly of functional preinitiation complexes at promoters containing consensus and non-consensus TATA elements. Consistent with this possibility, in studies of the interaction of TBP with wild-type and mutant AdML promoters, Lee *et al.* (1992) recently obtained evidence that TFIIA has the capacity to induce conformational changes in TBP that modulate its ability to interact with different promoter sequences.

Do the results presented here provide any insight into the

mechanism by which TFIIA stimulates transcription directed by holo-TFIID? Based on the observation that, in their transcription system, TFIIA stimulates holo-TFIID- but not TBP-dependent basal transcription, Cortes *et al.* (1992) proposed that TFIIA might function by displacing negative co-factors associated with the TBP subunit of holo-TFIID. According to this model, TFIIA does not exert a direct positive effect on transcription, but, rather, functions solely as an 'anti-repressor'. Support for this model comes from studies showing that TFIIA can prevent transcriptional inhibition by the negative co-factors NC1, NC2, Dr1 and Dr2 by competing with them for binding to TBP (Meisterernst and Roeder, 1991; Meisterernst *et al.*, 1991; Inostroza *et al.*, 1992). While we cannot entirely rule out the possibility that our highly purified preparations of RNA polymerase II and transcription factors contain some negative co-factor activity, our observation that TFIIA is capable of activating TBP-dependent transcription from promoters containing non-consensus TATA elements in our reconstituted transcription system argues that TFIIA could have the capacity to stimulate transcription by an additional mechanism that does not involve anti-repression.

Whether TFIIA functions as a positive activator, an anti-repressor or both, it is reasonable to propose that TFIIA stimulates both TBP-dependent transcription from non-consensus TATA promoters and holo-TFIID-dependent transcription from consensus and non-consensus TATA promoters by a common mechanism involving a direct interaction with TBP. If TFIIA does interact with both free TBP and the TBP subunit of holo-TFIID by a common mechanism, how is it possible that TFIIA inhibits TBP-dependent transcription from core promoters containing consensus TATA elements, but does not inhibit holo-TFIID-dependent transcription from the same promoters? One possibility is that TFIIA does in fact inhibit holo-TFIID-dependent transcription from consensus TATA promoters, but that this inhibition is compensated by a strong stimulation resulting from the removal of negative co-factors by TFIIA.

Alternatively, the observed differences in TFIIA activity in holo-TFIID- and TBP-directed transcription could be a direct consequence of the structure of TBP in holo-TFIID. As a subunit of holo-TFIID, TBP is physically associated with at least eight distinct polypeptides or TAFs, which could impose significant constraints on its conformation and affect its ability to interact with promoter sequences. Thus, a second possibility is that one or more TAFs simply prevents TFIIA from altering the conformation of TBP in ways that repress transcription from consensus TATA promoters but that, at least with free TBP, allow the stimulation of transcription from non-consensus TATA promoters. This model requires that TFIIA be capable of stimulating transcription through specific interactions with different conformations of TBP, since it proposes that TBP in holo-TFIID is in a different conformation than free TBP bound by TFIIA.

A third possibility is that TFIIA does not inhibit holo-TFIID-dependent transcription from consensus TATA elements because the TBP subunit of holo-TFIID is already constrained by TAFs to a 'repressed' conformation that is similar to the TBP conformation induced or trapped by the interaction with TFIIA, and that interacts with consensus TATA sequences more poorly than free TBP. In this case, the observed stimulation of TBP-dependent transcription from non-consensus TATA promoters and holo-TFIID-dependent transcription from all promoters would occur through interactions of TFIIA with the same conformation of TBP. We find this model attractive for two reasons. First, it is consistent with a variety of experimental evidence. Kokubo *et al.* (1993) have recently shown that the isolated 230 kDa TAF of *Drosophila* holo-TFIID negatively regulates the TATA box binding and transcriptional activities of TBP at a consensus TATA promoter; whether this TAF affects the activity of TBP similarly at all promoters or whether it has a differential effect on consensus and non-consensus TATA promoters is unknown. Furthermore, evidence from several studies indicates that holo-TFIID binds and directs transcription from a variety of promoters with consensus TATA elements much less efficiently than free TBP (Conaway *et al.*, 1990b, 1991; Nakatani *et al.*, 1990; Wobbe and Struhl, 1990; T.Aso, manuscript in preparation), suggesting that one or more TAFs can indeed alter TBP's ability to interact with the TATA region of promoters. Second, the proposal that TFIIA stimulates both TBP- and holo-TFIID-dependent transcription through interactions with the same conformation of TBP permits us to rationalize all of the effects of TFIIA on transcription directed by either TBP or holo-TFIID. According to this model, when TFIIA binds free TBP, it either induces TBP to assume, or traps TBP in, a conformation that is similar to the conformation of TBP in holo-TFIID and that is less able than free TBP to nucleate the assembly of productive preinitiation complexes at consensus TATA promoters. At these promoters any transcriptional stimulation by TFIIA would not be great enough to be detected above the background level of repressed transcription. As a subunit of holo-TFIID, TBP would already be constrained to the 'repressed' conformation by TAFs, and transcriptional stimulation by TFIIA would therefore be detectable at all promoters.

Materials and methods

Materials

Male Sprague–Dawley rats (200–300 g) were purchased from SASCO. Phenylmethylsulfonyl fluoride (PMSF) was obtained from Sigma and was dissolved in dimethyl sulfoxide to 1 M. Heparin was acquired from Sigma. Antipain and leupeptin were obtained from Boehringer Mannheim and dissolved in water to 25 mg/ml. Bovine serum albumin (Pentex fraction V) was purchased from ICN ImmunoBiologicals. Glycerol (spectra-analyzed grade) was acquired from Fisher. Schwarz/Mann ultra-pure sucrose and ammonium sulfate were bought from ICN Biomedicals, Inc. Unlabeled ultrapure ribonucleoside 5′-triphosphates were purchased from Pharmacia LKB Biotechnology Inc. [α-^{32}P]CTP (>650 Ci/mmol) was obtained from ICN.

Chromatography and buffers

Phosphocellulose (P11) and DEAE–cellulose (DE52) were purchased from Whatman. Spherogel TSK phenyl-5-PW, Spherogel TSK SP-5-PW, and Spherogel TSK DEAE-5-PW were obtained from Beckman Instruments, Inc. Bio-Gel HPHT (hydroxylapatite) was acquired from Bio-Rad, and Ultrogel AcA 34 was from IBF Biotechnics. HPLC was performed using a Beckman System Gold Chromatograph. Buffer B was 50 mM Tris–HCl, pH 7.9, 0.1 mM EDTA, 1 mM DTT, 20% (v/v) glycerol and 0.5 mM PMSF. Buffer C was 40 mM Tris–HCl, pH 7.9, 0.5 mM EDTA, 1 mM DTT and 10% (v/v) glycerol. Buffer D was 40 mM HEPES–NaOH, pH 7.9, 0.5 mM EDTA, 1 mM DTT and 10% (v/v) glycerol. Buffer I was 10 mM potassium phosphate, pH 7.5 and 1 mM DTT.

Purification of repressor/TFIIA

A 0.33 M $(NH_4)_2SO_4$ extract of crude rat liver nuclei (Fraction I) was prepared from the livers of 250 male Sprague–Dawley rats as previously described (Conaway *et al.*, 1991). All further operations were carried out at 4°C. Solid $(NH_4)_2SO_4$ (0.186 gm/ml) was added slowly to Fraction I with stirring. After the addition of 1 μl of 1 N NaOH per gram of $(NH_4)_2SO_4$, the suspension was stirred for an additional 30 min. The

precipitate was collected by centrifugation at 12 000 *g* for 45 min and dissolved in buffer B containing antipain and leupeptin at 10 μg/ml each. The resuspension was then diluted with buffer B until it reached a conductivity equivalent to that of 0.1 M $(NH_4)_2SO_4$ in buffer B and centrifuged at 7500 *g* for 15 min. The supernatant (Fraction II) was mixed with 1.0 l DEAE–cellulose pre-equilibrated with buffer B containing 0.1 M $(NH_4)_2SO_4$ in a 10.5 cm diameter column. The slurry was allowed to sit for 45 min with occasional stirring and then filtered at 1.0 l per hour. The column was washed at the same flow rate with buffer B containing 0.1 M $(NH_4)_2SO_4$ until the column eluate contained <0.05 mg/ml protein. Activity was then eluted at 2.0 l per hour with buffer B containing 0.5 M $(NH_4)_2SO_4$ (Fraction III). Solid $(NH_4)_2SO_4$ (0.33 g/ml) was added slowly to Fraction III with stirring. After the addition of 1 μl 1 N NaOH per gram of $(NH_4)_2SO_4$, the suspension was stirred for an additional 30 min. The precipitate was collected by centrifugation and dissolved to a final volume of 3 ml in buffer D containing 0.4 M KCl, 10 μg/ml antipain and 10 μg/ml leupeptin. The resuspension was dialyzed against buffer D until the conductivity was approximately equivalent to that of 0.5 M $(NH_4)_2SO_4$ in buffer D, and then centrifuged at 100 000 *g* for 30 min. The resulting supernatant was applied to an Ultrogel AcA 34 column (26 × 100 mm) equilibrated with buffer D containing 0.4 M KCl. The column was eluted at 5 ml/min and 5 ml fractions were collected. TBP-dependent repressor activity could not be assayed at this stage; fractions containing holo-TFIID-dependent stimulatory activity were pooled and dialyzed against buffer C to a conductivity equivalent to that of 0.07 M KCl in buffer C (Fraction IV). Fraction IV was centrifuged at 100 000 *g* for 30 min, and the supernatant was applied to a Spherogel TSK DEAE-5-PW HPLC column (21.5 × 150 mm) pre-equilibrated with buffer C containing 0.07 M KCl. Activity was eluted at 5 ml/min with a 500 ml linear gradient from 0.070 to 0.375 M KCl, and 10 ml fractions were collected. Active fractions were pooled and dialyzed against buffer D to a conductivity equivalent to that of 0.05 M KCl in buffer D (Fraction V). Fraction V was centrifuged at 100 000 *g* for 30 min, and the supernatant was applied at 1 ml/min to a Spherogel TSK SP-5-PW HPLC column (7.5 × 75 mm) pre-equilibrated with buffer D containing 0.05 M KCl. Flow-through fractions, which could be assayed for both holo-TIID-dependent stimulatory activity and TBP-dependent repressor activity, were pooled (Fraction VI), diluted 1:1 with buffer D containing 2.0 M $(NH_4)_2SO_4$ and centrifuged at 15 000 *g* for 15 min. The resulting supernatant was applied to a Spherogel TSK phenyl-5-PW HPLC column (7.5 × 75 mm) pre-equilibrated with buffer D containing 1.0 M $(NH_4)_2SO_4$. The column was eluted at 1 ml/min with a 30 ml linear gradient from 1.0 M $(NH_4)_2SO_4$ in buffer D to buffer D. Active fractions were pooled (Fraction VII), dialyzed against buffer I to a conductivity equivalent to that of buffer I, and centrifuged at 15 000 *g* for 15 min. The resulting supernatant was applied to a Bio-Gel HPHT HPLC column (7.8 × 100 mm) pre-equilibrated in buffer I. The column was eluted at 0.5 ml/min with a 27 ml linear gradient from 10 to 600 mM potassium phosphate, and 1 ml fractions were collected. Repressor and stimulatory activities eluted with ~200 mM potassium phosphate (Fraction VIII).

Preparation of RNA polymerase II and transcription factors

RNA polymerase II (Serizawa *et al.*, 1992), holo-TFIID (rat τ, TSK SW4000 fraction; Conaway *et al.*, 1990a) and initiation factor TFIIH (rat δ, TSK phenyl-5-PW fraction; Conaway *et al.*, 1992) were purified from rat liver nuclear extracts as previously described. Recombinant yeast TBP was expressed and purified to >95% homogeneity as described (Conaway *et al.*, 1991). Recombinant rat TBP was expressed in *Escherichia coli* using T7 expression vector pET-11a (Novagen) and was purified to >95% homogeneity from bacterial extracts by phosphocellulose chromatography performed as described (Conaway *et al.*, 1990a). Recombinant rat α(TFIIB) was expressed and purified to >90% homogeneity as described (Conaway *et al.*, 1987). Recombinant human TFIIE (Ohkuma *et al.*, 1991; Peterson *et al.*, 1991; Sumimoto *et al.*, 1991) purified to >80% homogeneity was prepared as described (Peterson *et al.*, 1991), except that the 56 kDa subunit was expressed in BL21(DE3). Recombinant RAP30/74 (TFIIF) was purified to >90% homogeneity by phosphocellulose chromatography (Conaway and Conaway, 1989) of whole cell extracts prepared from Sf21 cells co-infected with recombinant baculoviruses encoding human RAP74 (Aso *et al.*, 1992; Finkelstein *et al.*, 1992) and rat RAP30 (Garrett *et al.*, 1992); recombinant viruses were constructed using the BacPAK6 baculovirus expression system (Clontech). With this set of factors, transcription was >100-fold dependent on RNA polymerase II, the TATA factor (either holo-TFIID or TBP), TFIIB, TFIIE, TFIIF and TFIIH.

Assay of run-off transcription

Except where noted in the figure legends, assays were performed as described (Conaway *et al.*, 1991) with the indicated quantity of template DNA and ~10 ng of recombinant TFIIB, 15 ng of TFIIF, 15 ng of TFIIE, 25 ng of TFIIH (Conaway *et al.*, 1992), 80 ng of holo-TFIID or 20 ng of recombinant rat or yeast TBP, and 0.01 U of RNA polymerase II. Under these conditions reactions were saturated RNA polymerase II, TFIIB, TFIIF, TFIIE and TFIIH; TBP or holo-TFIID were near saturation. All steps were carried out at 60 mM KCl. Transcription was performed in the presence of 7 mM $MgCl_2$, 50 μM ATP, 10 μM CTP, 50 μM GTP, 50 μM UTP and 7 μCi [α-^{32}P]CTP. To limit transcription initiation to a single round of initiation, heparin was added to 10 μg/ml 1–3 min after the addition of ribonucleoside triphosphates. Run-off transcripts were analyzed by electrophoresis through 6% polyacrylamide/7.0 M urea gels. Transcription was quantitated by densitometry of autoradiograms using an LKB UltroScan XL laser densitometer.

DNA templates

Plasmids pN_4 (Lorch *et al.*, 1987); pDN-AdML, pDN-TAGA and pDN-MMTV (Conaway *et al.*, 1990b); pDN-IL3 (Conaway and Conaway, 1989); and pDN-β-Fib (Conaway *et al.*, 1991) were constructed as described. pDN-α-Fib, pDN-IgH and pDN-hDHFR were constructed by inserting double-stranded oligonucleotides containing the sequences TTAACCTAGT TTCCTGCAGG TTTAAGTAGG ATAGGAGCAG TGAGTGAAGT CAGTCCTCCT T, TGTGCGACTG TGATGATTAA TATAGGGATA TCCACACCAA ACATCATAT and CTCGCCTGCA CAAATAGGGA CGAGGGGGCG GGGCGGCCAC AATTTCGC, respectively, into the polylinker of pUC-18 between the *Kpn*I and *Xba*I sites. Plasmids were isolated from *E.coli* essentially as described using the Triton-lysozyme method (Davis *et al.*, 1986). Plasmid DNA was twice banded in CsCl-ethidium bromide density gradients, precipitated with ethanol and dissolved in 10 mM Tris–HCl pH 7.5, 1 mM EDTA. Template DNA fragments were purified from 1.5% agarose gels using GENECLEAN II (BIO 101 Inc.) according to the manufacturer's instructions.

Electrophoretic mobility shift assays

1 ng of a 5′ ^{32}P-labeled double-stranded oligonucleotide containing AdML promoter sequences from −50 to +10 of the transcriptional start site were incubated for 45 min at 28°C, with protein fractions as indicated in the legend to Figure 8, in 15 μl reaction mixtures containing 3 mM HEPES–NaOH (pH 7.9), 20 mM Tris–HCl (pH 7.9), 60 mM KCl, 7 mM $MgCl_2$, 10% glycerol, 0.5 mg/ml BSA and 0.2 μg poly dI-dC/poly dI-dC. Following incubations, reaction mixtures were applied to 4% polyacrylamide gels, which contained 3% glycerol and 0.5 × TBE and which were pre-electrophoresed for 2 h at 200 V. Electrophoresis was performed initially for 30 min at 100 V and finally at 200 V until the bromophenol blue dye in a marker lane just reached the bottom of the gel.

Protein determination

Protein concentrations were determined using the protein dye assay (Bio-Rad), with bovine serum albumin as standard.

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