An ATP-dependent inhibitor of TBP binding to DNA

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An activity in yeast nuclear extracts (termed ADI) is described that inhibits the binding of the TATA-binding protein (TBP) to DNA in an ATP-dependent manner. The effect is reversible, ATP specific, rapid, and is not promoter specific. ADI is specific for TBP because three other protein–DNA complexes are not affected by ADI. The action of ADI is blocked by association of TFIIA with the TBP–DNA complex. ADI activity at the adenovirus major late promoter requires a segment of DNA upstream from the TATA sequence, suggesting that ADI recognizes aspects of both TBP and DNA. The evolutionarily conserved carboxy-terminal domain of TBP is sufficient for ADI recognition, and amino acids in the basic region of TBP are required for ADI action. ADI can repress transcription in vitro in an ATP-dependent manner. In the presence of ADI, both TFIIA and TBP are required to commit a template to transcription. A model of ADI action is proposed, and possible roles of ADI in the regulation of the transcription complex assembly are discussed.

[Key Words: Transcription; TBP; ADI; yeast; inhibitor; ATP]

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The formation of a transcription complex capable of initiating the synthesis of mRNA requires several factors in addition to RNA polymerase II. These general factors include TFIIA, TFIIB, TFIID, TFIIE, TFIIIf, TFIIH, and TFIIJ (Sawadogo and Sentenac 1990; Cortes et al. 1992; Fischer 1992; Gileadi et al. 1992; Lu et al. 1992; Zawel and Reinberg 1992). For many genes, the first step in transcription complex formation is thought to involve the binding of TFIID to a TATA element located upstream of the start site of transcription (Davison et al. 1983; Van Dyke et al. 1988; Buratowski et al. 1989; Cortes et al. 1992). In mammalian cells, TFIID appears to consist of a TATA-binding protein (TBP) tightly complexed to a number of other polypeptides, at least some of which are required for activated transcription in vitro (Tanese et al. 1991 and references therein; Zhou et al. 1992). The gene encoding TBP has been cloned from several organisms (for review, see Greenblatt 1991), and the crystal structure of TBP has been determined (Nikolov et al. 1992). In yeast, the TFIIId fraction contains a monomeric TBP of ~27 kD, which does not appear to be tightly complexed to other proteins.

The binding of yeast TBP is relatively slow, but once it occurs the TBP–DNA interaction is stable (Hahn et al. 1989a), and this interaction is thought to commit a template to transcription (Buratowski et al. 1989). Therefore, the binding of TBP to DNA is likely a point at which transcription complex assembly is regulated. Evidence has accumulated suggesting that some transcription factors exert their positive effects either directly or indirectly by interaction with TFIIID (Sawadogo and Roeder 1985b, Abmayr et al. 1988; Horikoshi et al. 1988a,b, 1991; Pugh and Tjian 1990; Stringer et al. 1990; Lee et al. 1991), although other steps in complex assembly have been proposed as targets (Lin et al. 1991). Here, we describe the characterization and partial purification of a novel ATP-dependent inhibitor of TBP binding (ADI) to DNA. ADI is specific for TBP and can be antagonized by other general transcription factors that form higher order complexes with TBP and DNA. As a result, ADI is a good candidate for a cellular activity that regulates transcription complex assembly by modulating the binding of TBP to DNA.

Results

Assay of ADI

ADI was discovered during the initial purification and characterization of yeast TBP. After purification of yeast TBP by heparin and DEAE chromatography (Buratowski et al. 1988), it was observed that this crude TBP fraction gave a weak DNase I footprint at the adenovirus major late promoter TATA element. In an attempt to improve the DNase I footprinting activity, a number of conditions in the binding reaction were varied, including the addition of ATP. Unexpectedly, ATP was found to completely inhibit the binding of TBP. As demonstrated below, this inhibition was the result of an ADI. Inhibition of TBP binding was used as an assay to purify and characterize this factor.

For subsequent studies we have employed a homogeneous preparation of yeast TBP made in Escherichia coli (Reddy and Hahn 1991) and a partially purified preparation of ADI that is free of TBP, TFIIA, and TFIIIB (Table 1; Bio-Rex 70 fraction). The ADI in this Bio-Rex 70 fraction...
When ADI is added to a TBP–DNA-binding reaction in the absence of ATP, a small fraction of the TBP–DNA complex is shifted to a slower migrating complex (TBP–ADI–DNA) that depends on both TBP and the ADI fraction (Fig. 1A, cf. lanes 1 and 2). This slower mobility complex is not formed stoichiometrically with TBP on the adenovirus major late promoter under these incubation conditions, but it is reproducibly detected [also see Figs. 2 and 3C, below]. The supershifted complex is detected more easily using the LEU2 TATA element as a probe [see below]. We speculate that this complex results from the binding of ADI to the TBP–DNA complex, and evidence in support of this point is detailed below.

In the presence of ADI and 5 μM ATP, the TBP–DNA complex is eliminated almost completely (Fig. 1A, lanes 3, 9). The ADI fraction is required for this activity, as the addition of ATP to TBP alone has no effect on the TBP–DNA complex (lane 5). ADI may require ATP hydrolysis because the nonhydrolyzable ATP analog AMP–PNP does not substitute for ATP in this reaction (lane 4). Other ATP analogs, such as ATP–γ-S, and α,β- and β,γ-methyleneadenosine 5′-triphosphates, also fail to substitute for ATP in this reaction [not shown]. ADI alone has no specific DNA-binding activity [Fig. 1A, lane 8, and B]. A small amount of a complex that migrates faster than the TBP–DNA complex is also detected in reactions that contain ADI (lanes 2–4, 8–11). This complex does not require the presence of TBP [lane 8]; it is only formed on the major late promoter and not the LEU2 promoter [lanes 12–14; see below], and the peak of this binding activity does not coincide exactly with the peak of ADI activity [not shown] so it appears to be unrelated to ADI.

ADI does not alter or destroy TBP irreversibly because the ATP-dependent effect can be reversed with bacterial alkaline phosphatase [lane 10]. The effect of the phosphatase preparation is specific for the phosphatase enzyme itself, as inhibition of phosphatase with β-glycerophosphate reverses the effect of phosphatase and allows ADI to function [lane 11]. The phosphatase is capable of removing phosphate from ATP, as well as from proteins and DNA, and the $K_m$ of the phosphatase for ATP is apparently quite low. Preincubation of phosphatase and ATP in the binding reaction results in depletion of ATP; consequently, ADI does not function [not shown]. This, plus the fact that TBP is not phosphorylated by ADI [see below], indicates that the ability of the phosphatase to reverse the inhibitory effect is the result of its ATPase activity.

The $K_m$ for ATP in this reaction is ~1 μM, and the effect of ATP is rapid and stable; the result of adding ATP for 1 or 5 min is very similar to adding ATP during the entire 25-min incubation [Fig. 1A, cf. lanes 3 and 9; data not shown]. ADI is not promoter specific because complexes formed on either the major late promoter [lanes 1–11] or the yeast LEU2 TATA element [lanes 12–14] are equally dissociated by ADI. Note that the TBP–ADI–DNA complex is detected more easily on the LEU2 TATA element than on the major late promoter [cf. lanes 2 and 13] but that ADI dissociates TBP bound to either template in the presence of ATP [lanes 3,14]. ADI was also found to form ternary complexes, with TBP bound to the CYC1, HIS4, and DED1 promoters, and complexes formed on all of these templates were dissociated by ATP [not shown].

To rule out that the effect of ADI was specific to the gel shift assay, the effects of ADI were confirmed in DNase I footprinting experiments [Fig. 1B]. Purified recombinant yeast TBP was found to protect bases from nuclease digestion at positions −18 to −37, in agreement with previous results [Hahn et al. 1989c]. The ADI fraction alone results in no detectable protection of DNA from DNase I digestion; cleavage is enhanced at −9 and −10, but these changes are not affected by ATP or TBP. In the absence of ATP, ADI causes slight changes in the DNase I footprint of TBP. In particular, the addition of ADI protects positions −38 and −39 from DNase I digestion, a result discussed more fully below. In the presence of ATP, the TBP footprint largely disappears, indicating that TBP has been displaced from the TATA box.

### ADI recognizes the conserved domain of TBP

The effect of ADI on complexes formed between DNA and a 185-amino-acid carboxy-terminal fragment of TBP (ΔTBP) was tested. This truncated protein contains all essential TBP function in yeast [Cormack et al. 1991; Reddy and Hahn 1991]. Incubation of ΔTBP with ADI-containing Bio-Rex 70 fractions in the absence of ATP results in the appearance of a supershifted band [Fig. 2, ΔTBP–ADI]. In fractions 25 and 26, the ΔTBP–DNA complex has been supershifted almost completely to the upper complex, whereas only a portion of the ΔTBP–DNA complex has been supershifted in fractions 27 and 28. In the presence of ATP, the supershifted material is eliminated completely, as is the small amount of ΔTBP–DNA complex that was present in reactions with fractions 25 and 26. Thus, the quantity of supershifted complex parallels the elution profile of ADI activity, and this suggests that ΔTBP is capable of quantitatively forming a ternary complex with DNA and ADI in the absence of ATP.

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### Table 1. Purification of ADI

| Step         | Total protein (mg) | Total units* | Specific activity (U/mg) |
|--------------|--------------------|--------------|-------------------------|
| Nuclear extract | 735.0              | —            | —                       |
| DEAE         | 190.4              | —            | —                       |
| Phenyl–Sepharose | 16.0              | 55,000       | 3,437.5                 |
| Bio-Rex 70   | 0.72               | 24,000       | 33,333.0                |

*One unit of ADI activity is defined as the amount required to completely dissociate all of the TBP–DNA complexes detected in a standard gel shift assay performed as described in Materials and methods.
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Figure 1. ATP dependent inhibition of TBP binding to DNA. [A] A radiolabeled fragment of DNA spanning the adenovirus major late promoter TATA sequence (lanes 1–11) or the LEU2 TATA sequence (lanes 12–14) was incubated with purified recombinant yeast TBP and/or ADI (where indicated), and the TBP-DNA complex was then resolved from the free DNA by nondenaturing polyacrylamide gel electrophoresis as described in Materials and methods. The TBP–DNA and TBP–ADI–DNA complexes are indicated. The reactions in lanes 1–8 and 12–14 were incubated at room temperature for 25 min; 5 μM ATP or the nonhydrolyzable ATP analog AMP–PNP was then added for 5 min where indicated. The reaction in lane 9 contained ATP during the entire 25-min incubation. The reactions in lanes 10 and 11 were incubated for 25 min at room temperature, ATP was then added for 5 min to dissociate the TBP–DNA complexes, and bacterial alkaline phosphatase [0.14 units, Sigma (lane 10)] or phosphatase plus 5 mM β-glycerophosphate (lane 11) was then added for an additional 25-min incubation at room temperature before loading onto the gel. Reactions with ADI contained 1.0 μl (~1 unit) of the Bio-Rex fraction [see Table 1]. [B] DNase I footprinting reactions were performed by partial digestion of a uniquely end-labeled adenovirus major late promoter fragment preincubated with no protein (NP), TBP alone, ADI fraction alone, TBP plus the ADI fraction (TBP + ADI), or TBP plus ADI fraction plus 5 μM ATP (TBP + ADI + ATP) as described above for A and in Materials and methods. The reaction products were visualized by autoradiography of a 10% polyacrylamide sequencing gel. The TBP footprint extends from approximately −18 to −37. Note that these reactions contained 4.8-fold more TBP and 3.2-fold more ADI than was present in the reactions shown in A.
Figure 2. Effect of ADI on DNA complexes formed with the carboxy-terminal domain of TBP. Purified carboxy-terminal domain of TBP [amino acids 56-240; left] or full-length TBP [right] was incubated with radiolabeled adenovirus major late promoter DNA fragment and aliquots of fractions 24-29, as shown, across the Bio-Rex 70 column salt gradient, which includes the peak of inhibitor activity (25-27). ATP [5 μM] was also included where indicated. The reaction products were resolved on a 6% nondenaturing polyacrylamide gel as described in Materials and methods. ADI activity cofractionates with a new complex formed in the absence of ATP (ΔTBP–ADI), which we speculate is formed between TBP, DNA, and ADI [see Discussion]. Both this ternary complex and the ΔTBP–DNA complex (ΔTBP, arrow) are eliminated by ATP. The upper arrow (right) indicates the position of a similar very faint supershifted complex seen with full-length TBP; this is seen more easily in Fig. 1.

The right-hand panel of Figure 2 shows the results with full-length TBP and ADI fractions 25 and 27, which are very similar to results in Figure 1A; only a small fraction of the TBP–DNA complex is stably bound by ADI in the absence of ATP [marked with an arrow]. Although the TBP–ADI–DNA complex was detected more readily on the LEU2 promoter than on the major late promoter, incubation of the LEU2 promoter with the major late promoter, incubation of the LEU2 promoter with ΔTBP and ADI results in the formation of an even greater proportion of ΔTBP–ADI–DNA complexes than are formed under the same conditions using full-length TBP [not shown]. Thus, ADI binds more strongly to a ΔTBP–DNA complex than the full-length TBP–DNA complex. This suggestion is supported by the observation that ADI activity can be bound to a column containing ΔTBP bound to an immobilized TATA sequence but not to a column containing full-length TBP bound to a TATA sequence [not shown]. Finally, ADI activity can be quenched by the addition of excess ΔTBP, but not full-length TBP, to a gel shift reaction using the major late promoter [not shown].

As both the truncated TBP–DNA complex and the ternary complex are abolished by ADI in the presence of ATP, the conserved carboxy-terminal core of TBP contains the features that are recognized by ADI. Consistent with these results, ADI was found to dissociate human TBP from DNA in the gel shift assay [not shown]; a truncated form of human TBP has not been tested.

Specificity of ADI function

To establish the specificity of ADI action, we determined the effect of ADI on two other yeast DNA-binding complexes (Fig. 3). In gel shift assays, the binding of both purified GAL4–VP16 (Carey et al. 1990) and HAP2,3,4 proteins (Hahn and Guarente 1988; Forsburg and Guarente 1989) to their cognate DNA sequences was tested. There was no detectable effect of ADI on these complexes either in the presence or absence of ATP [Figure 3A,B]. ADI also had no effect on complexes formed between DNA and Myb, a protein whose DNA-binding activity was shown to be regulated by phosphorylation
[Lüscher et al. 1990; data not shown]. The failure of ADI to displace HAP2,3,4 from its DNA-binding site is not the result of a contaminant in the preparation that inactivates ADI (Figure 3C). ADI was found to dissociate TBP even in the presence of HAP2,3,4. Similar results were obtained in a control experiment using GAL4–VP16.

Mechanism of ADI action and the role of ATP

To determine whether TBP or proteins in the ADI fraction were covalently modified by ATP, these fractions were incubated with [α-32P]ATP, [γ-32P]ATP, or [2,8,5'-3H]ATP under the same conditions as in the gel shift assay (data not shown). Neither [α-32P]ATP nor [3H]ATP labeled TBP or proteins in the ADI fraction. The ADI fraction appears to contain a kinase, as several polypeptides in this fraction were labeled when incubated with [γ-32P]ATP, but TBP was not labeled and the phosphorylation of other proteins does not depend on the presence of either TBP or DNA. In a separate set of experiments it was found that ADI remains ATP dependent whether or not it has been preincubated with ATP before its addition to TBP–DNA complexes (not shown). Because no polypeptides were detected that were phosphorylated in a TBP- and DNA-dependent manner, these data suggest that ADI is not a kinase. A helicase might be able to unwind DNA and thereby dissociate TBP–DNA complexes; however, DNA unwinding activity has not been detected in any of the ADI fractions (not shown).

Effect of TFIIA and TFIIB on ADI action

The ability of ADI to dissociate complexes formed between TBP, TFIIA, or TFIIB was tested. As shown in Figure 4A, the addition of purified recombinant yeast TFIIA to a binding reaction containing TBP results in the formation of a TBP–TFIIA–DNA complex (lanes 3–7; Ranish et al. 1992). The binding reactions were performed with either a subsaturating amount (lanes 3,4) or a saturating amount (lanes 5–7) of TFIIA. In the presence of either subsaturating or saturating amounts of TFIIA, ADI reduces the TBP–TFIIA–DNA complex by, at most, twofold, as determined by densitometry. When both TBP–DNA and TBP–TFIIA–DNA complexes can be detected, the addition of ADI and ATP results in a sixfold reduction in the TBP–DNA complex but only a twofold reduction in the amount of the TBP–TFIIA–DNA complex (lanes 3,4). Thus, TFIIA appears to stabilize TBP to

Figure 4. Effects of TFIIA and TFIIB on ADI activity. (A) In lanes 1–6 yeast TBP, yeast TFIIA, ADI and ATP were incubated as indicated with a radiolabeled adenovirus major late promoter DNA fragment for 20 min at room temperature. In lane 7, TBP, ADI, DNA, and ATP were incubated together for 20 min followed by the addition of TFIIA for 20 min. The reactions in lanes 3 and 4 contained ~0.7 DNA-binding units of TFIIA, whereas the reactions in lanes 5–7 contained ~2 units of TFIIA. The reaction products were analyzed by nondenaturing gel electrophoresis as described in Materials and methods. The TBP–DNA and TBP–TFIIA–DNA complexes are indicated. (B) Yeast TBP, yeast TFIIB (SUA7), ADI, and ATP were incubated with radiolabeled adenovirus major late promoter DNA fragment (as above) for 30 min at room temperature, as indicated, and the various complexes were resolved on a 6% polyacrylamide gel as described in Materials and methods. In this gel system, neither TBP nor SUA7 alone are capable of forming a complex that is stable to gel electrophoresis. The position of the observed TBP–SUA7–DNA complex is shown. Note that a nonspecific complex (NS) is formed between DNA and a contaminant protein in the SUA7 preparation; this complex is present in all lanes that contain SUA7 (even lane 2 in which no TBP is present).
dissociation by ADI. Surprisingly, TFIIA can also reverse the effect of ADI. When TBP and DNA are first incubated with ADI and ATP to prevent the formation of TBP–DNA complexes, the subsequent addition of TFIIA still results in the formation of TBP–TFIIA–DNA ternary complexes (cf. lanes 6 and 7).

The effects of the addition of purified recombinant SUA7 protein [yeast TFIIB (Pinto et al. 1992)] on ADI function are shown in Figure 4B. A nonspecific protein–DNA complex attributable to a contaminating polypeptide in the recombinant SUA7 preparation is observed when SUA7 alone is added (Fig. 4B, NS). Under the gel conditions used, interactions between DNA and either SUA7 or TBP cannot be detected [lanes 1,2] (Maldonado et al. 1990). The incubation of both TBP and SUA7 together, however, results in the formation of a TBP–SUA7–DNA complex (lane 3). There is a twofold decrease in the abundance of the ternary complex when ADI is added (cf. lanes 3 and 4). This decrease is not ATP dependent [lanes 4,5]. Quantitation of the free DNA indicates that the decrease in the abundance of the ternary complex by ADI in the absence of ATP is not the result of the displacement of TBP and SUA7; rather, components of the ADI fraction supershift a small fraction of the TBP–SUA7–DNA complexes to new positions that are detected on longer exposure of the gel [not shown]. The nature of such supershifted complexes has not been investigated further. Like TFIIA, SUA7 can also reverse the effect of ADI. When TBP–DNA complexes are first formed and then dissociated by the addition of ATP and ADI, the subsequent addition of SUA7 results in the same level of TBP–SUA7–DNA complexes as are formed when ADI, ATP, and SUA7 are added to TBP and DNA together (cf. lanes 5 and 6).

Similar gel shift experiments were performed with human TFIIA and recombinant human TFIIB [data not shown]. The results obtained with the human and yeast factors are nearly identical, with the exception that human TFIIA is less able than yeast TFIIA to protect TBP from ADI action.

**ADI action requires DNA 5’ to the TATA sequence**

As ADI is specific for TBP–DNA complexes, perhaps a certain region of promoter DNA is required to mediate the dissociation reaction. Further insight into the mechanism of action of ADI was obtained by determining which regions of DNA are essential for inhibitor action. This was accomplished by performing the gel shift analysis using fragments of DNA encompassing various regions of the major late promoter. As shown in Figure 5, A and B, sequences 3’ to the boundary of the TBP footprint are dispensable for ADI action. Surprisingly, however, deletion of sequences 5’ to the footprint boundary (fragments d and e) result in complexes not dissociated by ADI. This suggests that ADI approaches a TBP–DNA

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**Figure 5. ADI activity requires DNA sequences 5’ to the TBP binding site.** (A) TBP was incubated with various radiolabeled adenovirus major late promoter DNA fragments (a–e) in the presence of the ADI fraction and ATP where indicated. The approximate positions of the TBP–DNA complexes are shown by the arrow. The relationships of the various DNA fragments to each other and a summary of the data in A are shown in B. The broken vertical lines at -38 and -18 indicate the approximate size of the TBP footprint. Note that fragments d and e which do not contain DNA 5’ to the TBP-protected footprint, fail to allow ADI to function.
complex in a stereospecific manner that involves recognition or interaction with a particular surface of TBP and DNA.

**ADI interacts with the basic region of TBP**

In binding reactions containing TBP, TFIIA, and ADI, a competition results between TFIIA and ADI for mutually exclusive interaction with TBP [Fig. 4A; data not shown]. This competitive behavior suggests that ADI and TFIIA might interact with a similar region of TBP. The conserved region of TBP consists of two 66- to 67-amino-acid direct repeats that are separated by a region rich in basic amino acids. Previously, it has been shown that the direct repeats contribute to DNA binding, whereas the basic region interacts with TFIIA [Reddy and Hahn 1991; Buratowski and Zhou 1992; Yamamoto et al. 1992]. To better define the interaction of TBP and ADI, two mutant TBPs were tested that each contain two amino acid changes in the basic region (Buratowski and Zhou 1992). Each of these mutants has been shown to be defective in interaction with TFIIA (Buratowski and Zhou 1992). As shown in Figure 6, the wild-type TBP forms TBP–ADI–DNA complexes in the absence of ATP, and the complexes are disrupted when ATP is added. [The TBP–ADI–DNA complex is a doublet in this experiment; we believe that certain preparations of ADI generate this doublet because ADI has been partially proteolyzed while still retaining its enzymatic activity.] The basic region mutants (K133,138L and K133,145L; Buratowski and Zhou 1992). Each of these mutants has been shown to be defective in interaction with TFIIA (Buratowski and Zhou 1992). As shown in Figure 6, the wild-type TBP forms TBP–ADI–DNA complexes in the absence of ATP, and the complexes are disrupted when ATP is added. The basic region mutants (K133,138L and K133,145L) bind DNA but are defective in formation of the TBP–ADI–DNA complex. ATP has little effect on the TBP–DNA complex formed with these proteins. The fact that the basic region mutants are defective in formation of the supershifted complex, as well as dissociation by ATP, provides additional support for the contention that the supershifted complex is composed of TBP, DNA, and ADI. Quantitation of the gel shows that K133,138L forms <1% of the TBP–ADI–DNA complex formed by wild-type TBP, and TBP–ADI–DNA complexes are not detectable using K133,145L. ADI plus ATP results in a 30-fold decrease in the abundance of the wild-type TBP–DNA complex, whereas the complex with K133,138L is reduced 1.6-fold and the complex with K133,145L is not altered detectably. As with TFIIA, the interaction between TBP and ADI is mediated, at least in part, by interactions with the basic region of TBP.

**An inhibitor of transcription in vitro**

The effect of ADI on transcription was addressed by performing template commitment experiments using yeast TBP and general transcription factors obtained from HeLa cells [Samuels et al. 1982]. Our fractionated yeast transcription system [Ranish et al. 1992] contains small amounts of TBP in several required fractions, so the HeLa system was used because of the well-characterized dependence on exogenously added TBP [Buratowski et al. 1988]. The experiments employed the adenovirus major late promoter fused to each of two G-less cassettes of different lengths. Following transcription in the absence of GTP and treatment by RNase T1, the two constructs yield transcripts of different lengths (Sawadogo and Roeder 1985a; Buratowski et al. 1988). The protocol for the experiments is shown in Figure 7C, and the results are displayed in Figure 7, A and B. Note that transcription using both templates in the preincubation step yields comparable amounts of RNA from each plasmid [Figure 7A, lane 1]. A limiting amount of yeast TBP was first incubated with template 1 for 25 min, and then ADI was added either in the presence or absence of ATP, TFIIA, or TFIIIB. Following a second incubation, the second template, the other general factors, RNA polymerase II, and nucleoside triphosphates were added. RNA synthesis was then allowed to proceed for 60 min, the reactions were terminated, and the RNA products were resolved on polyacrylamide gels. The experiment in Figure 7A was performed with a low level of ADI (~0.3 units of activity; see Materials and methods). Lane 2 demonstrates that preincubation of template 1 with TBP results in almost exclusive transcription from this template and very little from the challenging template 2. The differential transcription of the two templates is unaffected by the addition of ADI [lane 3]. The addition of ADI plus ATP, however, leads to an overall decrease in transcription [by about twofold; lane 4], and transcription occurs from both templates. The addition of TFIIA [lane 5] blocks ADI action, and transcription occurs primarily from template 1. Human TFIIIB also restores transcription to the same level as that obtained in the absence of ATP, but ADI has dissociated some of the TBP from template 1 allowing for increased transcription of tem-
TBP inhibitor

Figure 7. ADI affects template commitment and represses transcription in vitro in an ATP dependent manner. In vitro transcription reactions were performed using recombinant yeast TBP, human general transcription factors, and calf thymus RNA polymerase II as described in Materials and methods. Transcription from each of the two promoters employed can be distinguished because the templates contain different length G-less cassettes [Buratowski et al. 1988]. 

(A) Transcription using an equimolar amount of each of the two templates generates very similar levels of RNA from each template (lane 1), whereas preincubation of template 1 with a limiting amount of TBP results primarily in transcription from template 1 (lane 2). TBP alone is also able to commit template 1 to transcription in the presence of TBP and ADI (lane 3), but when ATP is added (lane 4) the overall level of transcription is reduced [by about twofold] and transcription occurs from both templates. The ATP dependent inhibitory effect can be blocked by TFIIA (lane 5), and to a small but reproducible extent by human TFIIB (lane 6). 

(B) In vitro transcription was performed essentially as in A, except that approximately three- to fourfold more ADI (-1 unit; see Table 1) was used in each reaction. Note that the addition of this increased amount of ADI plus ATP reduces transcription from both templates (cf. lane 1 with lane 2), and the effect is at least partially blocked by TFIIA and TFIIB (lanes 3,4). 

(C) The protocol for the experiments shown in A and B is diagramed.

Discussion

Mechanism of action of ADI

An activity in yeast nuclear extracts has been identified that specifically inhibits the binding of yeast TBP to DNA. This activity requires micromolar concentrations of ATP and acts to rapidly remove TBP from its cognate DNA-binding site. ADI does not bind DNA alone as judged by gel shift analysis and DNase footprinting (Figure 1A, B), nor does it appear to strongly bind full-length TBP in solution as measured by TBP affinity column chromatography (not shown). ADI can bind to a TBP-DNA complex formed on either the major late promoter or the LEU2 promoter, although it has an apparently higher affinity for a TBP-DNA complex formed on the LEU2 promoter. Regardless of the DNA template used, ADI binds more tightly to ATBP-DNA complexes than to complexes formed with full-length TBP. The specificity of ADI for TBP is demonstrated by its failure to dissociate other protein–DNA complexes and its failure to dissociate basic region mutant TBP–DNA complexes. We have not detected either phosphorylation or adenylation of TBP, or TBP-dependent, covalent modification of proteins in the ADI fraction.

One model of ADI action that is consistent with all of the data is that ADI is an ATPase that utilizes the energy of ATP hydrolysis to dissociate TBP from DNA. Complexes containing TFIIA are stable so TBP is sequestered...
from the action of ADI [Fig. 8]. TFIIIB also has some ability to prevent dissociation of TBP from DNA by ADI, but the effects of TFIIIB in either gel shift assays or transcription are less dramatic than that observed with TFIIA. It is possible that any protein that can form a complex with TBP and stabilize its binding to DNA will antagonize ADI action; the effectiveness of a factor in blocking ADI action could reflect the rate at which the factor binds a TBP–DNA complex and the stability of the ternary complex once it is formed. Because ADI does not tightly associate with wild-type TBP off the DNA, following the ATP dependent dissociation of a TBP–DNA complex the ADI molecule would be free to dissociate another TBP–DNA complex and the dissociated TBP would be free to rebind DNA. Rebinding of DNA by a displaced TBP can explain why TFIIA added after ADI and ATP can form a TBP–TFIIA–DNA complex (Fig. 4A, lane 7). An ATPase activity has been detected in the ADI fraction [not shown], but its direct association with ADI will not be possible until the ADI has been purified to homogeneity.

The effects of TFIIA and TFIIIB in countering the action of ADI are probably either attributable to a steric block of a surface of TBP recognized by ADI or to stabilization of TBP binding. The binding of TFIIA to a TBP–DNA complex protects DNA sequences 5′ to the TBP footprint, and DNA 5′ to the TBP binding site was shown to be essential for ADI action. Consistent with these results, DNase footprinting also showed that ADI protected two positions from DNase I digestion just upstream from the 5′ boundary of the TBP footprint. Because the DNA sequences of the promoters that were employed in the ADI assay do not contain conserved DNA sequences 5′ to the TBP footprint, it is possible that ADI makes nonspecific contacts with the DNA in this region, and these contacts may serve to either orient the inhibitor or to provide a “grip” with which TBP can be dissociated from the TATA sequence. However, ADI appears to bind more tightly to a TBP–DNA complex formed on the LEU2 promoter than on the major late promoter or any of the other promoters tested [not shown] so base-specific contacts may contribute to the affinity of ADI for a particular TBP–DNA complex.

Possible roles of ADI

In light of the data presented in this study, several possible functions of ADI can be imagined. First, ADI may be involved in maintaining promoter specificity. It has been shown that TBP will bind to multiple, diverse DNA sequences with comparable affinity [Hahn et al. 1989c; Singer et al. 1990]. While other sequences [e.g., upstream activating sequences (UASs)] help to determine which TBP-binding sites are ultimately utilized as promoters,
the stable binding of TBP to DNA may necessitate an activity that is capable of dissociating TBP from TATA-like sequences located throughout the genome at non-promoter sites. It is surprising that yeast cells are not expected to drive the binding of TBP to DNA and inappropriately activate transcription. Perhaps this inappropriate TBP binding is prevented by ADI. It is also possible that ADI regulates transcription in vivo at only a subset of promoters. The selective requirement of certain general transcription factors (i.e., TFIIA and TFIIB) and/or the rate at which they assemble with TBP could influence the degree to which ADI effects transcription initiation at specific promoters. Finally, TBP is also required for the transcription of genes transcribed by RNA polymerases I and III [Dahlberg and Lund 1991; Comai et al. 1992; Cormack and Struhl 1992, Schultz et al. 1992, White et al. 1992], and ADI may be involved in the regulation of genes transcribed by either of the other polymerases. Because ADI action is blocked by other factors that interact with TBP, it is less likely that TBP complexes formed on other promoters that do not contain TATA boxes would be affected by ADI. ADI may play a role, however, in regulating transcription from genes transcribed by RNA polymerase II that contain a TATA box (such as the U6 gene; Dahlberg and Lund 1991). In view of the high degree of conservation of the components of the RNA polymerase II transcription apparatus that have been described thus far, and the ability of ADI to function in conjunction with human factors, it is possible that similar inhibitor activities exist in other eukaryotic cells as well.

Materials and methods

Gel shift assay of ADI activity

Radiolabeled DNA fragment (0.5 nM, 20,000 cpm/ng; see below) was incubated at room temperature for 30 min with 10 nM recombinant yeast TBP (either full-length or truncated TBP) in a 20-μl binding reaction that contained 4 mM Tris-Cl (pH 8), 60 mM KCl, 5 mM MgCl2, 4% glycerol, 0.1% Brij 58, 100 ng of poly[dG-dC] and 100 μg/ml of BSA. One unit (~1 μl) of ADI activity from the Bio-Rex fraction [see Table I] and/or 5 μM ATP were included where indicated. The reaction products were analyzed on 6% polyacrylamide gels containing 25 mM Tris-glycine, 5 mM magnesium acetate, 2.5% (vol/vol) glycerol and 0.5 mM DTT in running buffer containing 25 mM Tris (pH 8.3), 190 mM glycine, and 5 mM magnesium acetate. Gels were run at 4°C. Binding reactions containing TFIIA and/or TFIIB were performed under the same conditions as for TBP alone. Complexes containing TFIIB were detected on gels as described above but that lacked glycerol and magnesium acetate and were run at room temperature in Tris-glycine buffer lacking magnesium acetate [Buratowski et al. 1991]. These are the only conditions we have found that allow the detection of complexes containing TFIIB in a gel shift assay. Quantitation of the levels of gel-shifted complexes was performed by either densitometry or PhosphorImager analysis.

The binding of GAL4-VP16 to DNA occurred under the same conditions as for TBP, but the reaction products were analyzed on 6% polyacrylamide gels in Tris-glycine buffer without magnesium acetate or EDTA. HAP2,3,4 protein was also bound to DNA in the same buffer as TBP, but the reaction products were analyzed on 6% polyacrylamide gels that were run in 10 mM Tris-Cl (pH 8), 1 mM EDTA.

DNA fragments

Most of the binding reactions contained a radiolabeled 106-bp EcoRI-XbaI fragment from plasmid pRW2 [Chodosh et al. 1986]. This fragment contains adenovirus major late promoter sequences from -50 to +33. The binding of TBP to the LEU2 TATA sequence utilized a radiolabeled 59-bp SalI-Aval fragment from pMS1 [Hahn et al. 1989b]. HAP2,3,4 protein was bound to a radiolabeled 89-bp Smal-XhoI fragment from p265up-1, which contains the HAP2,3,4 UAS2up-1 binding site [Hahn and Guarente 1988]. The binding of GAL4-VP16 to DNA was detected using a radiolabeled 107-bp XhoI-SacI fragment from pSK Gal4, which contains a single copy of a GAL4-binding site [Chasman et al. 1989] cloned into the EcoRI and BamHI sites of pSK II. Adenovirus major late promoter fragments of 72 and 51 bp in length [fragments a and b in Fig. 5] were obtained by digestion of pRW2 with XbaI and EarI (72 bp) or XbaI and HhaI (51 bp), followed by labeling at the XbaI site with the Klenow fragment of DNA polymerase and [α-32P]dCTP. The 39-bp fragment c [Fig. 5] was obtained by polymerase chain reaction using 20-base oligonucleotides complementary to the major late promoter sequence. Fragment d is 45 bp in length and e is 20 bp, both of these fragments were obtained by chemical synthesis of DNA.

Footprinting of TBP bound to DNA

The binding conditions were similar to those described above for the gel shift assay except that the DNA concentration was 5 nM, TBP was present at 48 nM and reactions contained 6.5 units of ADI activity where indicated. The DNA template consisted of the 106-bp EcoRI-XbaI major late promoter fragment uniquely labeled at the XbaI site. Following a 30-min incubation at room temperature, 5 ng of DNase I was added for 15–40 sec. The reactions were terminated by ethanol precipitation, and the reaction products were analyzed on 10% polyacrylamide-urea sequencing gels.

Recombinant proteins

TBP was purified from an E. coli strain overproducing TBP under control of the phage T7 promoter as described previously [Reddy and Hahn 1991]. Truncated TBP (ΔTBP) consists of TBP that has been digested proteolytically to remove the amino-terminal 55 amino acids and then purified further to eliminate the cleaved amino-terminal peptide. The truncated protein was the generous gift of J. Geiger (Yale University, New Haven, CT). The TBP preparations used in these studies had no detectable contaminants, as judged by silver staining of SDS-polyacrylamide gels. Plasmids encoding the TBP basic region mutants were obtained from S. Buratowski [Whitehead Institute, Cambridge, MA], and the proteins were purified from an E. coli overproducing strain as described [Buratowski and Zhou 1992]. TFIIA was purified from E. coli as described [Ranish et al. 1992]. GAL4-VP16 fusion protein was purified from an E. coli strain overproducing GAL4-VP16 under control of the tac promoter as
The inhibitor activity purified through the Bio-Rex 70 fraction unto was washed with 12 ml of loading buffer and then protein column equilibrated in HA + 0.1 M potassium acetate. The pooled protein (98%) was then loaded onto a 4.5-ml Bio-Rex 70 plus 0.1 M potassium acetate. A percentage (15.6 mg) of this step is estimated to be enriched from yeast cells. ADI eluted from ~0.47 to 0.55 mM potassium acetate.

In vitro transcription

The in vitro transcription reactions utilized recombinant yeast TBP (2.8 ng/reaction), human TFIIA (fraction AB), and other HeLa cell general transcription factors (fraction CB) isolated as described [Samuels et al. 1982] and kindly provided by S. Buratowski. RNA polymerase II was purified from calf thymus as described [Samuels et al. 1982]. The DNA templates (50 ng/reaction) consisted of the adenovirus major late promoter fused to G-less cassettes of different lengths, and transcription was performed essentially as described [Buratowski et al. 1988] except for the order in which the components were added and the lengths of the various preincubations that are depicted in Figure 7C. The reactions were performed in the presence of [α-32P]CTP, and the products were analyzed by autoradiography of 6% polyacrylamide–urea gels. Quantitation of the transcript levels was performed by either densitometry or PhosphorImager analysis.

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