Targeting TBP to a non-TATA box *cis*-regulatory element: a TBP-containing complex activates transcription from snRNA promoters through the PSE

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In the human small nuclear RNA (snRNA) promoters, the presence of a TATA box recognized by the TATA box-binding protein (TBP) determines the selection of RNA polymerase III over RNA polymerase II. The RNA polymerase II snRNA promoters are, therefore, good candidates for TBP-independent promoters. We show here, however, that TBP activates transcription from RNA polymerase II snRNA promoters through a non-TATA box element, the snRNA proximal sequence element (PSE), as part of a new snRNA-activating protein complex ($SNAP_c$). In contrast to the previously identified TBP-containing complexes SL1, TFIID, and TFIIIB, which appear dedicated to transcription by a single RNA polymerase, $SNAP_c$ is also essential for RNA polymerase III transcription from the U6 snRNA promoter. The U6 initiation complex appears to contain two forms of TBP, one bound to the TATA box and one bound to the PSE as a part of $SNAP_c$, suggesting that multiple TBP molecules can have different functions within a single promoter.

[*Key Words:* snRNA promoter; TATA box-binding protein; transcriptional activation; $SNAP_c$; proximal sequence element]

Received April 16, 1993; revised version accepted June 18, 1993.

In eukaryotes, transcription is carried out by three different RNA polymerases, none of which can recognize its target promoters directly. Instead, promoter elements are first recognized by specific transcription factors that then recruit the correct RNA polymerase. Because RNA polymerase I, II, and III promoters are generally very different in structure, it has long been assumed that RNA polymerase specificity is achieved through the binding of very distinct sets of transcription factors. Although it is now well established that the TATA box binding protein (TBP) participates in transcription from TATA-containing and TATA-less promoters by all three RNA polymerases, it does so as part of distinct complexes that are each dedicated to transcription by a single RNA polymerase (for review, see Hernandez 1993). Thus, SL1 (Comai et al. 1992), TFIID (for review, see Sawadogo and Sentenac 1990; Roeder 1991; Pugh and Tjian 1992; Zawel and Reinberg 1992,1993), and TFIIIB (Lobo et al. 1992; Simmen et al. 1992b; Taggart et al. 1992; White and Jackson 1992) participate in transcription by RNA polymerases I, II, and III, respectively.

The promoters of human small nuclear RNA (snRNA) genes are very similar in structure even though some of them are recognized by RNA polymerase II whereas others are recognized by RNA polymerase III. They therefore serve as a model to study how RNA polymerase specificity is achieved. The human U1 and U2 snRNA promoters are recognized by RNA polymerase II and consist essentially of two elements: A proximal sequence element (PSE) located upstream of position −40, which is essential and sufficient to direct basal levels of transcription, and a distal sequence element (DSE) located upstream of position −200, which serves as a transcriptional enhancer and is characterized by the presence of an octamer motif (for review, see Dahlberg and Lund 1988; Hernandez 1992). The human U6 snRNA promoter, which is recognized by RNA polymerase III, differs from most other RNA polymerase III promoters in that it does not contain any essential elements that are internal to the gene. Instead it contains, like the U1 and U2 promoters, a PSE and a DSE and, in addition, a TATA box located between positions −24 and −31. The TATA box is a dominant element that defines the U6 promoter as an RNA polymerase III promoter. If the TATA box is debilitated, the U6 promoter directs predominantly RNA polymerase II transcription. Conversely, if the U6 TATA box is introduced into the U2 promoter, the U2 promoter directs RNA polymerase III transcription (Lobo and Hernandez 1989; for review, see Hernandez 1992).

Unlike transcription from the RNA polymerase II snRNA promoters, which is difficult to reproduce in vitro, transcription from the U6 promoter can be obtained

readily in an in vitro transcription system. As a result, the factors required for U6 transcription have been separated into several fractions. One of these fractions contains a PSE-binding factor, termed PBP (Waldschmidt et al. 1991). Surprisingly, although PBP is a human factor, it binds much better to the PSE of the mouse U6 promoter than to the PSE of the human U6 promoter. However, this correlates with the relative transcriptional activities of these two genes in extracts from HeLa cells, strongly suggesting that PBP is involved in U6 transcription by RNA polymerase III (Waldschmidt et al. 1991; Simmen et al. 1992a). A second PSE-binding factor implicated in transcription from the 7SK promoter, a promoter related to the U6 promoter, has been termed PTF (Murphy et al. 1992). PTF binds to the 7SK PSE, as well as to the PSEs of several RNA polymerase II and III snRNA genes. Because the compositions of PTF and PBP are unknown, it is not clear whether these two factors are related.

RNA polymerase III transcription from the U6 promoter also requires TBP (Lobo et al. 1991; Simmen et al. 1991). TBP binds to the U6 TATA box but not to mutated versions that switch the specificity of the U6 promoter to RNA polymerase II, suggesting that in the context of the U6 promoter, the binding of TBP to the TATA box specifically selects RNA polymerase III (Lobo et al. 1991). These observations raise the possibility that the RNA polymerase II snRNA promoters represent a unique class of genes that does not require TBP for transcription. To examine this question, we have used a panel of monoclonal antibodies (mAb) directed against human TBP to deplete extracts and test their ability to sustain RNA polymerase II transcription of snRNA genes. We show that transcription from the U1 promoter requires a TBP-containing complex that we refer to as snRNA activating protein complex, or $SNAP_c$, which binds specifically to the PSE. In addition to TBP, this complex contains at least three polypeptides that can be cross-linked specifically to the PSE. Thus, in effect, these TBP-associated factors (TAFs) reprogram the binding specificity of TBP and direct it to a sequence that is unrelated to a TATA box. Remarkably, this complex appears to be also required for transcription of the RNA polymerase III U6 gene. Transcription from the U6 promoter seems to require at least two forms of TBP, one bound to the TATA box and the other bound to the PSE as part of $SNAP_c$. Thus, the RNA polymerase II and III initiation complexes formed on snRNA promoters may differ by the presence of one or two forms of TBP. These results raise the possibility that other types of initiation complexes may contain more than one form of TBP.

Results

RNA polymerase II transcription from the U1 and U2 snRNA promoters in vitro

RNA polymerase II transcription from vertebrate snRNA promoters is difficult to reproduce in vitro, even in extracts that are very active for transcription from mRNA promoters. However, Gunderson et al. (1990) showed that by using constructs in which the U1 promoter was fused to a G-less cassette (Sawadogo and Roeder 1985), they could detect accurate U1 transcription initiation in a nuclear extract. We therefore generated similar constructs with both the human U1 and U2 promoters, which are shown in Figure 1A. $pU1G^-$ and $pU2G^-$ contain the wild-type human U1 and U2 promoters fused to a G-less cassette, whereas in the constructs $pU1*G^-$ and $pU2*G^-$, the U1 and U2 PSEs were changed by site-directed mutagenesis to the PSE sequence of the mouse U6 gene. We reasoned that because the mouse U6 PSE has a higher affinity for the PSE-binding factors PBP and PTF than either the human U1, U2, or U6 PSEs (Murphy et al. 1992; Simmen et al. 1992a) and because the human U2 and U6 PSEs can be exchanged without effect on RNA polymerase specificity (Lobo and Hernandez 1989), the $pU1*G^-$ and $pU2*G^-$ constructs might be transcribed more efficiently in vitro. We also generated the construct $pU2*ABCG^-$, in which a mutation, referred to as the ABC mutation, alters 6 bp that are highly conserved in all mammalian PSEs (see Hernandez 1992) and debilitates PSE function in vivo as determined in a transient transfection assay (data not shown).

To transcribe the templates in vitro, each of these constructs was incubated in a whole cell extract (Maroney et al. 1990; see Materials and methods) in the absence of exogenously added GTP and in the presence of the chain terminator 3′-*O*-methyl GTP and RNase T1. 3′-*O*-methyl GTP competes with GTP present in the extract and thus decreases background transcription from cryptic promoters within vector sequences. RNase T1 cleaves RNA after G residues and therefore digests all RNA molecules initiated within vector sequences and reading through the G-less cassette to a discrete size. This size is 5 and 7 nucleotides longer than RNAs correctly initiated from the U1 and U2 promoters, respectively, because the first G residue upstream of the transcriptional start site is at position −6 in the U1 constructs and −8 in the U2 constructs (Fig. 1A). The results are shown in Figure 1B. The $pU1G^-$ and the $pU2G^-$ constructs each generated two products after RNase T1 digestion: one of the length expected for RNA correctly initiated at the U1 or U2 promoter (bands labeled U1 5′ and U2 5′), and one of the length expected for RNAs initiating upstream and reading through the G-less cassette (bands labeled RT, lanes 1,5). Consistent with the identities of these bands, the intensity of the RT band, but not that of the U1 5′ and U2 5′ bands, diminished with increasing concentrations of 3′-*O*-methyl GTP (data not shown), confirming that the RT bands resulted from transcription through sequences containing G residues. The synthesis of the U1 5′ and U2 5′ RNAs, as well as that of the RT RNAs, was sensitive to low levels of α-amanitin, indicating that it is directed by RNA polymerase II.

Importantly, synthesis of the U1 5′ and U2 5′ RNAs, unlike that of the RT RNAs, is dependent on the PSE. The $pU1*G^-$ and $pU2*G^-$ constructs, in which the PSEs are replaced by the mouse U6 PSE, directed higher levels of the U1 5′ and U2 5′ RNAs but similar levels of

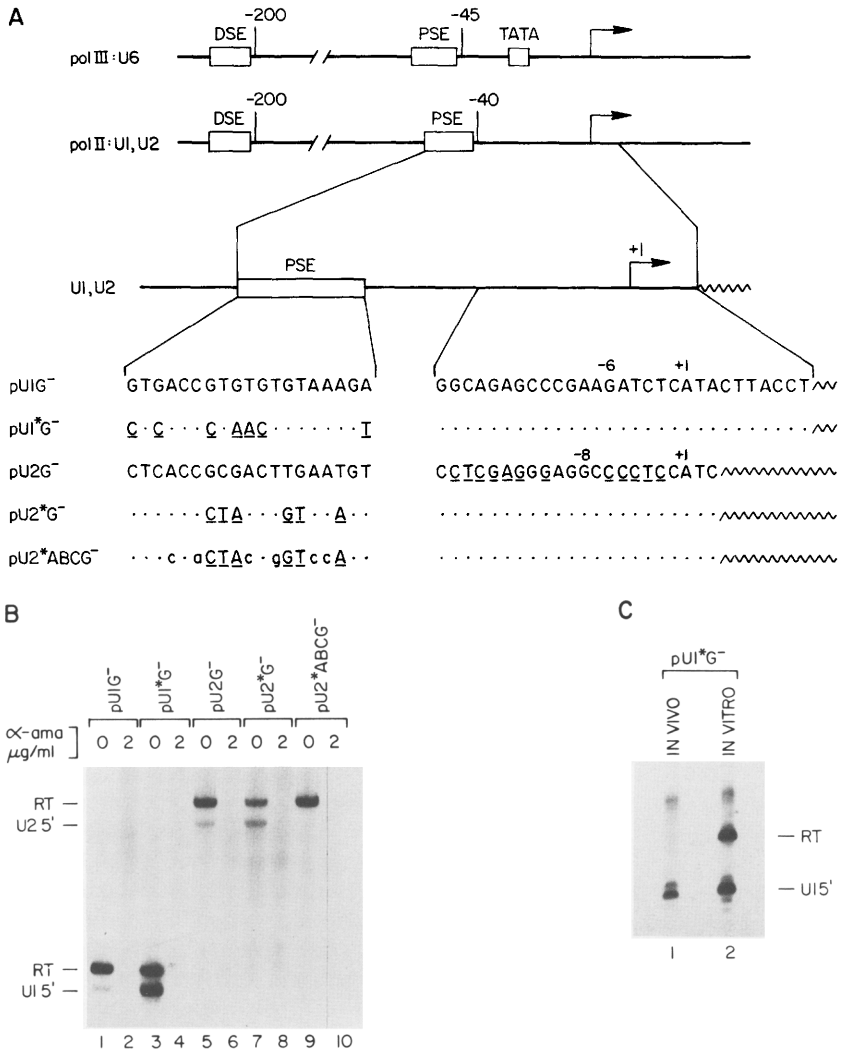

Figure 1. (*A*) Structure of the RNA polymerase III and II snRNA promoters and the pU1G⁻ and pU2G⁻ constructs and their derivatives. The sequence around the transcriptional start site is indicated, with the position of the first G residue upstream of the transcriptional start marked. In the U2G⁻ construct and its derivatives, several differences with the wild-type U2 sequence are indicated by underlined letters. pU1G⁻ and pU2G⁻ contain the wild-type human U1 and U2 PSE, respectively. In pU1*G⁻ and pU2*G⁻, point mutations, indicated by underlined letters, were introduced into the PSEs to recreate the sequence of the wild-type mouse U6 PSE. In pU2*ABCG⁻, the ABC mutation changes 6 nucleotides highly conserved in all human PSEs (Hernandez 1992) to the residues indicated in lowercase letters. This mutation debilitates the PSE as determined in transfection experiments (data not shown). (*B*) Activity of the pU1G⁻ and pU2G⁻ constructs and their derivatives in a whole cell extract. The constructs used as templates for transcription in vitro are indicated above the lanes. In the lanes labeled 2 (lanes *2,4,6,8,10*), 2 μg/ml of αa-amanitin was added to the transcription reactions to inhibit transcription by RNA polymerase II. The bands corresponding to RNA correctly initiated at the U1 and U2 promoters are labeled U1 5′ and U2 5′, respectively. The bands corresponding to RNA initiated upstream and reading through the G-less cassette are labeled RT. Note that the decrease of the RT band in lane 7 is not reproducible and is indicative of some sample loss in this lane. (*C*) Primer extension analysis of the U1 5′ and RT RNAs. (Lane *2*) A reaction similar to that shown in *B*, lane *3*, but performed in the absence of any labeled nucleotide was fractionated on a gel; the U1 5′ and RT bands were excised, and the RNAs were eluted from the gel and submitted to primer extension analysis. (Lane *1*) A similar primer extension was performed with RNA derived from HeLa cells transfected with the pU1*G⁻ construct.

the RT RNAs as compared with the parent constructs (cf. lane 3 with lane 1 and lane 7 with lane 5). Furthermore, the pU2*ABCG⁻ construct, in which the PSE is debilitated, did not produce any detectable U2 5′ RNA but produced similar levels of RT RNA as the parent construct (cf. lanes 9 and 5). Thus, basal transcription from the U1 and U2 promoters in vitro is dependent on the same element as transcription in vivo.

To ensure that transcription initiation was accurate, we excised both the U1 5′ and RT RNA bands from the gel, eluted the RNAs, and mapped their 5′ ends by primer extension. As shown in Figure 1C, the 5′ end of the RNA labeled U1 5′ is identical to that of RNA derived from the pU1G⁻ construct in transfected cells (cf. lanes 1 and 2). Moreover, the RNA labeled RT is 5 nucleotides longer than the RNA labeled U1 5′, as expected for an RNase T1 digestion product cleaved after the G residue at position −6 (lane 2; see also Fig. 1A). Thus, we could reproduce accurate RNA polymerase II transcription from both the human U1 and U2 promoters in vitro, and this transcription was more efficient when the U1 and U2 PSEs were replaced by the mouse U6 PSE. Therefore, in subsequent experiments, we used the pU1*G⁻ and pU2*G⁻ constructs to investigate the transcription factor requirements for RNA polymerase II snRNA transcription.

RNA polymerase II transcription from the U1 and U2 snRNA promoters requires TBP

With a soluble system capable of reproducing accurate RNA polymerase II transcription from snRNA promoters, we asked whether transcription from the human U1 and U2 snRNA promoters requires TBP. For this purpose, we used a panel of mAbs that we had raised previously against human TBP (Materials and methods; Lobo

et al. 1992). Amino acid sequence comparison of TBP from different species reveals a structure consisting of a highly conserved carboxy-terminal domain that is sufficient for binding to the TATA box and a hypervariable amino-terminal domain (Fikes et al. 1990; Gasch et al. 1990; Hoffmann et al. 1990a,b; Horikoshi et al. 1990; Kao et al. 1990; Peterson et al. 1990; for review, see Hernandez 1993). All of our mAbs recognize regions in the hypervariable amino-terminal domain, as detailed in Materials and methods. In the experiment shown in Figure 2, we depleted a whole cell extract with the anti-TBP mAb SL28 bound to protein G–Sepharose beads. As a control, we also depleted an extract with beads carrying mAb 12CA5 (Niman et al. 1983), which is directed against an irrelevant peptide derived from the influenza virus hemagglutinin protein. The depleted extracts were then programmed with the pU1*G$^-$ construct. As shown in lanes 1 and 2, the mAb 12CA5-depleted extract was as active as an untreated extract, whereas the mAb SL28-depleted extract generated reduced levels of both correctly initiated U1 RNA and readthrough RNA (lane 3). This result suggested that TBP is required for transcription of both types of RNAs.

We then attempted to restore U1 transcription by complementing the TBP-depleted extract with increasing amounts of either recombinant TBP (rTBP) or fractions enriched in the TBP-containing complexes TFIID and 0.38M–TFIIIB. TFIID is involved in transcription from RNA polymerase II mRNA promoters. Although TBP on its own can bind to the TATA box of mRNA promoters and direct basal RNA polymerase II transcription, the TAFs present in the TFIID complex are essential to mediate activation of both TATA-containing and TATA-less mRNA promoters by upstream binding factors (Dynlacht et al. 1991; Pugh and Tjian 1991; Tanese et al. 1991; Zhou et al. 1992; 1993; Hoey et al. 1993). 0.38M–TFIIIB is a TBP-containing complex that is part of the TFIIIB fraction (Lobo et al. 1992; see also Simmen et al. 1992b; Taggart et al. 1992; White and Jackson 1992) and is required for RNA polymerase III transcription from the adenovirus 2 (Ad2) VAI promoter (Lobo et al. 1992).

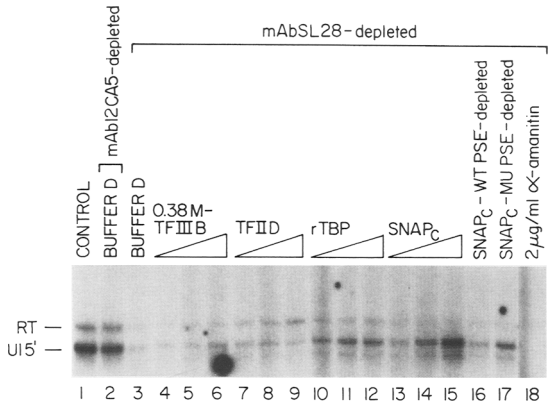

Figure 2. RNA polymerase II transcription from the U1 promoter requires TBP. A whole cell extract was either not depleted (lane *1*), mock depleted with the control mAb 12CA5 attached to protein G–Sepharose (lane *2*), or depleted with the anti-TBP mAb SL28 attached to protein G–Sepharose (lanes *3–18*). The depleted extracts were then complemented with buffer D (lanes *2,3*), or 0.5, 2, and 5 μl of a fraction enriched in the 0.38M–TFIIIB complex (lanes *4–6*), or 0.5, 2, and 5 μl of a fraction enriched in TFIID (lanes *7–9*), or 1, 2.5, and 5 ng of recombinant TBP expressed in *E. coli* (lanes *10–12*), or 0.5, 2, and 5 μl of a fraction enriched in SNAP$_c$ (lanes *13–15*). (Lanes *16,17*) The reaction was complemented with SNAP$_c$ fractions that had been depleted with oligonucleotides containing the wild-type mouse U6 PSE or the ABC mutated mouse U6 PSE, respectively (see Fig. 1A). (Lane *18*) 2 μg/ml of α-amanitin was added to a reaction complemented with 5 μl of the SNAP$_c$ fraction.

Although addition of recombinant TBP restored some synthesis of correctly initiated U1 RNA (Fig. 2, cf. lanes 10–12 with lane 3), addition of either the fraction enriched in 0.38M–TFIIIB or that enriched in TFIID had little or no effect (cf. lanes 4–9 with lane 3). However, the 0.38M–TFIIIB fraction was active in reconstituting RNA polymerase III transcription from the Ad2 VAI gene (data not shown; Lobo et al. 1992), and the TFIID fraction restored significant levels of readthrough RNA (lane 9), indicating that these fractions contain transcriptionally active TBP complexes. The activation of readthrough RNAs by TFIID and, to a lesser extent, by TBP, suggests that synthesis of these RNAs is directed by spurious mRNA-type RNA polymerase II promoters present in the vector.

We also tested the capacity of a fraction highly enriched in a PSE binding factor (see Materials and methods) to restore U1 transcription to the TBP-depleted extract. To our surprise, this fraction (labeled SNAP$_c$ in Fig. 2) was very active in restoring correctly initiated U1 transcription (cf. lanes 13–15 with lane 3), and this reconstituted transcription was sensitive to low levels of α-amanitin (lane 18). However, unlike recombinant TBP, this fraction did not restore readthrough transcription, suggesting that its activity was not attributable to contaminating TBP. To assess whether this activity was conferred by the PSE-binding factor, we depleted the fraction with oligonucleotides attached to beads and corresponding either to the mouse U6 PSE or to the mouse U6 PSE carrying the ABC mutation (see Fig. 1A). As shown in lanes 16 and 17, the fraction depleted with the wild-type U6 PSE beads lost significant activity, whereas the fraction depleted with the mutant PSE beads was as active as its nondepleted control fraction (cf. lane 17 with lane 14). A similar experiment performed with pU2*G$^-$ as a template gave identical results (data not shown). Together, these results indicate that RNA polymerase II transcription from the U1 and U2 promoters requires TBP. Furthermore, they suggest that TBP may be part of the PSE-binding factor.

The PSE-binding factor contains TBP

To determine directly whether TBP might be part of the PSE binding factor, we first established that TBP could

be detected by immunoblot in fractions highly enriched in PSE-binding factor (Fig. 3A). We then tested the effects of anti-TBP mAbs on the formation of a PSE-binding factor–DNA complex by the electrophoretic mobility-shift assay. As shown in Figure 3B, incubation of a radiolabeled probe containing the mouse U6 PSE with the fraction highly enriched in PSE-binding factor resulted in the formation of a single complex (lane 1). This complex was not affected by addition of the control mAb 12CA5 (lanes 2–5), and was not formed on a mouse U6 PSE containing the ABC mutation (cf. lanes 5 and 6). Similarly, this complex was not significantly affected by addition of some of our anti-TBP mAbs, for example, SL33 (lanes 21–24), as well as SL3, SL30, and SL35 (data not shown). However, when increasing amounts of the anti-TBP mAbs SL20, SL27, or SL28 (which was used for depletion in Fig. 2) were added, the complex was disrupted and several faster migrating complexes arose. These faster migrating complexes are specific, because they did not bind to the mouse U6 PSE containing the ABC mutation (lanes 11,20). Together, these observations strongly suggest that the PSE-binding factor is a multiprotein complex, which we refer to as $SNAP_c$, and that one of its components is TBP. The mAbs SL20, SL27, and SL28 recognize different TBP epitopes, thus virtually eliminating the possibility of cross-reaction with another protein. The observations that only some of the anti-TBP mAbs disrupt the complex and that none of them supershifts it suggest that TBP is embedded in the complex.

If the anti-TBP mAbs disrupt $SNAP_c$ by dislodging TBP, it should be possible to reconstitute $SNAP_c$ by adding back TBP. To explore this possibility, we disrupted $SNAP_c$ by the addition of the mAb SL27 and removed the antibody–TBP complexes by adsorption onto protein G–Sepharose beads. We then added increasing amounts of TBP to the mAb-depleted fraction and allowed the complexes to reform for 20 min at 30°C before performing the binding reaction. As shown in Figure 3C, addi-

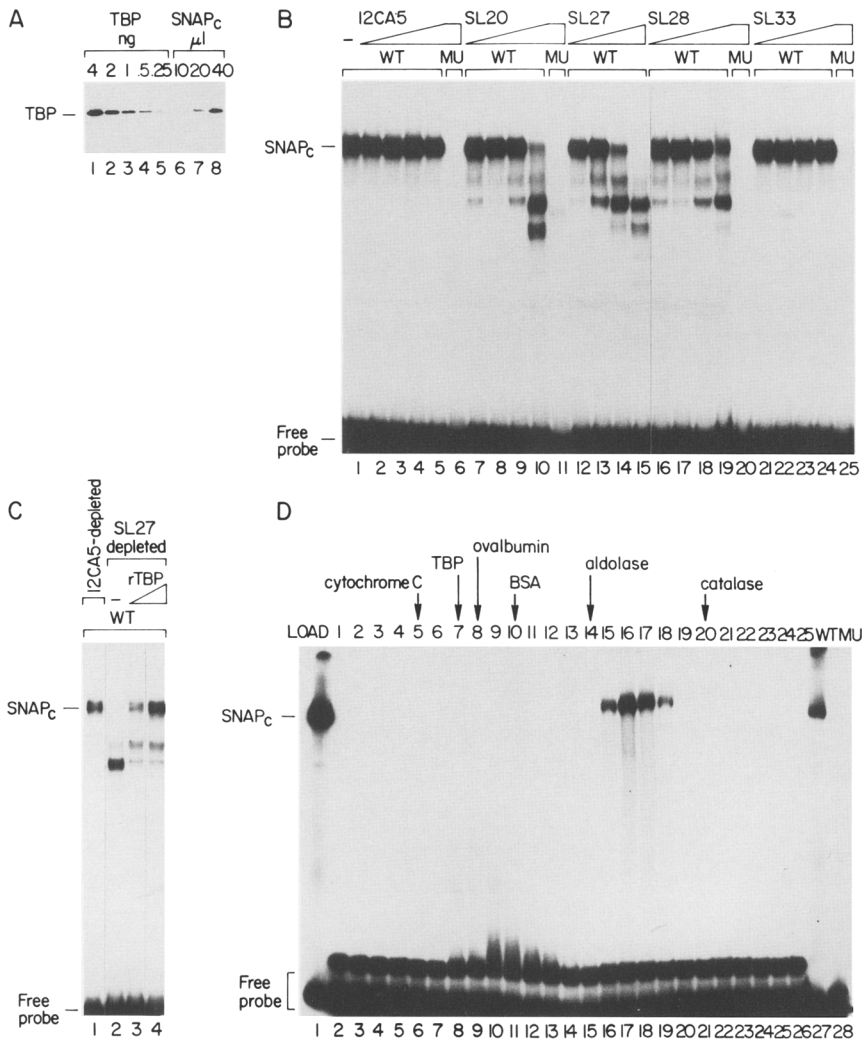

Figure 3. $SNAP_c$ contains TBP. (*A*) The indicated amounts of recombinant TBP (lanes *1–5*) or $SNAP_c$ fraction (lanes *6–8*) were fractionated on an SDS–polyacrylamide gel and analyzed by immunoblotting as described in Lobo et al. (1992) with mAbs SL20 and SL39. The $SNAP_c$ fraction contains ~12.5–25 pg of TBP/µl. (*B*) Amounts of 125 ng (lanes *2,7,12,16,21*), 250 ng (lanes *3,8,13,17,22*), 500 ng (lanes *4,9,14,18,23*), and 1 µg (lanes *5,6,10,11, 15,19,20,24,25*) of the mAbs indicated above the lanes were added to a fraction enriched in $SNAP_c$. These fractions were then incubated with probes containing either the wild-type mouse U6 PSE (WT) or the mouse U6 PSE carrying the ABC mutation (MU) and subjected to gel-mobility-shift analysis as detailed in Materials and methods. (*C*) mAb SL27 was added to a fraction enriched in $SNAP_c$. After a 30-min incubation at 4°C, the antibody–TBP complexes were removed by adsorption onto protein G–Sepharose beads. The depleted fractions were then complemented with either buffer (lane *2*) or 40 and 480 ng of recombinant TBP expressed in *E. coli* (lanes *3,4*). After a 20-min incubation at 30°C to allow $SNAP_c$ to reform, a standard binding reaction was performed with the probe containing the wild-type mouse U6 PSE and subjected to electrophoretic mobility-shift analysis. (*D*) Parallel 15–38% glycerol gradients were loaded with the $SNAP_c$ fraction, protein markers, and recombinant TBP, respectively. For the $SNAP_c$ fraction gradient, the loaded material (lane *1*), as well as gradient fractions 1 (*top*) to 25 (*bottom*) (lanes *2–26*) were analyzed by the electrophoretic mobility-shift assay with a probe containing the wild-type mouse U6 PSE. Lanes *27* and *28* show controls with probes containing the wild-type and mutant mouse U6 PSE, respectively. The sedimentation peaks of the marker proteins (cytochrome *c*, 12.5 kD; ovalbumin, 45 kD; BSA, 68 kD; aldolase, 158 kD; catalase, 240 kD) and recombinant TBP are indicated.

tion of increasing amounts of TBP resulted in a decrease in the intensity of the lower complexes and the reconstitution of the original $SNAP_c$ (cf. lanes 3 and 4 with lane 2). These results confirm the presence of TBP in $SNAP_c$. They also explain why depletions of nuclear extracts with mAbs SL3, SL30, SL33, and SL35, which do not disrupt $SNAP_c$ failed to inhibit U1 transcription (data not shown), as well as the observation that, in extract depleted with SL28, U1 and U2 transcription can be reconstituted by addition of TBP alone (see Fig. 2); because SL28, as with SL27, disrupts $SNAP_c$, TBP can reassociate with the other members of $SNAP_c$ that are left behind after depletion and, thus, regenerate a functional complex.

The observation that the PSE-binding activity is recovered in a single peak after chromatography over several columns (see Materials and methods) suggests that all the components of $SNAP_c$ are associated even when not bound to the PSE. To confirm this possibility, we subjected the $SNAP_c$ fraction to centrifugation through a glycerol gradient and tested each fraction for the presence of $SNAP_c$ by electrophoretic mobility-shift assay. As shown in Figure 3D, $SNAP_c$ sedimented in a single peak with a sedimentation coefficient of ~9.4S, which for a spherical protein would correspond to a molecular mass of ~200 kD. In contrast, uncomplexed, recombinant TBP sedimented with an apparent molecular mass of 25–50 kD. This suggests that all of the components of $SNAP_c$ exist as a complex in solution.

$SNAP_c$ contains at least three TAFs

The addition of anti-TBP mAbs to a binding reaction generates several faster migrating complexes in an electrophoretic mobility-shift assay (Fig. 3B), suggesting that $SNAP_c$ contains several polypeptides that bind specifically to the PSE. To characterize $SNAP_c$ further, we used UV cross-linking to identify those polypetides that are in close contact with the DNA. We prepared homogenously labeled probes substituted with bromodeoxyuridine as well as unlabeled competitors containing either the wild-type mouse U6 PSE or the mouse U6 PSE carrying the ABC mutation (for details, see Materials and methods), and tested them in the gel mobility-shift assay. As shown in Figure 4A, the mutant PSE probe did not form any complex (lane 1), whereas the wild-type PSE probe formed a complex that was competed by an excess of wild-type (lanes 2,4,6), but not mutant (lanes 3,5,7) competitor. Figure 4A also shows that the complex was not lost with irradiation of the binding reactions with two different doses of UV light (200 and 600 mJ, lanes 4–7). We then treated parallel binding reactions irradiated with 200 or 600 mJ of UV light with DNase I and micrococcal nuclease to remove any DNA fragment not cross-linked or in close contact with the DNA-binding proteins, and determined the approximate sizes of the cross-linked polypeptides by SDS-PAGE and autoradiography. As shown in Figure 4B, in addition to a number of weakly cross-linked proteins, three major proteins migrating with apparent molecular masses of ~53, 61, and 70 kD could be specifically cross-linked in a UV dose-dependent fashion to the wild-type (lanes 1,3) but not the mutant (lanes 2,4) PSE probes. The binding of these proteins was competed by an excess of unlabeled wild-type (lanes 5,7), but not mutant (lanes 6,8), PSE competitor. Together, these data suggest that $SNAP_c$ contains at least three TAFs that contact or are in close proximity to the DNA. These could correspond to different polypetides, to modified forms of the same polypetide, or both.

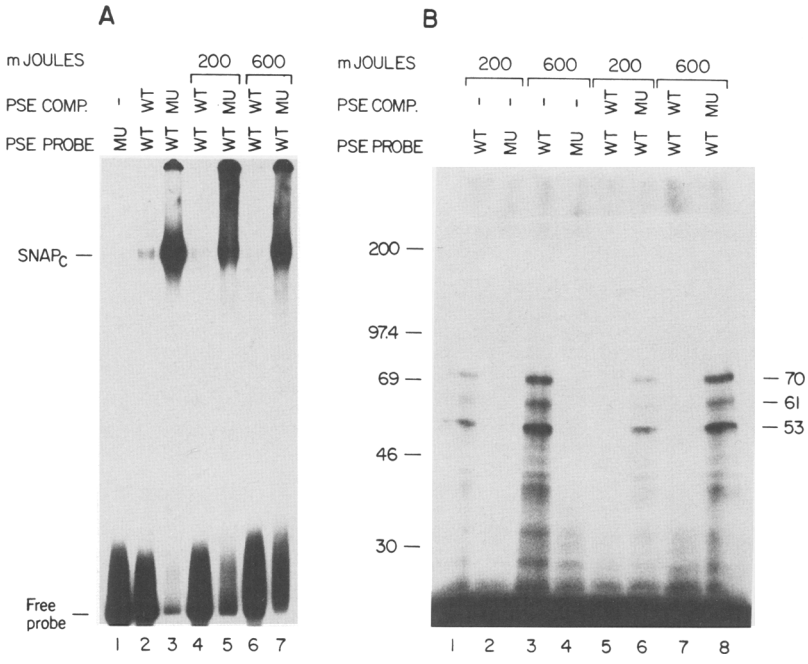

Figure 4. $SNAP_c$ contains at least three polypeptides that bind or are in close proximity to the DNA. (*A*) Homogenously labeled probes substituted with bromodeoxyuridine carrying either the wild-type mouse U6 PSE (WT) or the PSE with the ABC mutation (MU) were incubated with a fraction enriched in $SNAP_c$, either in the absence of any competitor (lane *1*) or in the presence of competitors corresponding to the wild-type mouse U6 PSE (lanes *2,4,6*) or to the U6 PSE with the ABC mutation (lanes *3,5,7*). (Lanes *4–7*) The binding reactions were irradiated with 200 (lanes *4,5*) or 600 (lanes *6,7*) mJ of UV light before being loaded on the gel. (*B*) Binding reactions performed as in *A*, but scaled up threefold, were treated with DNase I and micrococcal nuclease. Laemmli loading buffer (Harlow and Lane 1988) was then added, and the samples were boiled and fractionated on an SDS–polyacrylamide gel. The PSE-binding proteins were then visualized by autoradiography.

The TAFs in SNAP$_c$ are required for RNA polymerase II transcription from snRNA promoters

In an extract depleted with mAb SL28 (see Fig. 2 above), RNA polymerase II transcription from the U1 promoter could be reconstituted by addition of both recombinant TBP and SNAP complex. Because mAb SL28 disrupts SNAP$_c$ (see Fig. 3B) and because the complex can be reconstituted by addition of recombinant TBP (see Fig. 3C) it seemed likely that the ability of recombinant TBP to restore U1 transcription was attributable to it reassembling into SNAP complexes in vitro. To determine directly whether the TAFs in SNAP$_c$ are essential for snRNA transcription by RNA polymerase II, we depleted an extract of SNAP$_c$ with beads carrying double-stranded oligonucleotides corresponding to the wild-type mouse U6 PSE. As a control, we also depleted extracts with beads carrying the ABC mutated PSE. We then attempted to reconstitute transcription by addition of either recombinant TBP or SNAP$_c$.

As shown in Figure 5, extracts depleted with the wild-type PSE beads, but not with the mutant PSE beads, directed reduced levels of transcription from the U1 promoter (cf. lanes 1 and 10). Significantly, and in contrast to extracts depleted with anti-TBP antibodies (see Fig. 2), depletion with wild-type (or mutant) PSE beads did not affect the levels of readthough RNA, confirming that the synthesis of these RNAs is not dependent on SNAP$_c$. U1 transcription could be restored by addition of increasing amounts of SNAP$_c$ (lanes 2–5) but not by increasing amounts of recombinant TBP (lanes 6–9). In contrast, readthrough transcription was stimulated by addition of TBP but not by addition of SNAP$_c$. In the control extracts depleted with the mutant PSE beads, addition of the SNAP$_c$ fraction stimulated U1 transcription very weakly (lanes 11–14), suggesting that SNAP$_c$ is slightly limiting in these extracts. Addition of TBP had a very small stimulatory effect at low concentrations (lanes 15,16) but, for reasons that are unclear, inhibited U1 transcription at high concentrations (lanes 17,18). In contrast, as in the extract depleted with wild-type PSE beads, addition of TBP stimulated readthrough transcription (lanes 15–18). Thus, SNAP$_c$ is essential for U1 transcription but not for transcription of the readthrough RNA.

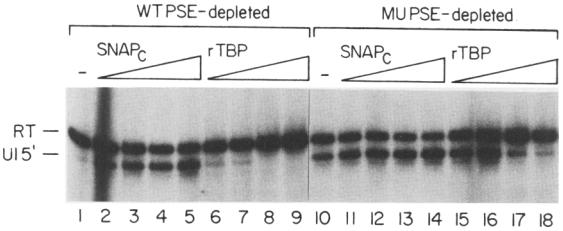

Figure 5. The TAFs in SNAP$_c$ are required for RNA polymerase II transcription from snRNA promoters. A whole cell extract was depleted with beads carrying either the wild-type mouse U6 PSE (lanes *1–9*) or the mouse U6 PSE with the ABC mutation (lanes *10–18*). The depleted extracts were then complemented with either buffer (lanes *1,10*), 1.5 (lanes *2,11*), 3 (lanes *3,12*), 4.5 (lanes *4,13*), and 6 (lanes *5,14*) μl of the fraction enriched in the SNAP complex or 10 (lanes *6,15*), 20 (lanes *7,16*), 30 (lanes *8,17*), and 40 (lanes *9,18*) ng of recombinant TBP produced in *E. coli*, and used for in vitro transcription of the pU1*G$^-$ construct.

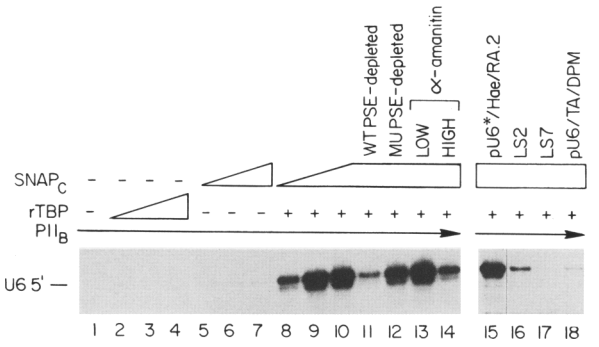

Figure 6. RNA polymerase III transcription from the U6 promoter requires SNAP$_c$. Combinations of 10 μl of the P11$_B$ fraction alone (lane *1*), or 10 μl of the P11$_B$ fraction with 1 (lane *2*), 5 (lane *3*), and 10 (lane *4*) ng of recombinant TBP, or 10 μl of the P11$_B$ fraction with 1.5 (lane *5*), 4 (lane *6*), and 14 (lane *7*) μl of the fraction enriched in SNAP$_c$ or 10 μl of the P11$_B$ fraction with 5 ng of recombinant TBP and 1.5 (lane *8*), 4 (lane *9*), and 14 (lane *10–18*) μl of the fraction enriched in SNAP$_c$ were tested for their ability to reconstitute U6 transcription. (Lanes *1–15*) The template was a derivative of pU6/Hae/RA.2 (Lobo and Hernandez 1989) in which the human U6 PSE was changed by site-directed mutagenesis to the mouse U6 PSE. (Lanes *16,17*) The constructs LS2 and LS7 (Lobo and Hernandez 1989), which contain clustered point mutations in the PSE and the TATA box, respectively, were used as templates. (Lane *18*) The construct pU6/TA/DPM (Lobo et al. 1991), which contains a double point mutation in the U6 TATA box, was used. (Lanes *11,12*) The fraction enriched in SNAP$_c$ was depleted with beads carrying the wild-type mouse U6 PSE and the mouse U6 PSE with the ABC mutation, respectively, before being added to the reactions. (Lanes *13,14*) The reactions contained 2 and 300 μg/ml of α-amanitin, respectively.

RNA polymerase III transcription from the U6 promoter requires the SNAP$_c$ fraction

The human U2 and U6 PSEs can be exchanged with no effect on RNA polymerase specificity, suggesting that RNA polymerase II and III snRNA promoters use the same PSE-binding factor (Lobo and Hernandez 1989). To determine whether this is the case, we attempted to reconstitute U6 transcription with a combination of a phosphocellulose B fraction (P11$_B$), which provides RNA polymerase III and TFIIIB, and either TBP or the SNAP$_c$ fraction, or both. As shown in Figure 6, addition of TBP alone (lanes 2–4) or the SNAP$_c$ fraction alone (lanes 5–7) to the P11$_B$ fraction did not result in detectable levels of U6 transcription. However, addition of increasing amounts of the SNAP$_c$ fraction in the presence of added recombinant TBP reconstituted efficient U6 transcription (lanes 8–10). Depletion of the SNAP$_c$ fraction with wild-type PSE beads, but not mutant PSE beads, significantly reduced reconstituted U6 transcription, indicat-

ing that the $SNAP_c$ is a functional component of this fraction (cf. lanes 11 and 12 with lane 10). Reconstituted transcription was diminished [although, as observed previously (Lobo et al. 1991,1992), not abolished] by high, but not low, levels of α-amanitin, confirming that it is directed by RNA polymerase III (cf. lanes 13 and 14). Furthermore, the reconstituted transcription was reduced by mutations in both the TATA box (cf. lane 15 with lanes 17 an 18) and the PSE (cf. lanes 15 and 16), indicating that it depends on the promoter elements that are required for basal U6 transcription in vivo (Lobo and Hernandez 1989). These results suggest that RNA polymerase III transcription from the U6 promoter, as with RNA polymerase II transcription from the U1 promoter, requires $SNAP_c$.

The RNA polymerase III U6 initiation complex may contain two forms of TBP, one bound to the TATA box and one bound to the PSE

The observation that reconstitution of U6 transcription requires both TBP and $SNAP_c$ (Fig. 6) suggests that the U6 initiation complex contains two forms of TBP. In contrast, in a TBP-depleted extract, U1 transcription can be reconstituted by addition of $SNAP_c$ alone (see Fig. 2), suggesting that the U1 initiation complex contains only the TBP present in $SNAP_c$. To explore this possibility further, we depleted extracts with beads carrying oligonucleotides that contained a TATA box or an irrelevant sequence. Each depleted extract was then tested for both U1 and U6 transcription. As shown in Figure 7A, depletion with increasing amounts of TATA box beads (lanes 1–3) or control beads (lanes 4–6) had no effect on U1 transcription or, interestingly, on transcription of the readthrough RNA. The latter observation suggests that TFIID was not efficiently depleted in this protocol. However, Figure 7B shows that the lowest amount of TATA box beads used in Figure 7A was sufficient to reduce U6 transcription to undetectable levels (lane 1), whereas transcription was still detectable in extracts depleted with the control beads (lane 7). Significantly, high levels of U6 transcription could be restored by addition of TBP (lanes 2,3), but not by addition of $SNAP_c$ (lanes 4,5). These results clearly show that the TBP requirements for RNA polymerase II and III transcription from snRNA genes differ. Although both require the TBP-containing $SNAP_c$, only the U6 gene requires in addition TBP bound to the TATA box. This is confirmed by the observation that depletion of an extract with mAbs SL3, SL30, SL33, and SL35, which do not disrupt $SNAP_c$ (see Fig. 3B; data not shown), do not affect U1 transcription (data not shown) but strongly reduce transcription from the U6 promoter (Lobo et al. 1992). Thus, the U6 initiation complex appears to contain two forms of TBP, one bound to the TATA box and the other bound to the PSE as part of $SNAP_c$, whereas the U1 initiation complex contains only the latter form.

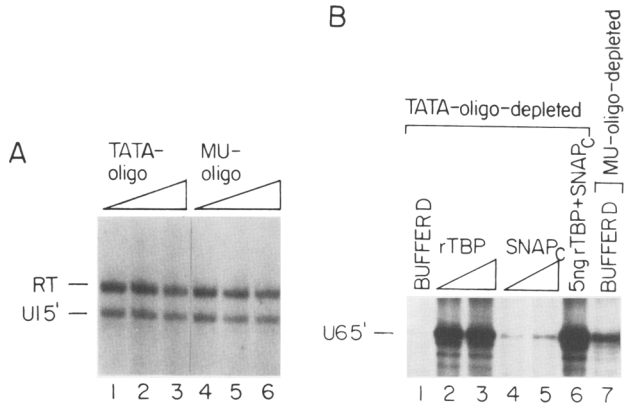

Figure 7. The RNA polymerase III U6 initiation complex contains two forms of TBP, one bound to the TATA box and one bound to the PSE. (*A*) Whole cell extract (750 μg) was depleted with 5 (lanes *1,4*), 10 (lanes *2,5*), or 15 (lanes *3,6*) μl of beads carrying either a TATA box (lanes *1–3*) or an irrelevant sequence (lanes *4–6*), and 200-μg aliquots were tested for their ability to direct RNA polymerase II transcription from the U1 promoter. (*B*) Aliquots of 50 μg of whole cell extract depleted with 5 μl of beads carrying either the TATA box (lanes *1–6*) or the irrelevant sequence (lane 7) were complemented with buffer (lanes *1,7*), or 5 (lane 2) and 20 (lane 3) ng of recombinant TBP, or 1 (lane *4*) and 4 (lane *5*) μl of the fraction enriched in $SNAP_c$, or both 5 ng of TBP and 4 μl of the fraction enriched in $SNAP_c$ (lane *6*) and tested for their ability to direct U6 transcription.

Discussion

TBP is required for transcription by all three RNA polymerases (for review, see Hernandez 1993). Thus, it has been shown to participate in RNA polymerase I transcription as part of the SL1 complex (Comai et al. 1992), RNA polymerase II transcription of mRNA-encoding genes as part of the TFIID complex (for review, see Sawadogo and Sentenac 1990; Roeder 1991; Pugh and Tjian 1992; Zawel and Reinberg 1992,1993), and RNA polymerase III transcription of TATA-less genes as part of the TFIIIB complex (Huet and Sentenac 1992; Kassavetis et al. 1992; Lobo et al. 1992; Simmen et al. 1992b; Taggart et al. 1992; White and Jackson 1992). TBP is also required for RNA polymerase III transcription of the TATA-containing U6 snRNA gene (Lobo et al. 1991; Simmen et al. 1991); and as for TATA-containing RNA polymerase II mRNA promoters, uncomplexed TBP is sufficient to direct basal levels of U6 transcription (this study; Lobo et al. 1992). Here, we show that TBP is also required for RNA polymerase II transcription of the snRNA genes, suggesting that at least in higher eukaryotes, TBP plays a central role in transcription initiation of all genes. The TBP present in the RNA polymerase II snRNA initiation complexes is part of a novel TBP-containing complex, $SNAP_c$, that does not bind to the TATA box but instead specifically recognizes the PSEs of snRNA promoters. Remarkably, $SNAP_c$ appears to be also required for transcription of the RNA polymerase III U6 snRNA gene. Thus, RNA polymerase II and III snRNA initiation complexes may differ by the presence of one versus two forms of TBP.

$SNAP_c$ and other PSE-binding factors

In addition to PBP (Waldschmidt et al. 1991) and PTF (Murphy et al. 1992), another factor termed PSE1 has been reported to bind specifically to the PSE. PSE1 has been shown to bind to the U1 PSE (Knuth et al. 1990) and to restore partially U1 transcription in an extract depleted with anti-PSE1 antibodies (Gunderson et al. 1990). PSE1 is heterodimeric with subunits of apparent molecular masses of 83 and 73 kD. It is recognized by anti-Ku antibodies (Knuth et al. 1990), suggesting that it is related or identical to the Ku protein, a common autoantigen in patients with systemic lupus erythematosus and related disorders (Mimori and Hardin 1986; Mimori et al. 1986). $SNAP_c$ is clearly different from PSE1; it is composed of TBP and several additional polypetides, and it is not recognized by anti-Ku antibodies (data not shown). In contrast, $SNAP_c$ shares properties displayed by PBP and PTF. Thus, as with PBP (Waldschmidt et al. 1991) and PTF (Murphy et al. 1992), it binds with higher affinity to the mouse U6 PSE than to the human U1, U2, and U6 PSEs; and as described for PTF (Murphy et al. 1992), its binding to the human U2 and U6 PSEs is potentiated by Oct-1 binding to an adjacent octamer-binding site (data not shown). However, in glycerol gradients, PBP sediments with an apparent molecular mass of 90 kD (Waldschmidt et al. 1991), whereas $SNAP_c$ sediments with an apparent molecular mass of ~200 kD (Fig. 3D). Thus, at present, it is not clear whether and how these factors are related.

$SNAP_c$ TAFs target TBP to a new cis*-regulatory element*

$SNAP_c$ displays two novel properties relative to previously characterized TBP-containing complexes. First, unlike SL1, TFIID, and TFIIIB, each of which appears to be required for transcription by only one RNA polymerase, $SNAP_c$ is required for transcription of snRNA genes by two different RNA polymerases. Second, although TFIID recognizes the same element as TBP, namely the TATA box, and although SL1 and TFIIIB have very low or no affinity for DNA in the absence of other factors, $SNAP_c$ binds specifically to the mouse U6 PSE as well as to the PSEs of the human U1, U2, and U6 promoters, but with reduced affinity (data not shown). Two experiments clearly indicate that $SNAP_c$ components other than TBP contact the DNA directly. First, removal of TBP from $SNAP_c$ by antibody depletion results in the appearance of new, faster migrating protein–PSE complexes in an electrophoretic mobility-shift assay, indicating that $SNAP_c$ components other than TBP bind specifically to the PSE (Fig. 3B). Second, UV cross-linking experiments identify at least three polypeptides with apparent molecular masses of ~53, 61, and 70 kD that interact specifically with the PSE (Fig. 4B). Thus, the $SNAP_c$ TAFs effectively target TBP to a new DNA sequence that is completely unrelated to the TATA box.

We cannot formally eliminate the possibility that TBP is not an integral part of $SNAP_c$ but, rather, is recruited after the binding of other $SNAP_c$ components to the PSE. However, several observations argue against this possibility. First, TBP and $SNAP_c$ copurify over several columns (see Materials and methods), suggesting that TBP is associated with $SNAP_c$. Second, a complex that contains TBP (Fig. 3B,C) sediments in a glycerol gradient with an apparent molecular mass of 200 kD (Fig. 3D), whereas uncomplexed TBP sediments with an apparent molecular mass of 25–50 kD under the same conditions. And finally, unlike uncomplexed TBP, the $SNAP_c$ fraction does not reconstitute readthrough transcription in a TBP-depleted extract (Fig. 2) or U6 transcription in an extract depleted with TATA box beads (Fig. 7B), indicating that it does not contain free TBP.

Determination of RNA polymerase specificity in the human snRNA promoters

The first step in the assembly of an initiation complex is the recognition of promoter elements by specific transcription factors. As shown in Figure 8, the RNA polymerase III promoter of the Ad2 VAI gene, which consists of a gene internal A and B box, is recognized by TFIIIC. TFIIIC then recruits TFIIIB to the promoter (for review, see Geiduschek and Kassavetis 1992). We have shown previously that the TFIIIB activity can be divided chromatographically into two functionally distinct components (Lobo et al. 1992). One of these, referred to as 0.38M–TFIIIB, is a TBP-containing complex (see also Simmen et al. 1992b; Taggart et al. 1992; White and Jackson 1992), whereas the second, referred to as 0.48M–TFIIIB, does not contain any detectable TBP. Both of these fractions are required for transcription of the VAI gene and are therefore indicated in the model. Transcription from the U6 promoter has been shown to be independent of TFIIIC (Reddy 1988; Waldschmidt et al. 1991). We imagine that the TFIIIC function in the U6 promoter is conferred by the combination of $SNAP_c$ and TBP, both of which can bind directly to the DNA and are required for U6 transcription. In this model, then, the U6 initiation complex contains two forms of TBP. We cannot disprove the possibility that the U6 PSE binds only some of the TAFs in $SNAP_c$ and that the U6 initiation complex contains only TBP bound to the TATA box. However, the observation that during chromatographic purification of $SNAP_c$, the PSE-binding activity peaks exactly in the same fractions as both U1 and U6 transcriptional activity (see Materials and methods) argues against this possibility. Purification of $SNAP_c$ to homogeneity should resolve this question.

Because the spacing between the PSE and the TATA box is highly conserved in RNA polymerase III promoters of the U6 type (see Hernandez 1992) and must be maintained to preserve RNA polymerase III transcription (Lobo and Hernandez 1989; Lescure et al. 1991), $SNAP_c$ and TBP bound to the TATA box are likely to interact. The resulting protein surface presumably allows the binding of 0.48M–TFIIIB. We have shown previously that although U6 transcription requires a component present in the TFIIIB fraction, this component is

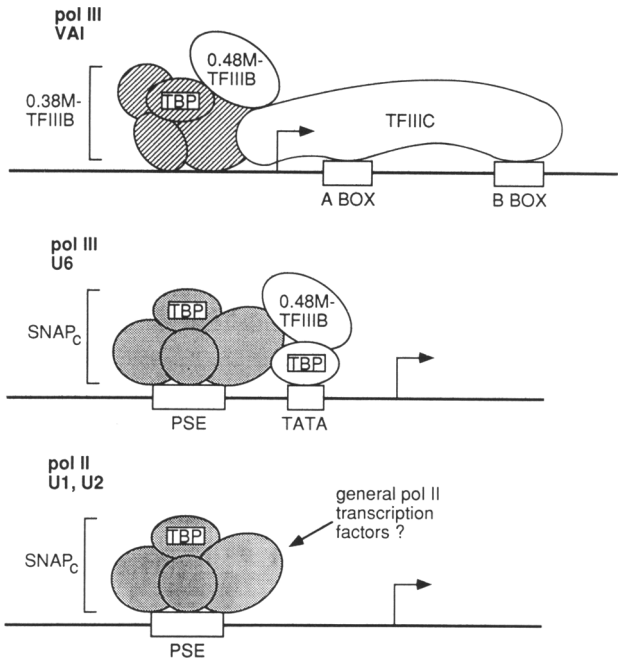

Figure 8. Model showing known and hypothetical components of initiation complexes formed on the VAI, U6, and U1 or U2 promoters. On the TATA-less RNA polymerase III VAI promoter, TFIIIC binds first to the internal A and B boxes and then recruits TFIIIB (for review, see Geiduschek and Kassavetis 1992). The exact composition of mammalian TFIIIB is not known, but it is clear that TFIIIB contains a TBP complex (Lobo et al. 1992; Simmen et al. 1992b; Taggart et al. 1992; White and Jackson 1992), referred to here as 0.38M–TFIIIB, and a second, uncharacterized factor, referred to as 0.48M–TFIIIB (Lobo et al. 1992). On the TATA-containing RNA polymerase III U6 promoter, $SNAP_c$ and TBP can both bind directly to the DNA. The combination of the two factors is presumed to recruit 0.48M–TFIIIB. The 0.38M–TFIIIB complex is not required for basal U6 transcription (Lobo et al. 1992). On the RNA polymerase II U1 and U2 promoters, $SNAP_c$ binds to the PSE and presumably recruits members of the general RNA polymerase II transcriptional machinery. $SNAP_c$ contains at least three proteins that bind to or are in close contact with the PSE. TBP is not cross-linked or is cross-linked only weakly to the DNA and may, therefore, not contact DNA directly.

not 0.38M–TFIIIB (Lobo et al. 1992). Because, at least in yeast, the TFIIIB factor is an essential RNA polymerase III factor that is sufficient to direct several rounds of RNA polymerase III transcription from tRNA and 5S promoters (Kassavetis et al. 1990), it seems reasonable to assume that the component in the TFIIIB fraction required for U6 transcription is part of the TFIIIB factor and corresponds to 0.48M–TFIIIB.

In the basal RNA polymerase II snRNA promoters, which consist of just the PSE, only $SNAP_c$ binds directly to the DNA. Because there is no TBP bound to a TATA box just downstream, the resulting protein surface is different from that obtained in the case of the U6 promoter and, thus, presumably recruits some of the general RNA polymerase II transcription factors rather than TFIIIB. Because the RNA polymerase II snRNA promoters, but not the RNA polymerase II mRNA promoters, direct the formation of transcription complexes that recognize a termination signal (the 3′ box) specific to snRNA genes, the snRNA transcription complexes must differ from the mRNA transcription complexes (Hernandez and Weiner 1986; Neuman de Vegvar et al. 1986; Hernandez and Lucito 1988; Neuman de Vegvar and Dahlberg 1989). Perhaps the RNA polymerase II snRNA transcription complexes lack one of the general transcription factors associated with RNA polymerase II, or they may contain a specific termination factor.

The observation that the U6 initiation complex appears to contain two forms of TBP raises the possibility that this is a more general occurrence. TBP has been shown to interact with a bewildering array of factors, including TAFs, general transcription factors, and transcriptional activators. Perhaps in some initiation complexes, these interactions are distributed among several molecules of TBP.

Materials and methods

Constructs

To generate the constructs pU1G$^-$ and pU1*G$^-$, a *Bam*HI–*Taq*I fragment containing the U1 promoter and part of the U1-coding sequence was excised from the construct pNS11Eco$^+$-SalXba (Hernandez 1985), blunt-ended with Klenow, and inserted into the construct pHIV-1/R (Sheldon et al. 1993) cleaved with *Eco*RI and *Bam*HI and blunt-ended with Klenow. This reconstituted a *Bam*HI site at the *Taq*I–*Bam*HI junction. This construct was then cleaved with *Bgl*II, which cleaves at −6 relative to the U1 transcriptional start site, and *Bam*HI, and the resulting vector fragment containing the U1 promoter was ligated to a G-less cassette. The G-less cassette was generated by PCR amplification of the pML(C_2AT) construct (Sawadogo and Roeder 1985) with two primers: The upstream one contained a *Bgl*II site and 23 nucleotides hybridizing to the 5′ region of the G-less cassette present in pML(C_2AT), whereas the downstream primer contained a *Bam*HI site followed by a *Sna*BI site and 16 nucleotides hybridizing to the 3′ region of the G-less cassette. The PCR fragment was cleaved with *Bgl*II and *Bam*HI before ligation into the vector. Finally, a double-stranded oligonucleotide containing the U1 3′ end and 3′ box was inserted into the *Sna*BI site to create pU1G$^-$. pU1*G$^-$ was constructed by site-directed mutagenesis of pU1G$^-$. The resulting constructs contain a 174-nucleotides G-less cassette downstream of the transcriptional start site followed by the U1 3′ box. The 3′ box was not functional in the in vitro system.

To generate the pU2G$^-$, pU2*G$^-$, and pU2*ABCG$^-$ constructs, an *Eco*RI–*Bam*HI fragment containing the U2 promoter from position −240 was excised from the construct LS−11/−20 (Hernandez and Lucito 1988) and inserted into pHIV-1/R (Sheldon et al. 1993) cleaved with *Eco*RI and *Bam*HI. This construct was then cleaved with *Xho*I, which cleaves at position −17 upstream of the U2 transcriptional start site, and *Bam*HI, and the resulting vector fragment containing the U2 promoter was ligated to a G-less cassette. The G-less cassette was generated as described above with two primers: The upstream primer contained an *Xho*I site and 30 nucleotides hybridizing to the 5′ region of the G-less cassette, whereas the downstream primer was identical to the one used above. The PCR fragment was cleaved with *Xho*I and *Bam*HI before ligation to the vector.

Finally, a double-stranded oligonucleotide containing the U1 3′ end and 3′ box was inserted into the *Sna*BI site to create pU2G$^-$. pU2*G$^-$ and pU2*ABCG$^-$ were constructed by site-directed mutagenesis of pU2G$^-$. The resulting constructs contain a 212-nucleotide G-less cassette downstream of the transcriptional start site followed by the U1 3′ box. The 3′ box in pU2G$^-$ was not functional in the in vitro system.

Fractionation/purification of SNAP$_c$

Whole cell extract used in transcription reactions was prepared according to the method described by Maroney et al. (1990), except that the starting material was HeLa cells washed twice in isotonic phosphate-buffered saline. The resulting extract had a protein concentration of 30 mg/ml. The source of material for all of the fractionation was HeLa cell extract prepared by the method of Dignam et al. (1983). The 0.38M–TFIIIB and TFIID fractions used in Figure 2 were prepared as described in Lobo et al. (1992). Recombinant TBP was purchased from Promega.

SNAP$_c$ was purified from HeLa cell S100 (1000 ml, 10 mg protein/ml), first by a 18–32% ammonium sulfate precipitation. The precipitate was resuspended in 100 ml of buffer D [20 mM HEPES (pH 7.9), 0.2 mM EDTA, 15% glycerol, 3 mM DTT, 0.5 mM PMSF] containing 100 mM KCl, dialyzed against the same buffer and loaded on a P11 (Whatman) phosphocellulose column (8 mg of protein/ml of packed resin). The column was washed with three column volumes of buffer D + 100 mM KCl and then step eluted with five volumes of buffer D + 350 mM KCl, followed by five volumes of buffer D + 500 mM KCl. The 350–500 mM KCl step (P11$_C$ fraction) was loaded directly onto a 15-ml Cibacron blue Affi-gel column (Bio-Rad). The column was washed with 50 ml of CB buffer [20 mM HEPES (pH 7.9), 0.1% Tween 20, 0.2 mM EDTA, 3 mM DTT, 0.5 mM PMSF] containing 0.5 M NaCl and 8% ethylene glycol, and eluted with a 0.5 M NaCl/8% to 2.5 M NaCl/40% ethylene glycol gradient in CB buffer. The fractions were dialyzed against buffer Q [20 mM HEPES (pH 7.9), 0.5 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.01% Tween 20, 5% glycerol, 100 mM KCl] and tested for activity in a PSE mobility-shift assay. The peak fractions were pooled and loaded onto a Mono Q HR 10/10 column (Pharmacia). The column was washed with 15 ml of buffer Q, and the bound material was eluted with a linear gradient of 100 mM to 1 M KCl in buffer Q. The fractions were dialyzed against buffer D − 100 mM KCl and tested for activity in a PSE mobility-shift assay for their ability to reconstitute U1 and U6 transcription. The peaks of activity in the three assays coincided perfectly, strongly suggesting that they were the result of the same factor. The peak fractions were pooled and contained ~40 protein bands in a silver-stained gel. This pooled fraction was the material used for all of the experiments, with the exception of the experiment shown in Figure 3B and C, which had a mixture of this fraction with a slightly cruder fraction in which the Cibacron blue column step had been omitted. In Figure 3D, 200 μl of the Mono Q peak was loaded on a 4.8-ml 15–38% glycerol gradient in 25 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 0.01% Tween 20, 2 mM DTT, and 0.25 mM PMSF. Parallel gradients were loaded with protein markers and recombinant TBP, respectively. The gradients were centrifuged for 21 hr at 49,000 rpm and 4°C in a SW55Ti rotor. Fractions of 200 μl were collected. SNAP$_c$ gradient fractions were tested in an electrophoretic mobility-shift assay for specific binding to the mouse U6 PSE as described below, protein marker gradient fractions were analyzed by SDS-PAGE, and TBP gradient fractions were analyzed by immunoblot with anti-TBP mAbs, as described in Lobo et al. (1992).

The phosphocellulose B fraction (0.1–0.35 M KCl) used in Figure 6 was obtained from nuclear extracts as detailed in Lobo et al. (1991).

Determination of TBP epitopes required for recognition by the SL mAbs

The epitopes required for recognition by the mAbs of the SL series were determined as detailed in Lobo et al. (1992). Essentially, full-length human TBP protein, as well as three amino-terminally truncated proteins containing 282 (282C), 244 (244C), and 198 (198C) carboxy-terminal amino acids were synthesized in rabbit reticulocyte lysates and used in immunoprecipitations with different SL mAbs. SL20, SL27, and SL33 precipitated only the full-length protein, indicating that they require an epitope at the extreme amino terminus of TBP, whereas SL28 precipitated full-length TBP and 282C, indicating that it requires the glutamine stretch present in 282C but not in 244C.

Immunodepletions

SL mAbs were purified from ascites by chromatography on protein A–Sepharose in high salt as described previously (Lobo et al. 1992) and dialyzed against buffer D, and the concentrations were determined by the Bradford protein assay and confirmed by visualization on Coomassie-stained gels. Equal dilutions of either 12CA5 or SL28 mAb (in buffer D) were mixed with one volume of whole cell extract (200 μg) at 4°C for 30 min. The mAb extract mixture was then added to protein G–Sepharose (prewashed five times in buffer D) and mixed at 4°C for 30 min. The beads were spun gently, and the supernatant was used in transcription reactions.

Oligodepletions

DNA affinity columns containing the wild-type mouse U6 PSE and the mouse U6 PSE with the ABC mutation were constructed from the following oligonucleotides:

WT-upper, 5′-GAGGAAACTCACCCTAACTGTAAAGTAATTGTGCTC-3′; WT-lower, 5′-CTCGAGCACAATTACTTTACAGTTAGGGTGAGTTTC-3′; MU-upper, 5′-GAGGAAACTCCCACTACCGGTCCAGTAATTGTGCTC-3′; and MU-lower, 5′-CTCGAGCACAATTACTGGACCGGTAGTGGGAGTTTC-3′. The TATA DNA affinity column was constructed from the complementary oligonucleotides TATA-upper, 5′-GATTTCTTTGGCTTTATATATGACTCGAG-3′and TATA-lower, 5′-AGTCATATATAAAGCCAAAGAAATCCTCG-3′. The oligonucleotides were kinased, annealed, ligated, and coupled to CNBr-activated Sepharose CL4B (Kadonaga and Tjian 1986). All columns contained ~100 μg of DNA/ml of resin. For the PSE depletions, the resin was washed five times in binding buffer [60 mM KCl, 20 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF] and incubated with either whole cell extract or the SNAP$_c$ fraction for 15 min at ambient temperature with mixing. For TATA depletions, the resin was washed five times in buffer D and incubated with whole cell extract (in the presence of 5 mM $MgCl_2$) for 30 min at 30°C with mixing. Mutant PSE beads were used as the control for the TATA depletions. After depletion, the beads were spun gently and the supernatant was used in transcription reactions.

Transcription reactions

In vitro transcription reactions for U1 and U2 G-less constructs were done in a total volume of 25 μl containing 60 mM KCl, 20 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 1 mM Spermidine, 1 mM DTT, 400 μM rATP, 400 μM rCTP, 0.26 μM [α-^{32}P]UTP (20 μCi), 1.2 mM 3′-

O-methyl-GTP, and 12% glycerol and included 1.2 μg of supercoiled template, 0.5 units of RNase T1, and 200–400 μg of whole cell extract. Transcription reactions were incubated for 90 min at 30°C and were terminated by the addition of stop mix (0.3 M sodium acetate, 0.5% SDS, 0.2 mM EDTA, 5 μg/ml of tRNA) and 60 μg of proteinase K. The reactions were incubated for 30 min at 37°C, processed as described in Lobo and Hernandez (1989), and run on a 4% sequencing gel. The dried gel was quantitated using a PhosphorImager (Fuji) and exposed for autoradiography. Primer extension analyses were performed as described in Lobo and Hernandez (1989), except that the RNA was excised from a sequencing gel loaded with unlabeled in vitro transcription reactions (and flanking hot reactions as markers), eluted, and precipitated with ethanol. In vivo RNA used as control RNA for the primer extension was isolated from HeLa cells transfected with the pU1*G$^-$ construct according to the transfection protocol described in Lobo et al. (1991).

U6 was transcribed in whole cell extract under the conditions described in Lobo and Hernandez (1989). In Figure 6, the transcription reactions were performed in a total volume of 40 μl with the fractions indicated in the legend as described in Lobo et al. (1991), except that 250 ng of template was used in the reactions. The RNA transcripts were analyzed by RNase T1 protections with the probe U6/RA.2/143 as described in Lobo and Hernandez (1989).

Mobility-shift assay

The oligonucleotides used for DNA affinity columns and containing the wild-type mouse U6 PSE or the mouse U6 PSE with the ABC mutation were annealed, ligated, cut with *Xho*I, and inserted into the *Sal*I site of the pUC 119 polylinker to generate the constructs pUC118/mU6PSE$^-$ and pUC118/mU6PEDPMABC$^-$. The probes used in mobility-shift assays were generated by PCR amplification of these two constructs with 5′ end-labeled universal sequencing primer and reverse sequencing primer. Because the same labeled primer was used to generate wild-type and mutant probes, this protocol ensured that the two probes had equal specific activities. The binding reactions were performed in a total volume of 20 μl containing 5 μl of the SNAP$_c$ fraction and final concentrations of 60 mM KCl, 20 mM HEPES (pH 7.9), 5 mM $MgCl_2$, 0.2 mM EDTA, and 10% glycerol. A standard binding reaction involved an incubation of fraction with 0.05 μg/μl of dIdC and 0.05 μg/μl of pUC119 as nonspecific competitor for 30 min at room temperature, followed by incubation with 25,000 cpm of either wild-type mouse U6 PSE or mutant mouse U6 PSE probe for another 30 min. The samples were then fractionated on a 5% nondenaturing polyacrylamide gel (40 : 1) in 1 x TGE running buffer (50 mM Tris base, 380 mM glycine, 2 mM EDTA); the gel was dried and exposed to autoradiography.

UV cross-linking

The probes were generated by PCR amplification of two derivatives of pBSM13$^+$, pBS1xmU6PE$^+$ and pBS1xU6PEDPMABC$^+$, which contained the wild-type mouse U6 PSE or mutant mouse U6 PSE inserted into the *Sal*I site of the polylinker, with the universal sequencing primer and the reverse sequencing primer. The PCR reaction was performed in a total volume of 10 μl and contained the following nucleotides: 16 μl of [α-^{32}P]dATP (dried) and 16 μl of [α-^{32}P]dGTP (dried), both at 800 Ci/mmole, 1 μl of 5 mM dCTP and 1 μl of 5 mM 5-bromo-2′-deoxyuridine 5′-triphosphate (BrdUTP) substituted for dTTP. Unlabeled competitors were generated by similar PCR amplifications with unlabeled deoxynucleotide triphosphates.

Two parallel sets of binding reactions were performed. In the first set, 2,000,000 cpm of either the wild-type or mutant BrdU-substituted probe was incubated with 5 μl of the SNAP$_c$ fraction in a final volume of 20 μl, either in the absence or in the presence of cold competitor as indicated in the figures. In the second set, the binding reactions were scaled up three times. The binding reactions were performed as described for the mobility-shift assay, and some of the reactions (as indicated in the figures) were exposed to either 0, 200, or 600 mJ of UV light in a Stratalinker (Stratagene, Inc.). The first set of reactions was then submitted to the electrophoretic mobility-shift assay described above, whereas the reactions from the second set were treated with DNase I (5 units) and micrococcal nuclease (2 units) in the presence of 25 mM $CaCl_2$ and 25 mM $MgCl_2$ for 90 min at 37°C. The reactions were then mixed with Laemmli loading buffer (see Harlow and Lane 1988), boiled, and loaded on a 12.5% SDS–polyacrylamide gel. After electrophoresis, the gel was fixed, dried, and exposed to autoradiography.

Acknowledgments

We thank R. Burgess and M. Knuth for their generous help with our initial attempts in setting up the in vitro transcription system for RNA polymerase II transcription of snRNA genes. We thank M. Sullivan for HeLa cell extracts and expert technical help; C. Bautista for help in raising the mAbs; H. Sadowski for advice and discussion throughout the course of this work; and W. Herr, H. Sadowski, and B. Stillman for helpful comments on the manuscript. We also thank J. Wiggins and R. Whitaker for cell culture and J. Duffy and P. Renna for artwork and photography. This work was supported by National Institutes of Health grant RO1GM38810. N.H. is a Rita Allen Foundation Scholar.

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Genes Dev. 1993, **7:**
Access the most recent version at doi:10.1101/gad.7.8.1535