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The Small Nuclear RNA-activating Protein 190 Myb DNA Binding Domain Stimulates TATA Box-binding Protein-TATA Box Recognition*

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Human U6 small nuclear RNA (snRNA) gene transcription by RNA polymerase III requires cooperative promoter binding involving the snRNA-activating protein complex ($SNAP_c$) and the TATA-box binding protein (TBP). To investigate the role of $SNAP_c$ for TBP function at U6 promoters, TBP recruitment assays were performed using full-length TBP and a mini-$SNAP_c$ containing SNAP43, SNAP50, and a truncated SNAP190. Mini-$SNAP_c$ efficiently recruits TBP to the U6 TATA box, and two $SNAP_c$ subunits, SNAP43 and SNAP190, directly interact with the TBP DNA binding domain. Truncated SNAP190 containing only the Myb DNA binding domain is sufficient for TBP recruitment to the TATA box. Therefore, the SNAP190 Myb domain functions both to specifically recognize the proximal sequence element present in the core promoters of human snRNA genes and to stimulate TBP recognition of the neighboring TATA box present in human U6 snRNA promoters. The SNAP190 Myb domain also stimulates complex assembly with TBP and Brf2, a subunit of a snRNA-specific TFIIIB complex. Thus, interactions between the DNA binding domains of SNAP190 and TBP at juxtaposed promoter elements define the assembly of a RNA polymerase III-specific preinitiation complex.

Transcription in eukaryotic organisms occurs by three different RNA polymerases that transcribe different classes of genes. The recruitment of a specific RNA polymerase to a given promoter is dictated by the nature of the preinitiation complex (1–4); however, the molecular determinants for RNA polymerase specificity are not known. One possibility is that recruitment of TBP[1] complexes containing distinct cadres of TBP-associated factors (TAFs) may confer polymerase specificity. For example, the TBP complexes SL1, TFIID, and TFIIIB are multiprotein TBP·TAF complexes that are required for transcription by RNA polymerases I, II, and III, respectively (for review, see Refs. 5–7). These various TBP·TAF complexes play crucial roles by serving as targets for regulatory proteins and providing specific promoter recognition functions. Once recruited to a promoter, TBP·TAF complexes further provide an important structural façade during preinitiation complex assembly to recruit other general transcription factors dedicated to transcription by a specific RNA polymerase.

In contrast to the well characterized TBP complexes that function for transcription of most genes, how TBP is recruited to human small nuclear (sn) RNA gene promoters is understood less well. None of the aforementioned TBP·TAF complexes appears to function at these genes (8, 9). Human snRNA genes are unusual because they have similar promoters, and yet some snRNA genes are transcribed by RNA polymerase II (*e.g.* U1) and others by RNA polymerase III (*e.g.* U6) (for review, see Refs. 10 and 11). Thus, these genes serve as an important model for understanding the mechanisms of polymerase specificity (12). Each gene contains a proximal sequence element (PSE) in the core promoter which recruits the general transcription factor $SNAP_c$ (9), which is also known as proximal sequence element transcription factor (13) or proximal sequence element binding protein (14). Substoichiometric levels of TBP copurify with $SNAP_c$ during the biochemical fractionation of $SNAP_c$ from HeLa cell nuclear extracts (15), and fractions enriched for $SNAP_c$ restore U1 snRNA transcription by RNA polymerase II to HeLa cell extracts that have been immunodepleted of endogenous TBP (9). Thus, $SNAP_c$ may play an important role in recruiting TBP to the TATA-less promoters of RNA polymerase II-transcribed snRNA genes. In contrast, the TBP present in the $SNAP_c$-enriched fractions does not support U6 transcription by RNA polymerase III (9). One key difference between RNA polymerase II- and III-transcribed snRNA genes is the presence of a TATA box in the core promoter of RNA polymerase III-transcribed snRNA genes. TBP binds to this TATA box only poorly but is stimulated by $SNAP_c$. Furthermore, the combination of the PSE and TATA box directs transcription by RNA polymerase III, and mutation of either promoter element switches transcription to RNA polymerase II (16, 17). These observations suggest that $SNAP_c$-mediated recruitment of TBP or TBP complexes acting at the TATA box is a key step in assembling the specific preinitiation complex that preferentially recruits RNA polymerase III. Thus, depending upon the promoter context, $SNAP_c$ may recruit a different TBP complex that determines the type of RNA polymerase recruited to a snRNA promoter.

For most RNA polymerase III-transcribed genes, the TFIIIB complex, which is composed of TBP with the tightly associated TFIIB-related factor (Brf1) and a loosely associated factor called Bdp1 (originally named B″) (18), nucleates preinitiation complex assembly (19–26). Human U6 gene transcription by

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[1] The abbreviations used are: TBP, TATA-box binding protein; EMSA, electrophoretic mobility shift assay; IDP, inhibitory DNA binding; PSE, proximal sequence element; rTBP, recombinant TBP; SNAP, snRNA-activating protein; $SNAP_c$, snRNA-activating protein complex; snRNA, small nuclear RNA; TAF, TBP-associated factor; Pou, Pit1/Oct1/Oct2/Unc86.

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RNA polymerase III similarly requires both TBP and Bdp1; however, these genes do not require Brf1 (9, 24, 27–30). In contrast, transcription of these genes depends upon other TFIIIB-like complexes that contain Brf1-related factors. Two different Brf-like factors have been proposed to function for human snRNA gene transcription by RNA polymerase III. One Brf-related factor, called Brf2 (originally named BRFU/TFIIIB50) (18), shows limited similarity to Brf1, including the zinc ribbon domain and the TFIIB-related repeats, and functions specifically for transcription of human U6 genes (30, 31). Another related factor, called Brf1_v2 (originally named Brf2) (18), is an alternatively spliced form of Brf1 which contains the TFIIB-related repeat 2 and additional carboxyl-terminal sequences that are unique to Brf1_v2 and also acts to support human U6 transcription (32). Both Brf2 (33) and Brf1_v2 (32) can interact with TBP through the TFIIB-related repeat 2. Neither Brf2 nor Brf1_v2 appears to be tightly associated with TBP or Bdp1 in the absence of DNA binding by TBP (30, 32). However, Brf2 does stimulate TBP binding to the TATA box in electrophoretic mobility shift assays (EMSAs), and it may make DNA contacts surrounding the TATA box (33). The circumstances whereby Brf2, Brf1_v2, or both factors are used for human U6 transcription are currently unclear.

To understand how TBP is specifically recruited to human U6 snRNA gene promoters, we have examined those proteins within $SNAP_c$ which are important for interactions with TBP and for cooperative DNA binding of the U6 promoters by both $SNAP_c$ and TBP. We find that three subunits of $SNAP_c$ (SNAP43, SNAP45, and SNAP190) can interact individually with the TBP DNA binding domain. Thus, $SNAP_c$ may make extensive contacts with TBP. Further analysis of SNAP43 and SNAP190 indicate that the SNAP190 Myb DNA binding domain plays a pivotal role in TBP recruitment and stimulates assembly of a complex containing TBP and the TFIIIB factor Brf2. The juxtaposition of the PSE and TATA elements at human U6 promoters suggests that the DNA binding domains of SNAP190 and TBP are well positioned to facilitate these interactions during the early stages of preinitiation complex assembly. The unique architecture of SNAP190 and TBP at human U6 promoters thus may define whether RNA polymerase III-specific TFIIIB factors are specifically recruited to these genes.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Inserts encoding amino- or carboxyl-terminal truncations of $SNAP_c$ proteins or human TBP were generated by PCR amplification and cloned into pET11c-derived vectors. GST-SNAP190 RaRbRcRd contains amino acids 283–518, and GST-SNAP190 RcRd contains amino acids 390–518. Internal deletions were generated using the QuikChange site-directed mutagenesis protocol from Stratagene.

Protein Expression and Purification—Proteins used in the EMSAs were expressed as GST fusions in *Escherichia coli* BL21-CodonPlus-RIL cells (Stratagene). Proteins were purified from crude lysates on glutathione-Sepharose beads (Amersham Biosciences), and if necessary, the GST moiety was removed by digestion with thrombin. Proteins were purified further by ion exchange chromatography. Brf2 was expressed and purified as described previously (30). For Fig. 6, mini-$SNAP_c$ was assembled and purified by ion exchange chromatography prior to use in EMSA. Purified proteins were dialyzed against buffer D80 containing 20 mM HEPES pH 7.9, 20% glycerol, 80 mM KCl, 0.2 mM EDTA, 0.1% Tween 20, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM sodium metabisulfite, and 1 μM pepstatin A.

GST Pulldown Assays—GST pulldown assays were done essentially as described previously (34).

EMSAs—Mini-$SNAP_c$ was assembled for 2 h at room temperature using ~200 ng of each individual wild-type or mutant SNAP43, SNAP50, and SNAP190 (1–505) protein. Approximately 3 μg of SNAP19 was included in reactions to assemble complexes containing SNAP190 (1–505) proteins with internal deletions. Approximately one-third of each assembly reaction was then used in EMSA using DNA probes containing a wild-type or mutant mouse U6 PSE with a wild-type or mutant human U6 TATA box as described previously (9, 35). All DNA binding reactions were performed in a 20-μl total volume. DNA binding reactions using only $SNAP_c$ were performed in a buffer containing 60 mM KCl, 20 mM HEPES pH 7.9, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, 0.5 μg of poly(dI-dC), and 0.5 μg of pUC119 plasmid. Reactions were incubated for 20 min at room temperature after which 5,000 cpm of probe was added, and reactions were incubated an additional 20 min. Samples were fractionated on a 5% nondenaturing polyacrylamide gel (39:1) in TGE running buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA). Reactions containing both $SNAP_c$ and TBP were performed in a buffer containing 100 mM KCl, 20 mM HEPES pH 7.9, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.07% Tween 20, 0.2 μg of poly(dG-dC), and 0.2 μg of pUC119 plasmid. Reactions with SNAP190 RcRd and TBP also contained 0.5 μl of fetal bovine serum and 50 mM NaF but lacked KCl. The samples were fractionated on a 5% nondenaturing polyacrylamide gel (39:1) in TGEM running buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA, 5 mM $MgCl_2$). Reactions also containing Brf2 were performed in a buffer containing 10 mM HEPES pH 7.9, 20 mM Tris pH 8.4, 60 mM KCl, 7.5 mM $MgCl_2$, 10% glycerol, 6 mM β-mercaptoethanol, 12 mM dithiothreitol, 0.2 μg of poly(dG-dC), and 0.2 μg of pUC119 plasmid. Reactions were incubated for 20 min on ice after which 5,000 cpm of probe was added, and reactions were incubated for an additional 30 min at 30 °C. The samples were fractionated on a 4% nondenaturing polyacrylamide gel (39:1) containing 1× TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA) and 2.5% glycerol in 0.5 × TBE running buffer.

RESULTS

Mini-$SNAP_c$ Recruits TBP to the U6 snRNA TATA Box—Recruitment of TBP to the TATA box is a crucial early step in preinitiation complex assembly at human U6 snRNA promoters (for review, see Refs. 12 and 36). This step is facilitated by $SNAP_c$, which has two important roles in this process. First, interactions between $SNAP_c$ and TBP relieve negative regulation by the amino-terminal domain of TBP and allow the carboxyl-terminal DNA binding domain of TBP to bind efficiently to the TATA box. Second, cooperative interactions between $SNAP_c$ and TBP increase promoter occupancy by both $SNAP_c$ and TBP (35). Naturally occurring $SNAP_c$ contains at least five proteins SNAP19, SNAP43, SNAP45, SNAP50, and SNAP190 (15, 37–42). However, a baculovirus-expressed mini-$SNAP_c$ with only three proteins, SNAP43, SNAP50, and SNAP190 containing amino acids 1–514 is sufficient for recruiting TBP to a U6 snRNA TATA box (43). To investigate further the role of $SNAP_c$ for TBP recruitment to human U6 promoters, mini-$SNAP_c$ containing SNAP43, SNAP50, and SNAP190 (1–505) was assembled from bacterially expressed proteins and tested for DNA binding ability and recruitment of TBP to the U6 TATA box. As shown in Fig. 1*A*, none of the individual bacterially expressed $SNAP_c$ proteins could effectively bind to DNA containing a wild-type PSE (*lanes 2–4*). Similarly, little DNA binding activity was observed for reactions containing pairwise combinations of SNAP50 plus SNAP190 (1–505), SNAP43 plus SNAP190 (1–505), or SNAP43 plus SNAP50 (*lanes 5*, *9*, and *13*, respectively). However, as increasing amounts of SNAP43 (*lanes 6–8*), SNAP50 (*lanes 10–12*), or SNAP190 (1–505) (*lanes 14–16*) were added to reactions containing the remaining two proteins, significant levels of DNA binding activity were observed. The minicomplex specifically recognizes the PSE because no DNA binding activity was observed on DNA probes containing a mutant PSE (*lane 17*). Therefore, efficient PSE-specific DNA binding activity requires all three proteins, SNAP43, SNAP50, and SNAP190 (1–505).

To determine whether mini-$SNAP_c$ can efficiently recruit TBP to the U6 TATA box, EMSAs were performed with mini-$SNAP_c$ and full-length human TBP (Fig. 1*B*). As expected, full-length human TBP alone did not bind appreciably to a probe containing both a PSE and a TATA box (*lane 2*) or to a similar probe containing mutations in the TATA box (*lane 18*).

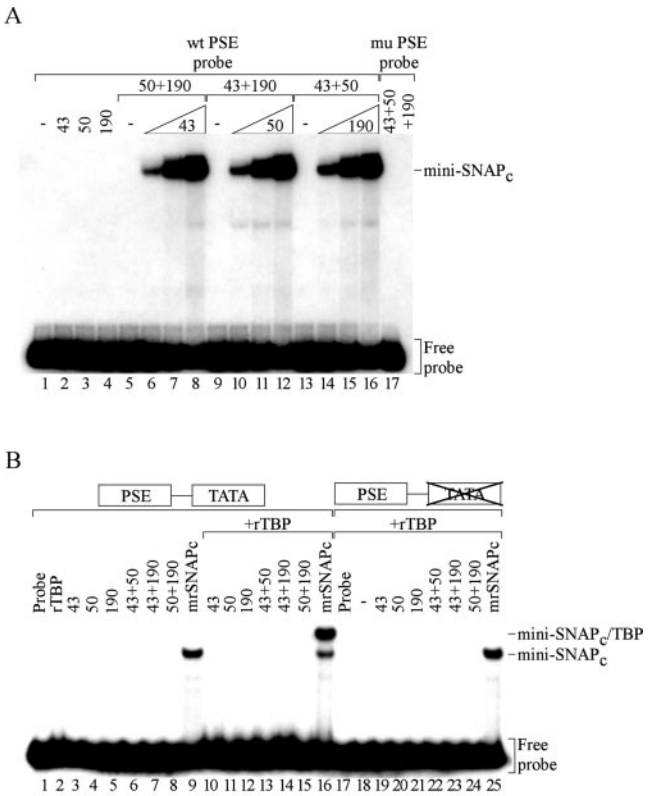

FIG. 1. **A mini-SNAP complex containing the SNAP43, SNAP50, and SNAP190 (1–505) proteins is sufficient to recruit human TBP to the U6 TATA box.** *A*, EMSAs were performed using ~63 ng of each $SNAP_c$ protein either individually (*lanes 2–4*) or in pairwise combination (*lanes 5*, *9*, and *13*) with a DNA probe containing a high affinity mouse U6 PSE. Approximately 7, 21, and 63 ng of SNAP43 (*lanes 6–8*), SNAP50 (*lanes 10–12*), or SNAP190 (1–505) (*lanes 14–16*) was added to reactions containing 63 ng each of the remaining two proteins as indicated. The positions of free probe and mini-$SNAP_c$ bound to DNA are indicated. *Lane 1* contains the probe alone, and *lane 17* contains a probe with a mutant PSE but is otherwise identical to *lane 16*. *B*, EMSA using individual $SNAP_c$ proteins (*lanes 3–5*, *10–12*, and *19–21*), pairwise combinations of $SNAP_c$ proteins (*lanes 6–8*, *13–15*, and *22–24*), or mini-$SNAP_c$ (*lanes 9*, *16*, and *25*) performed in the absence (*lanes 3–9*) or presence (*lanes 10–25*) of recombinant human TBP. DNA binding reactions contained about 30 ng of each $SNAP_c$ protein and 100 ng of TBP. The DNA probes used contain a high affinity mouse U6 snRNA PSE and either a wild-type (*lanes 1–16*) or mutant (*lanes 17–25*) human U6 snRNA TATA box. *Lanes 1* and *17* contain DNA probes alone, and *lanes 2* and *18* contain probes with recombinant TBP alone. The positions of the free probe, mini-$SNAP_c$, and mini-$SNAP_c$·TBP complex are indicated.

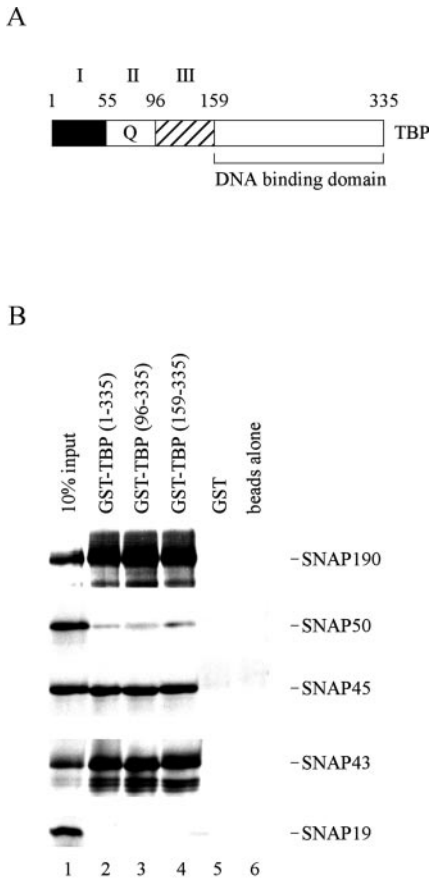

FIG. 2. **Three $SNAP_c$ proteins interact directly with the TBP DNA binding domain.** *A*, schematic of human TBP. The nonconserved NH_2 terminus that is divided into three segments (*I*, *II*, and *III*) and the highly conserved COOH terminus that contains the DNA binding domain of TBP are indicated. Segment II contains a stretch of glutamine residues (*Q*) from amino acids 55–95. *B*, $SNAP_c$ proteins were labeled with [^{35}S]methionine and used in GST pulldown assays with GST-TBP (1–335) (*lane 2*), GST-TBP (96–335) (*lane 3*), GST-TBP (159–335) (*lane 4*), GST protein alone (*lane 5*), or glutathione-Sepharose beads alone (*lane 6*). The SNAP190 protein contains amino acids 1–719. 10% of the total input for each protein is shown in *lane 1*.

None of the $SNAP_c$ proteins either alone (*lanes 3–5*) or in pairwise combination (*lanes 6–8*) showed significant DNA binding to a probe with a wild-type PSE. The combination of all three proteins (labeled $mrSNAP_c$) resulted in significant DNA binding to the wild-type probe (*lane 9*) but not to a probe containing mutations in the PSE (data not shown). Recombinant human TBP was then added to these $SNAP_c$ proteins to examine their ability to recruit TBP to the U6 TATA box. When incubated with recombinant TBP, none of the $SNAP_c$ proteins either alone or in pairwise combination showed significant DNA binding to probes containing a wild-type PSE and either a wild-type (*lanes 10–15*) or mutant (*lanes 19–24*) TATA box. In contrast, mini-$SNAP_c$ efficiently recruited human TBP to a probe containing both a wild-type PSE and TATA box as evidenced by the formation of a complex that migrated more slowly than that observed with mini-$SNAP_c$ alone (compare *lane 16* with *lane 9*). Recruitment of TBP by $SNAP_c$ was cooperative as evidenced by the dramatic increase in DNA binding by TBP in the presence of mini-$SNAP_c$ (compare the *upper* complex in *lane 16* with *lane 2*). Mutations in the TATA box did not affect DNA binding by mini-$SNAP_c$ but abolished formation of the mini-$SNAP_c$·TBP·DNA complex (*lane 25*). Thus, as demonstrated with recombinant mini-$SNAP_c$ expressed in insect cells by baculovirus infection (43), mini-$SNAP_c$ assembled from bacterially expressed proteins is able to recruit TBP to the U6 snRNA TATA box, and these interactions are dependent upon both the PSE and TATA box promoter elements. Furthermore, these results show that SNAP19, SNAP45, or other eukaryotic proteins that may associate with recombinant $SNAP_c$ are not required for either PSE-specific binding or TBP recruitment to the U6 TATA box.

Multiple $SNAP_c$ Subunits Can Interact with TBP—As a first step to determine which $SNAP_c$ proteins are required for TBP recruitment, interactions between individual $SNAP_c$ proteins and human TBP were analyzed by GST pulldown assays. Previously, it was demonstrated that the TBP COOH-terminal DNA binding domain and two regions of the NH_2 terminus are important for cooperative DNA binding by $SNAP_c$ and TBP (35). These regions from the NH_2 terminus include amino acids 1–54 and the Q-rich region containing amino acids 55–95 (Fig. 2*A*). Therefore, we also tested interactions between $SNAP_c$ proteins and TBP proteins that lacked these regions of the NH_2 terminus. As shown in Fig. 2*B*, three $SNAP_c$ subunits, SNAP190 (1–719), SNAP45, and SNAP43, interacted well with

full-length GST-TBP (1–335) in these assays (*lane 2*), and these interactions were specific because no interaction was observed with GST protein (*lane 5*) or beads alone (*lane 6*). In similar assays, full-length SNAP190 (1–1469) also interacted well with full-length TBP (data not shown). Moreover, the DNA binding domain of TBP was sufficient for interactions with all three $SNAP_c$ proteins because no significant difference for interactions involving full-length GST-TBP (1–335) (*lane 2*), GST-TBP (96–335) (*lane 3*), or GST-TBP (159–335) (*lane 4*) was observed. These results suggest that TBP makes extensive contacts with $SNAP_c$ and that the COOH-terminal DNA binding domain of TBP is important for interactions with $SNAP_c$. Although interactions involving both SNAP43 and SNAP45 with TBP have been described previously (15, 40, 41), the current observations reveal that a previously uncharacterized interaction between SNAP190 and TBP may contribute to $SNAP_c$ function for human snRNA gene transcription.

No Single Region of SNAP43 Is Required for TBP Recruitment to the U6 TATA Box—Of the proteins present within mini-$SNAP_c$, both SNAP43 and SNAP190 protein interacted well with TBP (Fig. 2*B*). To characterize further the interactions between SNAP43 and TBP, regions of SNAP43 which are important for mediating interactions with TBP were mapped by GST pulldown assays. SNAP43 proteins containing NH_2- or COOH-terminal truncations were tested for interactions with GST-TBP (1–335) and also with GST-SNAP50. The data for separate experiments testing interactions between SNAP43 proteins and either GST-TBP or GST-SNAP50 are shown in Fig. 3, *A* and *B*, respectively, and a summary of the results is shown in Fig. 3*C*. As expected, significant interactions of full-length SNAP43 (1–368) with both GST-TBP and GST-SNAP50 were observed. Interestingly, SNAP43 containing only the NH_2-terminal 168 amino acids (1–168) interacted well with SNAP50 but not with TBP. In contrast, the COOH-terminal region of SNAP43 containing amino acids 169–368 interacted well with both TBP and SNAP50. These interactions are specific because the SNAP43 proteins did not interact with GST protein (Fig. 3, *A* and *B*, *lanes 3*) or the beads alone (Fig. 3, *A* and *B*, *lanes 4*). Ma and Hernandez (44) did not detect an interaction between SNAP50 and the COOH-terminal region of SNAP43 using coimmunoprecipitation assays, suggesting that this interaction is weak. These observations suggest that SNAP50 makes extensive interactions with at least two separate regions of SNAP43. Furthermore, a major region for TBP interaction with SNAP43 is contained within amino acids 169–368.

To determine the contribution of SNAP43 for TBP recruitment to the U6 TATA box we then took advantage of the observation that both halves of SNAP43 could interact with SNAP50 and SNAP190 (1–505) (Fig. 3*B* and data not shown) and could therefore potentially form a mini-$SNAP_c$. First, the ability of each half of SNAP43 to form DNA-binding competent complexes with SNAP50 and SNAP190 (1–505) was tested using an EMSA, and the results are shown in Fig. 3*D*. As with full-length SNAP43 (1–368) (*lane 4*), both SNAP43 (1–168) and SNAP43 (169–368) assembled into complexes capable of efficient DNA binding (*lanes 7* and *10*, respectively). In all cases, DNA binding by these complexes was PSE-specific because no DNA binding was observed using a probe with a mutant PSE (data not shown). These observations suggest that SNAP43 is essential for DNA binding by mini-$SNAP_c$, but no single region of SNAP43 is critical. Thus, it appears that SNAP43 coordinates DNA binding by SNAP50 and SNAP190, and either half of SNAP43 is sufficient for this activity. In contrast to strong DNA binding by mini-$SNAP_c$, full-length human TBP (1–335) was unable to bind DNA efficiently in these assays (*lanes 2* and *3*). As expected, TBP is recruited to DNA efficiently by mini-$SNAP_c$ containing SNAP43 (1–368) (*lanes 5* and *6*). Complexes assembled with SNAP43 containing either the NH_2-terminal region (amino acids 1–168) or COOH-terminal region (amino acids 169–368) were capable of recruiting TBP to the U6 snRNA TATA box (*lanes 8*, *9* and *11*, *12*, respectively). This result indicates that no single region of SNAP43 is essential for TBP recruitment to the TATA box under the conditions of this assay. This was unexpected in light of the results of the GST pulldown experiments in Fig. 3, *A* and *B*, and suggests that interactions other than those involving SNAP43 are sufficient for TBP recruitment to the U6 snRNA TATA box.

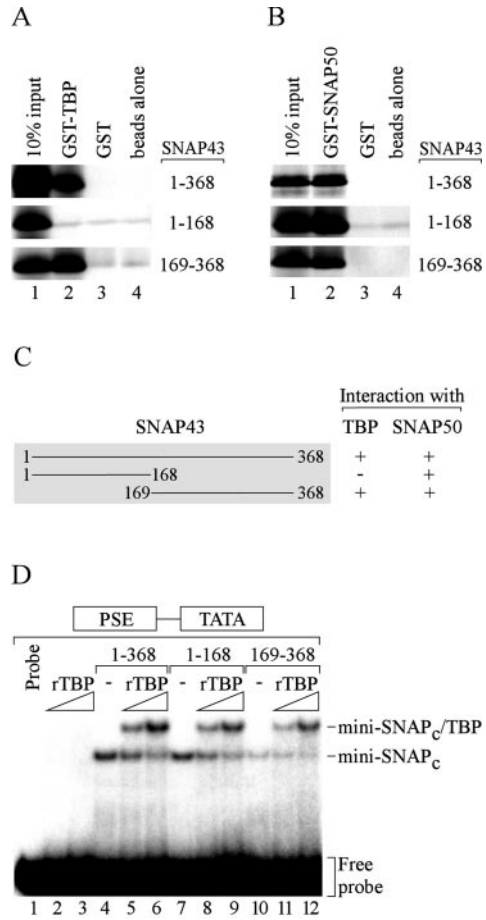

FIG. 3. **No single region of SNAP43 is required for TBP recruitment to the U6 TATA box.** *A*, full-length SNAP43 protein or truncated SNAP43 proteins containing amino acids 1–168 or 169–368 were used in GST pulldown assays with GST-TBP (*lane 2*), GST (*lane 3*), or glutathione-Sepharose beads alone (*lane 4*). 10% of the total input for each protein is shown in *lane 1*. *B*, same as *A* except GST-SNAP50 was used instead of GST-TBP. *C*, summary of results for the GST pulldown assays between the SNAP43 proteins and GST-TBP or GST-50. A (+) indicates that a direct interaction was observed; (−) indicates that no interaction was detected. *D*, EMSA containing ~30 ng each of SNAP50, SNAP190 (1–505), and either full-length SNAP43 (*lanes 4–6*), SNAP43 (1–168) (*lanes 7–9*), or SNAP43 (169–368) (*lanes 10–12*) in the absence (*lanes 4*, *7*, and *10*) or presence (*lanes 5*, *6*, *8*, *9*, *11*, and *12*) of recombinant human TBP. The DNA probe used contains a high affinity mouse U6 snRNA PSE and a human U6 snRNA TATA box. The positions of free probe, mini-$SNAP_c$, and mini-$SNAP_c$·TBP complex are indicated. *Lane 1* contains DNA probe alone, and *lanes 2* and *3* contain recombinant TBP alone.

The SNAP190 RcRd Myb Repeats Are Sufficient for TBP Recruitment to the U6 TATA Box—Both SNAP43 and SNAP190 are capable of interacting with TBP; however, no single region of SNAP43 is essential for recruitment of TBP to the U6 TATA box, and thus it seems possible that interactions between SNAP190 and TBP are important for TBP recruit-

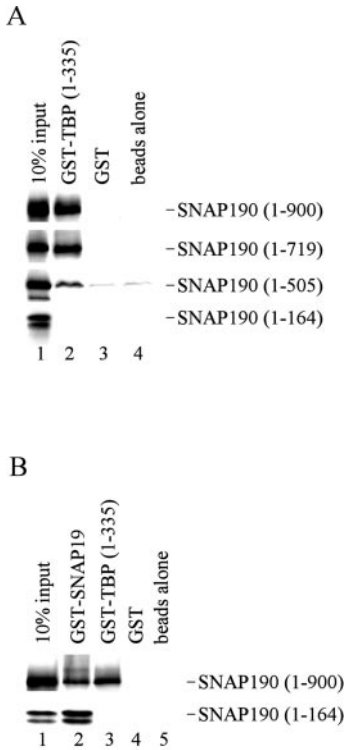

FIG. 4. **SNAP190 (1–505) interacts directly with human TBP.** *A*, full-length or COOH-terminally truncated SNAP190 proteins were used in GST pulldown assays with GST-TBP (1–335) (*lane 2*), GST (*lane 3*), or glutathione-Sepharose beads alone (*lane 4*). 10% of the input for each protein is shown in *lane 1*. *B*, SNAP190 (1–900) and SNAP190 (1–164) were used in GST pulldown assays with GST-SNAP19 (*lane 2*), GST-TBP (1–335) (*lane 3*), GST (*lane 4*), or glutathione-Sepharose beads alone (*lane 5*). 10% of the total input for each protein is shown in *lane 1*.

ment. First, to determine whether SNAP190 (1–505) can interact with TBP, interactions between truncated SNAP190 proteins and GST-TBP were tested in GST pulldown experiments. Significant interactions between both SNAP190 (1–900) and SNAP190 (1–719) with GST-TBP (1–335) were observed (Fig. 4*A*, *lane 2*). Importantly, SNAP190 (1–505) also interacted well with TBP. This interaction was reduced compared with that observed for longer versions of SNAP190, suggesting that amino acids 506–719 influence TBP recruitment, but it still was significantly greater than that observed for SNAP190 (1–164) (compare *lane 2* with *lane 1* for each SNAP190 protein). As shown in Fig. 4*B*, SNAP190 (1–164) is capable of interacting with GST-SNAP19 (*lane 2*) but is not capable of interacting with GST-TBP (1–335) (*lane 3*). In contrast, SNAP190 (1–900) interacts well with both GST-SNAP19 (*lane 2*) and GST-TBP (1–335) (*lane 3*). Together, these observations indicate that SNAP190 interacts with TBP specifically, and the region of SNAP190 contained within amino acids 164–505, which encompasses the SNAP190 Myb DNA binding domain, is important for this interaction.

The above observations raise the interesting possibility that interactions between the SNAP190 DNA binding domain and TBP may be important for TBP recruitment to the U6 TATA box. To determine whether SNAP190 contributes to TBP recruitment, SNAP190 (1–505) proteins with internal deletions were assembled into a mini-$SNAP_c$ and tested for both PSE-specific binding and TBP recruitment by EMSA. A schematic representation of the mutant SNAP190 proteins tested is shown in Fig. 5*A*. SNAP190 (1–505) contains an NH_2-terminal region located between amino acids 84 and 133, which is required for interactions with SNAP19 and SNAP43 (44), and an unusual Myb DNA binding domain containing four and one-half Myb domain repeats located between amino acids 263 and 503. The SNAP190 Myb repeats are referred to as Rh, Ra, Rb, Rc, and Rd (38). When tested with SNAP43 and SNAP50, complexes containing the mutant SNAP190 (1–505) proteins were either unable to bind (Δ165–259, Δa, and Δb) or bound poorly (Δh) to DNA (data not shown). However, it was shown previously that mini-$SNAP_c$ containing SNAP190 with deletions of amino acids 1–84 and 134–262 failed to bind DNA, but binding was stimulated by the addition of SNAP19 (44). Therefore, mini-$SNAP_c$ was assembled in the presence of SNAP19 and tested again for DNA binding. As shown in Fig. 5*B*, all of the mini-SNAP complexes containing SNAP190 (1–505) proteins with internal deletions were able to bind DNA efficiently in the presence of the SNAP19 (*lanes 3*, *5*, *7*, and *9*). Binding was PSE-specific and was not observed on DNA with mutant PSE sites (data not shown). These complexes were also all able to recruit TBP to a TATA box (*lanes 4*, *6*, *8*, and *10*). Therefore, no single region of SNAP190 between amino acids 164 and the RcRd Myb repeats is essential for TBP recruitment to the U6 TATA box under these conditions, although amino acids in these regions may contribute to efficient TBP recruitment by the full-length SNAP190.

Although the SNAP190 Rh, Ra, and Rb repeats are dispensable for DNA binding, both SNAP190 Rc and Rd repeats are crucial for PSE-specific binding (38, 43), and therefore mutant SNAP190 lacking RcRd could not be tested directly in the above assays. However, even though SNAP190 (1–505) requires SNAP43 and SNAP50 for efficient DNA binding (see Fig. 1), the SNAP190 RcRd Myb repeats alone are sufficient for DNA binding, but with reduced specificity (38, 45). To examine directly whether the SNAP190 RcRd Myb repeats can recruit TBP to the U6 snRNA TATA box in the absence of any other $SNAP_c$ protein, an EMSA was performed (Fig. 5*C*). The DNA probes used in these experiments contained a wild-type PSE and TATA box (*lanes 1–4*), a wild-type PSE and mutant TATA box (*lanes 5–8*), a mutant PSE and wild-type TATA box (*lanes 9–12*), or a mutant PSE and mutant TATA box (*lanes 13–16*).

Little binding to any of the probes was observed with either SNAP190 RcRd (*lanes 3*, *7*, *11*, and *15*) or TBP (*lanes 2*, *6*, *10*, and *14*) proteins alone. Lack of detectable binding by the SNAP190 RcRd protein alone was somewhat surprising, but binding was detectable on gels lacking $MgCl_2$ (45, and data not shown). Similarly, no binding was observed with both proteins when the PSE and TATA box were both mutated (*lane 16*). Interestingly, the SNAP190 RcRd protein was able to recruit TBP to the U6 snRNA TATA box (compare *lane 4* with *lane 2*). TBP was present on the DNA because antibodies directed against TBP supershifted the complex (data not shown). Recruitment of TBP was dependent on a wild-type TATA box because the SNAP190 RcRd protein was unable to recruit TBP when the TATA box was mutated (*lanes 5–8*). SNAP190 RcRd and TBP bound to the DNA probe with a mutant PSE albeit more weakly than to the probe containing a wild-type PSE (compare *lane 12* with *lane 4*), suggesting that TBP can recruit SNAP190 RcRd in the absence of a strong PSE. Similar results were obtained with a GST-tagged SNAP190 RcRd protein (data not shown). Thus, the SNAP190 RcRd Myb DNA binding domain is sufficient for TBP recruitment to the U6 promoter.

As shown above, the Myb DNA binding domain of SNAP190 can stimulate TBP recruitment to the TATA box in DNA binding assays. To determine whether the SNAP190 RcRd protein can interact with TBP, GST pulldown experiments were performed. As shown in Fig. 5*D*, full-length TBP interacts well with GST-SNAP190 (1–505) and with GST-SNAP190 RcRd (*lanes 2* and *3*, respectively) but not with the negative control

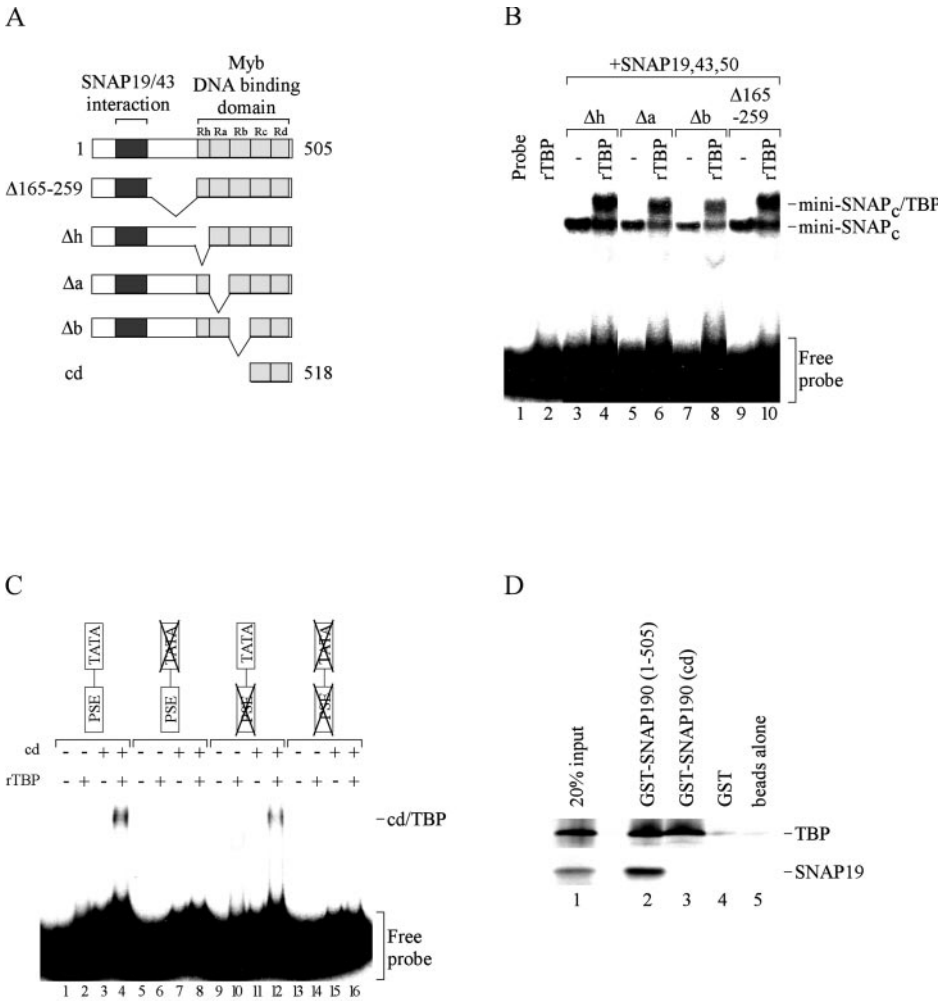

FIG. 5. **The SNAP190 RcRd Myb repeats are sufficient to recruit TBP to the U6 TATA box.** *A*, schematic of the wild-type and mutant SNAP190 proteins. The region of SNAP190 required for interaction with SNAP19 and SNAP43 and the Myb DNA binding domain that contains four (Ra through Rd) and a half (Rh) Myb repeats are indicated. *B*, EMSAs containing SNAP19, SNAP43, SNAP50, and either SNAP190 (1–505) Δh (*lanes 3* and *4*), SNAP190 (1–505) Δa (*lanes 5* and *6*), SNAP190 (1–505) Δb (*lanes 7* and *8*), or SNAP190 (1–505) Δ165–259 (*lanes 9* and *10*) in the absence (*lanes 3*, *5*, *7*, and *9*) or presence (*lanes 4*, *6*, *8*, and *10*) of recombinant human TBP. The DNA probe used contains a high affinity mouse U6 snRNA PSE and a human U6 snRNA TATA box. *Lane 1* contains DNA probe alone, and *lane 2* contains probe with recombinant TBP alone. *C*, EMSAs were performed with 60 ng of rTBP (*lanes 2*, *6*, *10*, and *14*), 1 μg of SNAP190 RcRd (*lanes 3*, *7*, *11*, and *15*), or rTBP and SNAP190 RcRd (*lanes 4*, *8*, *12*, and *16*). The DNA probes used contain a high affinity mouse U6 snRNA PSE and a human U6 snRNA TATA box (*lanes 1–4*), a wild-type PSE and mutant TATA box (*lanes 5–8*), a mutant PSE and wild-type TATA box (*lanes 9–12*), or a mutant PSE and mutant TATA box (*lanes 13–16*). *Lanes 1*, *5*, *9*, and *13* contain probe DNA alone. The presence (+) or absence (−) of rTBP or SNAP190 RcRd is indicated above each *lane*. The positions of free probe and SNAP190 RcRd and rTBP complexes are indicated (*−cd/TBP*). *D*, human TBP or SNAP19 proteins were used in GST pulldown assays with GST-SNAP190 (1–505) (*lane 2*), GST-SNAP190 RcRd (*lane 3*), GST (*lane 4*), or glutathione-Sepharose beads alone (*lane 5*). 20% of the total input for each protein is shown in *lane 1*.

GST or beads alone samples (*lanes 4* and *5*, respectively). To examine further the specificity of this interaction, SNAP19 binding was also tested. As was observed with TBP, SNAP19 interacts well with GST-SNAP190 (1–505). However, SNAP19 did not interact with GST-SNAP190 RcRd (*lane 3*). This result was expected because SNAP19 interacts with the NH_2-terminal region of SNAP190 (44). Importantly, this result supports the notion that the SNAP190 RcRd interaction with TBP is specific.

$SNAP_c$ Stimulates TFIIIB Assembly at Human U6 Promoters—$SNAP_c$ binding to the PSE is a crucial early event during the assembly of a functional preinitiation complex at human U6 promoters. As described, one function of $SNAP_c$ is to stimulate TBP recruitment to the TATA box. However, additional events are required, including the assembly of additional TFIIIB components at these promoters. As a marker for TFIIIB assembly, we followed the binding of Brf2 to human U6 promoter probes in EMSAs. As shown in Fig. 6, neither TBP alone (*lanes 2*, *10*, *18*, and *26*) nor Brf2 alone (*lanes 3*, *11*, *19*, and *27*) bound efficiently to any of the U6 promoter probes. In contrast, mini-$SNAP_c$ alone bound efficiently to wild-type PSE probes (*lanes 4* and *20*) but not to mutant PSE probes (*lanes 12* and *28*) as expected. When recombinant TBP was added to reactions containing mini-$SNAP_c$, modest formation of a slower migrating complex was observed (*lane 5*, labeled *mini-$SNAP_c$ + rTBP*), and surprisingly, complex formation was not affected by TATA box mutation (*lane 21*). This complex was inferred to contain both mini-$SNAP_c$ and TBP because it was only observed in the presence of both mini-$SNAP_c$ and TBP. $SNAP_c$ stimulation of TBP binding was also less than that observed in the experiments presented in Figs. 1 and 3 presumably because these experiments were performed using different electrophoresis conditions. Interestingly, a slower migrating complex was observed when Brf2 was added to reactions containing mini-$SNAP_c$ (*lane 6*, labeled *mini-$SNAP_c$ + Brf2*). This complex contained Brf2 because antibodies directed against the histidine tag on Brf2 were able to supershift this complex (data not shown). Mutation of the PSE severely debilitated $SNAP_c$·Brf2

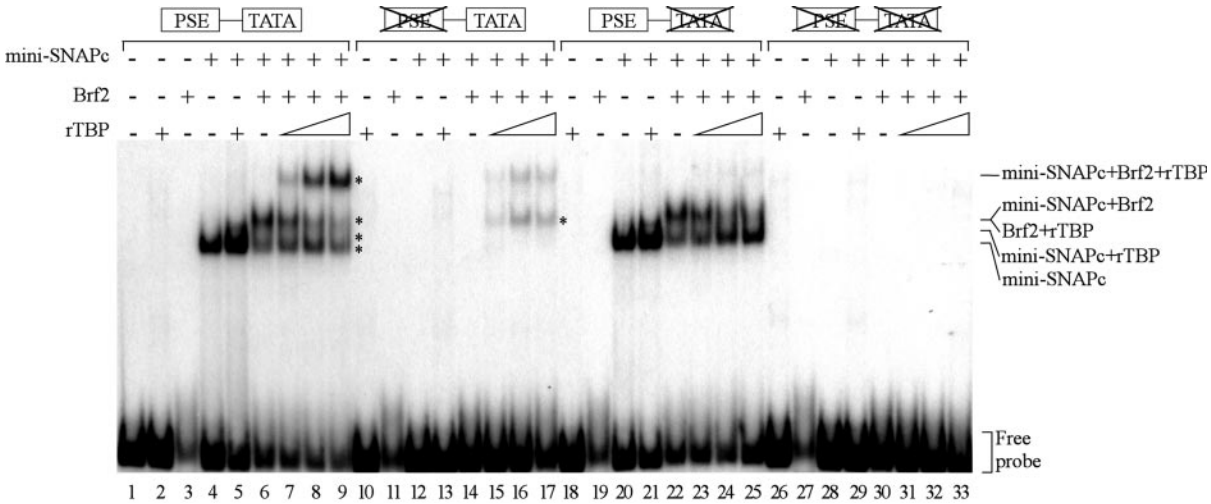

FIG. 6. **Mini-SNAP$_c$ can recruit Brf2 to a U6 snRNA promoter.** EMSAs were performed with 1 μg of rTBP (*lanes 2*, *10*, *18*, and *26*), 120 ng of Brf2 (*lanes 3*, *11*, *19*, and *27*), 10 ng of mini-SNAP$_c$ (*lanes 4*, *12*, *20*, and *28*), rTBP and mini-SNAP$_c$ (*lanes 5*, *13*, *21*, and *29*), Brf2 and mini-SNAP$_c$ (*lanes 6*, *14*, *22*, and *30*), or Brf2, mini-SNAP$_c$, and increasing amounts of rTBP (0.1, 0.3 and 1.0 μg; *lanes 7–9*, *15–17*, *23–25*, and *31–33*, respectively). The DNA probes used contain a high affinity mouse U6 snRNA PSE and a human U6 snRNA TATA box (*lanes 1–9*), a mutant PSE and wild-type TATA box (*lanes 10–17*), a wild-type PSE and mutant TATA box (*lanes 18–25*), or a mutant PSE and mutant TATA box (*lanes 26–33*). *Lane 1* contains probe alone. The presence (+) or absence (−) of rTBP, Brf2, and mini-SNAP$_c$ is indicated above each *lane*. The positions of free probe and protein·DNA complexes are indicated.

complex formation (*lane 14*), whereas TATA box mutations did not affect this complex (*lane 22*). Thus, mini-SNAP$_c$ can recruit Brf2 to a U6 promoter in a PSE-dependent manner. Next, the ability of SNAP$_c$·Brf2 to recruit TBP was tested. As increasing amounts of TBP were added to reactions containing mini-SNAP$_c$ and Brf2 (*lanes 7–9*), formation of a new complex was observed (labeled *mini-SNAP$_c$ + Brf2 + rTBP*). The presence of Brf2 in this new complex was confirmed using antibody supershift assays (data not shown). In these reactions, the levels of the SNAP$_c$·Brf2 complex was reduced concomitantly with added TBP, whereas SNAP$_c$ binding was unchanged, suggesting that TBP preferentially recognized the SNAP$_c$·Brf2 complex to form the SNAP$_c$·Brf2·TBP complex. Mutation of the PSE severely impaired, but did not completely abrogate, the formation of this new complex (*lanes 15–17*). This observation suggests that TBP and Brf2 together can help stabilize SNAP$_c$ binding to a weak PSE. In these reactions, a faster migrating complex is also observed, which comigrates with the SNAP$_c$·Brf2 complex. However, this complex likely corresponds to a complex containing Brf2 and TBP because the SNAP$_c$·Brf2 complex does not form on probes containing a mutated PSE (*lane 14*). Indeed, in subsequent experiments the SNAP$_c$·Brf2 and TBP·Brf2 complexes were observed to comigrate (data not shown). As expected, when mutations were introduced into both the PSE and TATA box, DNA binding by all factors was abolished (*lanes 26–33*). Therefore, SNAP$_c$ can recruit Brf2 in a PSE-specific manner, and together, these factors further stimulate TBP recruitment to a human U6 promoter.

The SNAP190 Myb Domain Stimulates Preinitiation Complex Assembly with TFIIIB—To determine whether the SNAP190 DNA binding domain can facilitate TFIIIB assembly at human U6 promoters the ability of GST-SNAP190 RcRd to form higher order complexes with TBP and Brf2 was tested using an EMSA. As shown in Fig. 7, no significant DNA binding was observed for TBP (*lane 2*), Brf2 (*lane 3*), or GST-SNAP190 RcRd (*lane 4*) in reactions containing DNA probes with wild-type PSE and TATA sequences. Similarly, no significant DNA binding was observed in reactions containing GST-SNAP190 RcRd and TBP (*lane 5*), which is consistent with the previous observation that mini-SNAP$_c$ recruits TBP poorly under these particular conditions (see Fig. 6). GST-SNAP190 RcRd also did not facilitate efficient Brf2 binding (*lane 6*). This

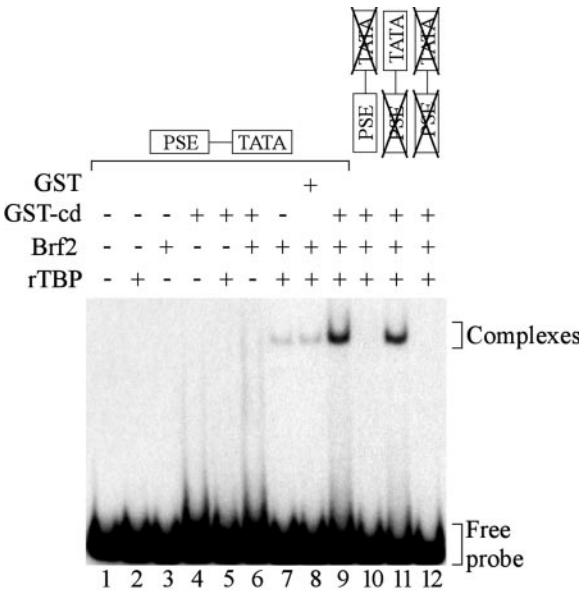

FIG. 7. **The SNAP190 RcRd Myb repeats stimulate binding of TBP and Brf2 to the U6 promoter.** The indicated proteins individually (*lanes 2–4*), in pairwise combinations (*lanes 5–7*), or rTBP, Brf2, and either GST (*lane 8*) or GST-cd (*lanes 9–12*) were used in EMSAs. *Lane 1* contains probe alone. The DNA probes used are represented schematically. The positions of free probe and protein·DNA complexes are indicated.

result is distinct from that observed in Fig. 6 wherein mini-SNAP$_c$ supported efficient Brf2 recruitment, suggesting that an additional region of SNAP190 or other SNAP$_c$ subunits is important for this function. In these experiments, Brf2 and TBP bound DNA cooperatively as evidenced by the greater DNA binding by both factors (*lane 7*) compared with that observed with either factor alone. This result is consistent with that described previously (33) and supports the notion that Brf2 contributes to stable TBP binding to these promoters. Interestingly, a significant increase in complex formation was observed when GST-SNAP190 RcRd was added to reactions containing Brf2 and TBP (compare *lane 9* with *lane 7*), which suggests that SNAP190 also cooperates with TBP and Brf2 for

promoter binding. This is specific for SNAP190 RcRd because comparable amounts of GST did not stimulate TBP recruitment (*lane 8*). Mutations in the TATA box completely abolished the formation of the SNAP190·Brf2·TBP complex (*lane 10*), but surprisingly, mutations in the PSE had only a modest effect on binding (*lane 11*). The presence of GST-SNAP190 RcRd in this complex was confirmed by antibody supershift experiments (data not shown). Thus, it appears that Brf2 and TBP can help stabilize SNAP190 RcRd association with the promoter in the absence of strong DNA binding by SNAP190. This result was unexpected but is consistent with the data shown in Fig. 6 wherein mini-$SNAP_c$·Brf2·TBP complex formation was reduced, but not abolished, on probes containing a wild-type TATA box with a mutant PSE. Altogether, these results indicate that interactions between the SNAP190 Myb domain and TBP are important for promoter recognition by TBP and for initial stages of preinitiation complex assembly with TFIIIB.

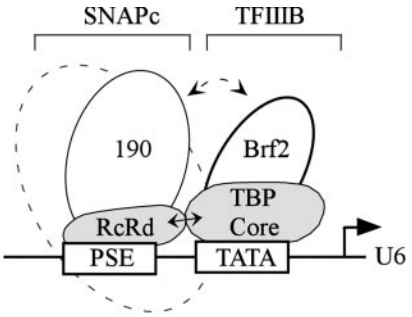

FIG. 8. **Schematic for promoter recognition by $SNAP_c$ and TFIIIB.** Efficient PSE recognition by $SNAP_c$ is mediated by the SNAP190 Myb RcRd DNA binding domain, which also functions to interact with the TBP DNA binding domain to recruit a snRNA-specific TFIIIB complex. For simplicity, the remaining $SNAP_c$ subunits are represented as a *dotted ellipse*. Additional interactions between Brf2 and $SNAP_c$ (*dashed arrow*) contribute to complex stability at these promoters. Other TFIIIB components are not shown.

DISCUSSION

Human U6 gene transcription depends upon a PSE and a TATA box that together specify recruitment of RNA polymerase III to these promoters. Earlier studies revealed that a strict spacing between the PSE and TATA box is required for efficient transcription (46), which suggested that proteins that bind to these sites interact with each other. Indeed, $SNAP_c$ interacts cooperatively with TBP to bind to the U6 snRNA promoter (35). The data presented herein suggests that three subunits, SNAP43, SNAP45, and SNAP190 interact directly with the conserved COOH-terminal DNA binding domain of TBP. Thus, $SNAP_c$ can make extensive contacts with TBP, and these interactions may be important for TBP function at human snRNA promoters. However, neither SNAP43 nor SNAP45 is critical for TBP recruitment to a human U6 TATA box. One possibility is that these factors participate in TBP function for RNA polymerase II transcription of human snRNA genes that contain only a PSE and not a TATA box as is present in human U1 genes, for example. This scenario seems more plausible for SNAP43 than SNAP45 because SNAP45 is not essential for $SNAP_c$-dependent transcription *in vitro* (43).

Coordinated Interactions of Juxtaposed SNAP190 and TBP DNA Binding Domains—SNAP190 interacts well with TBP via the TBP DNA binding domain, and a TBP interaction region within SNAP190 was mapped to the Myb DNA binding domain. Interestingly, the SNAP190 Myb domain alone is sufficient to recruit TBP to a human U6 TATA box (see Fig. 5*C*). Full-length human TBP by itself does not bind well to a TATA box because of an inhibitory effect of its NH_2-terminal domain. Therefore, the SNAP190 Myb RcRd DNA binding domain is sufficient to overcome the inhibitory affect of the TBP NH_2 terminus on TBP-TATA box binding by the TBP DNA binding domain. TBP binding to TATA box elements is a multistep mechanism that involves initial binding to unbent DNA and conversion to a more stable form containing bent DNA (47, 48). Recent experiments suggest that the role of the NH_2-terminal region of TBP is to inhibit DNA bending rather than DNA binding (46). The function of the NH_2-terminal region is also linked to a surface-exposed region of the TBP DNA binding domain called the inhibitory DNA binding (IDP) surface. The IDP surface regulates both initial TATA box binding and subsequent DNA bending, and it has been posited that the NH_2-terminal region directly contacts the IDP surface to regulate negatively the transition to a TBP complex containing bent DNA (47). Thus, an intriguing possibility is that the SNAP190 Myb domain may directly contact the IDP surface to relieve negative regulation by the TBP NH_2 terminus and thus stimulate TBP conversion to a stable complex containing bent DNA. In this context, the SNAP190 DNA binding domain has two functions. One function is to recognize the PSE present in the core promoters of human snRNA genes, and a second function is to stimulate TBP recognition of the neighboring TATA box present in human U6 snRNA gene promoters.

TBP Complex Recruitment to Human U6 snRNA Promoters—In addition to regulating TBP function at human U6 promoters, the SNAP190 Myb DNA binding domain might be sufficient to recruit a TBP-containing complex that functions at these promoters. The observation that the SNAP190 Myb domain facilitates formation of a complex containing TBP and the TFIIIB factor Brf2 (see Fig. 7) is consistent with this idea. One possibility is that the SNAP190 Myb RcRd repeats may function through direct protein interactions with factors in the TFIIIB complex other than TBP. However, the SNAP190 Myb RcRd domain alone does not recruit Brf2 efficiently, suggesting that higher order complex formation observed for SNAP190 RcRd with Brf2 and TBP is mediated predominantly by the SNAP190-TBP interaction. It is interesting that mini-$SNAP_c$ containing SNAP190 (1–505), SNAP43, and SNAP50 can recruit Brf2 in a TATA box-independent fashion (see Fig. 6). This observation suggests that additional cross-talk between Brf2 and other regions of $SNAP_c$ is likely important for efficient complex assembly with TFIIIB factors. If so, these regions must reside within SNAP43, SNAP50, or SNAP190 (1–505) because these proteins are sufficient to reconstitute $SNAP_c$ function *in vitro*. In addition, the strict spacing arrangement between the PSE and TATA box may select which TBP complex can bind to the U6 promoter. Because the two DNA elements must be positioned close to each other to allow interactions between the DNA binding domains of SNAP190 and TBP, their close proximity might present steric limitations to which TBP·TAF complex can recognize the U6 TATA box. For example, the RNA polymerase II-specific TFIID complex that contains TBP and at least 10 TAFs may be unable to contact the U6 TATA box when $SNAP_c$ is bound to the PSE. In contrast, a smaller complex such as one containing TBP, Bdp1, and a Brf protein might present a better fit.

Assembly of an RNA Polymerase III Transcription Complex at Human U6 snRNA Promoters—The upstream DSE in human U6 snRNA promoters contains an octamer element that binds the transcription factor Oct-1. Once bound to the octamer element the Oct-1 POU DNA binding domain interacts directly with the SNAP190 protein to recruit $SNAP_c$ to the U6 PSE (43, 49, 50). In the context of the chromosome, $SNAP_c$ recruitment is dependent upon a nucleosome positioned between the octamer site and the PSE (51, 52) which effectively loops out the intervening DNA to juxtapose these promoter elements. When the nucleosome is absent, the POU domain is unable to recruit

$SNAP_c$ probably because the distance between the two sites is too great for the POU domain to stabilize $SNAP_c$ binding (51). After or concomitant with $SNAP_c$ binding to the PSE, an RNA polymerase III-specific TBP complex is recruited to the U6 TATA box. It seems unlikely that the TATA box alone is sufficient to recruit a specific TBP complex. Indeed, TBP can efficiently recruit Brf2 to a TATA box; however, this recruitment is not specific for the U6 TATA box (33). In contrast, $SNAP_c$ is sufficient to recruit and stabilize TBP binding to the U6 TATA box (Fig. 1 and Ref. 43), and the SNAP190 Myb RcRd DNA binding domain alone is sufficient for TBP and TBP·Brf2 recruitment via interactions with the TBP core region. Additional interactions involving amino acids 34–83 of SNAP190 with TBP may bolster TBP recruitment to the U6 TATA box (45). These observations suggest a model for the $SNAP_c$-mediated recruitment of a snRNA-specific TFIIIB complex to the U6 snRNA promoter as shown in Fig. 8. Binding of $SNAP_c$ to the U6 PSE leads to recruitment of TBP through interactions with the SNAP190 Myb RcRd DNA binding domain and Brf2 through interactions with other $SNAP_c$ proteins. Binding of TBP and Brf2 to the TATA box further stabilizes $SNAP_c$ on the U6 promoter leading to formation of a stable preinitiation complex. Thus, the unique arrangement of core promoter elements at the U6 gene coordinates interactions involving $SNAP_c$, TBP, and Brf2 for assembly of a RNA polymerase III-specific preinitiation complex.

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