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Cloning and characterization of SNAP50, a subunit of the snRNA-activating protein complex $SNAP_c$

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The human RNA polymerase II and III snRNA promoters share a common basal element, the proximal sequence element (PSE), which is recognized by a complex we refer to as the snRNA-activating protein complex ($SNAP_c$). Biochemical purifications suggest that $SNAP_c$ is composed of at least four polypeptides of 43, 45, 50 and 190 kDa, as well as variable amounts of the TATA box binding protein, TBP. cDNAs encoding the 43 and 45 kDa subunits, SNAP43 and SNAP45, have been isolated, but there is no evidence that either of these subunits contacts DNA. Here we report the isolation of cDNAs encoding the 50 kDa subunit of $SNAP_c$, SNAP50. The open reading frame predicts a 411 amino acid protein, which contains two potential zinc finger motifs. Depletions with anti-SNAP50 antibodies inhibit RNA polymerase II and III snRNA gene transcription *in vitro*. SNAP50 interacts with SNAP43 in co-immunoprecipitation experiments, but not with SNAP45 or TBP. UV cross-linking experiments suggest that SNAP50 contacts DNA in the SNAP complex. These results are consistent with the same core SNAP complex recognizing the PSEs of RNA polymerase II and III snRNA promoters, and provide an initial view of the architecture of the SNAP complex.

Keywords: proximal sequence element/RNA polymerase/SNAP50/snRNA transcription/TBP

Introduction

Transcription in eukaryotic cells is accomplished by three different RNA polymerases, which are specialized in the transcription of different subsets of genes. None of these enzymes can recognize their target promoter sequences directly. Rather, they are recruited to the promoter with the aid of polymerase-specific accessory transcription factors, some of which bind directly to promoter elements. However, each type of pre-initiation complex contains a common factor, the TATA box binding protein TBP.

TBP is recruited to RNA polymerase I, II and III promoters as part of different complexes (reviewed in Hernandez, 1993; Goodrich and Tjian, 1994; Struhl, 1994; Zawel and Reinberg, 1995; Burley and Roeder, 1996). The RNA polymerase II TBP-containing complex, TFIID, is composed of TBP and at least eight TBP-associated factors or TAFs. TBP, as part of TFIID, directly recognizes the TATA box of many mRNA-type promoters and, *in vitro*, this event initiates the assembly of the general RNA polymerase II transcription factors and RNA polymerase II into a functional initiation complex. TFIID is also required for the transcription of those mRNA promoters that lack TATA boxes, but in these cases it is less clear how TFIID is recruited to the DNA (for reviews, see Weis and Reinberg, 1992; Smale, 1994). However, there is evidence that components of TFIID can contact sequences overlapping the start site of transcription (Kaufman and Smale, 1994; Purnell *et al.*, 1994; for reviews, see Weis and Reinberg, 1992; Smale, 1994), and recombinant $TAF_{II}150$ from *Drosophila* can recognize promoter sequences directly (Verrijzer *et al.*, 1994, 1995).

The RNA polymerase I TBP-containing complex SL1 consists of TBP and three TAFs. SL1 on its own has little affinity for the RNA polymerase I core promoter element, the USE. It is recruited to the USE with the help of the upstream sequence factor (USF), which binds to the upstream portion of the USE. Which SL1 subunits contact the DNA in the USF–SL1 complex is not known (reviewed in Hernandez, 1993; Goodrich and Tjian, 1994; Struhl, 1994; Zawel and Reinberg, 1995; Burley and Roeder, 1996). The RNA polymerase III TBP-containing complex TFIIIB consists, in yeast, of TBP, a tightly associated TAF (Brf, Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Lopez-De-Leon *et al.*, 1992) and a loosely associated TAF (TFC5, Kassavetis *et al.*, 1995; Roberts *et al.*, 1996; Ruth *et al.*, 1996) that can easily be dissociated from the complex (Kassavetis *et al.*, 1991; Huet *et al.*, 1994). *In vitro*, yeast TFIIIB can associate with the DNA either through protein–protein interactions with the DNA binding transcription factor TFIIIC or, in the TATA-containing RNA polymerase III promoter, through a TFIIIC-independent pathway that involves recognition of the TATA box by the TBP subunit of TFIIIB. In both cases, however, Brf and TFC5 also contact the DNA, although with little sequence specificity (Huet *et al.*, 1994; Joazeiro *et al.*, 1994, 1996; Kassavetis *et al.*, 1994; Whitehall *et al.*, 1995). Much less is known about human TFIIIB, although it is clear that it contains TBP and a tightly associated homolog of yeast Brf (Wang and Roeder, 1995; Mital *et al.*, 1996).

The human snRNA promoters are highly unusual. Some recruit RNA polymerase II while others recruit RNA polymerase III, yet they are more similar to each other than they are to either typical RNA polymerase II or RNA polymerase III promoters (reviewed in Hernandez, 1992; Lobo and Hernandez, 1994). The RNA polymerase II snRNA promoters, such as the U1 and U2 promoters, contain essentially one basal promoter element, the proximal sequence element (PSE). The RNA polymerase

III U6 snRNA promoter also contains a PSE and, in addition, a TATA box. The PSEs of the RNA polymerase II and III snRNA promoters are interchangeable, which suggests that they may recruit the same factor. RNA polymerase specificity is determined, in large part, by the presence or absence of the TATA box (Mattaj *et al.*, 1988; Lobo and Hernandez, 1989).

Transcription from both the RNA polymerase II and III human snRNA promoters requires TBP (Lobo *et al.*, 1991; Simmen *et al.*, 1991; Bernues *et al.*, 1993; Sadowski *et al.*, 1993), but how TBP is recruited to these promoters is not clear. An activity that recognizes specifically the PSE was first detected by Waldschmidt *et al.* (1991) and named the PSE binding protein (PBP). Subsequently, PSE binding activities named PTF (Murphy *et al.*, 1992) and $SNAP_c$ (Sadowski *et al.*, 1993) were identified. Both PTF and $SNAP_c$ contain four subunits of 43, 45, 50 and ~190 kDa (Henry *et al.*, 1995; Yoon *et al.*, 1995). The 43 and 45 kDa subunits have been cloned from both complexes and found to be identical (Henry *et al.*, 1995; Sadowski *et al.*, 1996; Yoon and Roeder, 1996). However, unlike PTF, $SNAP_c$ preparations contain various amounts of TBP (Sadowski *et al.*, 1993; Henry *et al.*, 1995), suggesting that a fraction of the SNAP complexes contains TBP. Indeed, immunodepletion of a transcription extract with an anti-TBP antibody inhibits U1 and U2 transcription, and transcription can be reconstituted very efficiently with $SNAP_c$ fractions (Sadowski *et al.*, 1993). On the RNA polymerase III U6 promoter, $SNAP_c$ and TBP bind highly cooperatively to the PSE and the TATA box, respectively (Goomer *et al.*, 1994; V.Mittal and N.Hernandez, unpublished results), suggesting that, in this case, TBP is recruited through interactions with both $SNAP_c$ and the DNA.

To understand exactly how TBP is recruited to RNA polymerase II and III snRNA promoters, and how this might result in the recruitment of different RNA polymerases, we have begun the systematic characterization of all the subunits of $SNAP_c$. Here we report the isolation of cDNAs encoding the 50 kDa subunit of $SNAP_c$ and the initial characterization of this protein. We show that, as for SNAP43 and SNAP45, immunodepletions with anti-SNAP50 antibodies inhibit both RNA polymerase II and III snRNA gene transcription *in vitro*. This suggests that the SNAP complexes that recognize the PSE in the RNA polymerase II and III snRNA promoters contain at least three common subunits. Co-immunoprecipitation and UV cross-linking experiments suggest that SNAP50 directly associates with SNAP43 but not with SNAP45 or TBP, and contacts DNA within the SNAP complex.

Results

Isolation of cDNAs encoding SNAP50

The purification of $SNAP_c$ has been described previously (Henry *et al.*, 1995). We obtained protein sequence information for several peptides derived from the protein band migrating with an apparent M_r of ~50 kDa. We first cloned a cDNA encoding some of these peptides, but further experiments revealed that this open reading frame (ORF) did not encode a subunit of $SNAP_c$. Database searches then revealed a partial cDNA sequence (GenBank accession No. T19314) containing two of the four

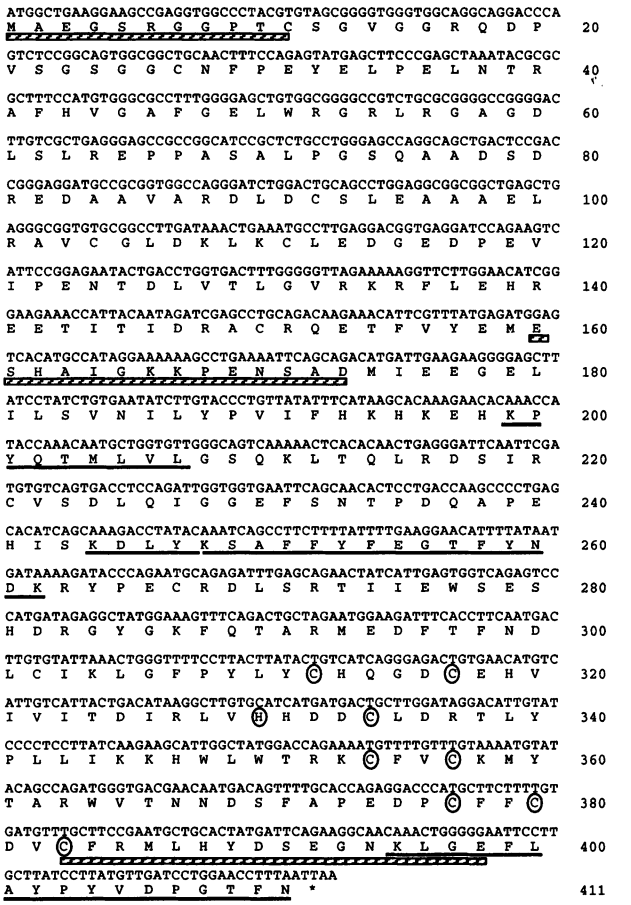

Fig. 1. Nucleotide sequence and predicted amino acid sequence for SNAP50. The peptide sequences obtained from the purified protein are indicated by single underlines. The sequences corresponding to the synthetic peptides used to raise rabbit polyclonal antibodies are shown as stippled underlines (C-terminal peptide, CSH482; internal peptide, CSH483; N-terminal peptide, CSH484). The cysteines and histidines of the putative zinc fingers are circled. The database sequence accession No. is U71300.

remaining peptides, which we used to design oligonucleotide primers for PCR from human cDNA. A specific amplification product was used to select six λ phage recombinants. DNA sequence analysis revealed an ORF encoding a putative protein of 411 amino acids with a calculated M_r of 47 kDa. The amino acid sequence is shown in Figure 1, and contains the four peptides we microsequenced (underlined with single lines) that were not encoded by the first p50 cDNA we isolated. Database searches revealed the 411 amino acid protein to have little similarity to other characterized protein sequences contained in the databases. It is an extremely charged protein with a predicted isoelectric point of 4.90. At pH 7.4, the p50 protein is predicted to have a total net charge of –22.3, in sharp contrast to the predicted charge of –4.1 for SNAP45 and +17.0 for SNAP43 at the same pH. Motif analysis of the amino acid sequence revealed a cysteine–histidine cluster with two potential zinc finger motifs (for review, see Berg and Shi, 1996) in the carboxy-terminal region of the protein. Zinc finger 1 (amino acids 312–334) is a C2HC type reminiscent of the TFIIIA family of zinc fingers, but with a histidine instead of a cysteine at the third potential metal coordination site. Zinc

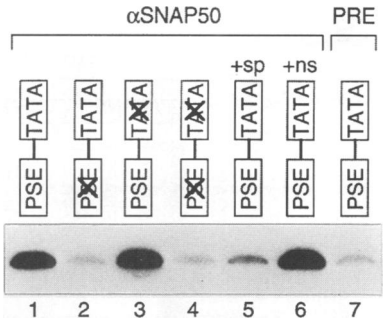

Fig. 2. SNAP50 is a component of the SNAP complex. DNA is co-immunoprecipitated specifically by anti-SNAP50 antibodies when $SNAP_c$ is bound to the PSE. Fractions enriched for $SNAP_c$ were bound to ^{32}P-radiolabeled probes containing either a wild-type or mutant PSE sequence in combination with a wild-type or mutant TATA box sequence as indicated above the lanes. Then 20 μl binding reactions were diluted to 200 μl with buffer D and immunoprecipitated using 20 μl of anti-SNAP50 antibody beads in the absence (lanes 1–4) or presence of specific (CSH482, lane 5) or non-specific (CSH483, lane 6) peptide. Immunoprecipitations were also performed using 20 μl of rabbit pre-immune antibody beads (lane 7). Antibody beads were washed extensively and subsequently boiled in formamide loading buffer to release bound DNA. Samples were fractionated on a 6% polyacrylamide + 8 M urea gel and the results observed by autoradiography.

finger 2 (amino acids 354–383) has a C-X2-C-X19-C-X2-C-X2-C motif. Neither zinc finger shows general sequence conservation with other previously described zinc finger proteins contained in protein databases.

To determine whether the p50 protein encodes a member of the SNAP complex, we raised antibodies directed against peptides derived from the predicted amino acid sequence (stippled underlining, Figure 1). Rabbit pre-immune serum and anti-p50 sera were tested in an electrophoretic mobility shift assay (EMSA) as previously described (Henry *et al.*, 1995), but the anti-p50 antibodies disrupted rather than supershifted the SNAP complex (data not shown). To obtain more convincing evidence that p50 is part of $SNAP_c$, we therefore coupled the antibodies to protein A–Sepharose beads, and used them to immunoprecipitate $SNAP_c$ from DNA binding reactions performed with purified $SNAP_c$ (mono Q fraction, Henry *et al.*, 1995) and radiolabeled probes containing wild-type or mutant PSE and TATA box. The precipitates were analyzed for the presence of radiolabeled DNA by polyacrylamide gel electrophoresis (McKay, 1981; see Materials and methods). As shown in Figure 2, the anti-p50 immunoprecipitates contained much more radiolabeled DNA (9-fold more) when the binding reaction was performed with a wild-type PSE probe than when it was performed with a mutant PSE probe (compare lanes 1 and 3 with lanes 2 and 4). In contrast, the presence of a functional or mutated TATA box had no significant effect on the amount of co-precipitated DNA (compare lanes 1 and 3). The effect was blocked by pre-incubation of the anti-p50 antibodies with specific peptide (CSH482, lane 5), but not by pre-incubation with non-specific peptide (lane 6). Additionally, rabbit pre-immune antibodies could not precipitate DNA effectively (lane 7). These results indicate that the cDNA clones obtained encode a protein, present in the biochemically purified $SNAP_c$ fractions, that associates (directly or indirectly) with the PSE. This strongly suggests that

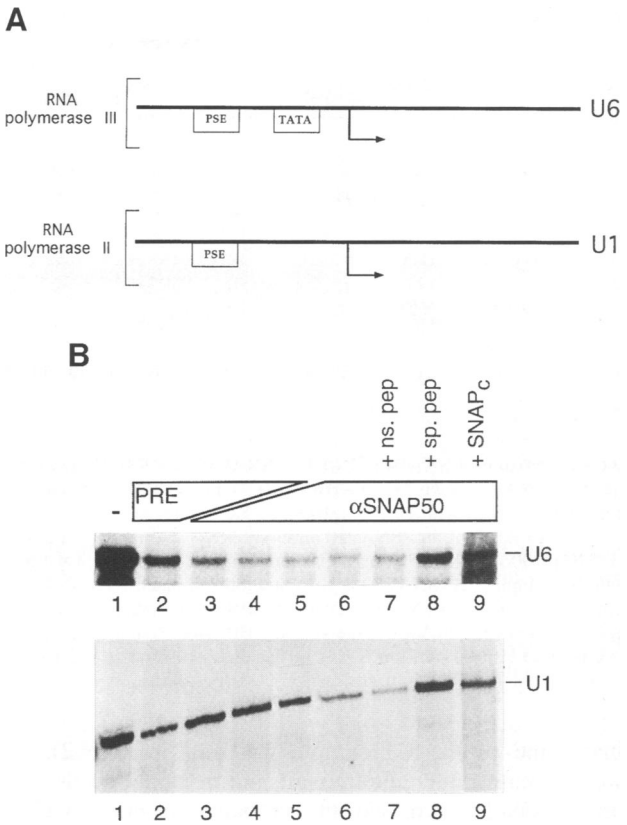

Fig. 3. SNAP50 is required for transcription by both RNA polymerases III and II. (**A**) Promoter schematic showing basal promoter elements for the human U6 and U1 snRNA genes. (**B**) Whole cell extracts were tested for their ability to support U6 transcription by RNA polymerase III or U1 transcription by RNA polymerase II (lane 1). Forty μl of whole cell extracts were depleted with a total of 40 μl of antibody beads containing only pre-immune antibody beads (lane 2) or pre-immune beads and 10 (lane 3), 20 (lane 4), 30 (lane 5) or only 40 μl of anti-SNAP50 antibody beads (lane 6). Depletions with the highest amount of anti-SNAP50 beads were also performed in the presence of 4 μl of non-specific peptide (CSH483, lane 7) or specific peptide (CSH482, lane 8), each at 1 mg/ml. In lane 9, whole cell extracts depleted with 40 μl of anti-SNAP50 beads were complemented with 4 μl of purified $SNAP_c$. For the U1 transcription experiments, depletions were done only once. For the U6 transcription experiments, depleted extracts were subjected to a second round of depletion under similar conditions as described. The bands labeled U1 and U6 correspond to the correctly initiated transcripts.

p50 is a member of the SNAP complex. We therefore refer to this protein as SNAP50.

SNAP50 is required for both RNA polymerase II and III snRNA gene transcription

To determine whether SNAP complexes required for RNA polymerase II and III snRNA gene transcription contain SNAP50, we depleted an extract with anti-SNAP50 antibodies covalently coupled to protein A–Sepharose beads and tested these depleted extracts for RNA polymerase II transcription from the U1 snRNA promoter and RNA polymerase III transcription from the U6 snRNA promoter. These promoters are depicted schematically in Figure 3A; they both contain a PSE, and the RNA polymerase III U6 promoter contains, in addition, a TATA box. As shown in Figure 3B, treatment of the extracts with pre-immune beads resulted in a non-specific inhibition of U6 transcription but had little effect on U1 transcription (lanes 1 and 2, note

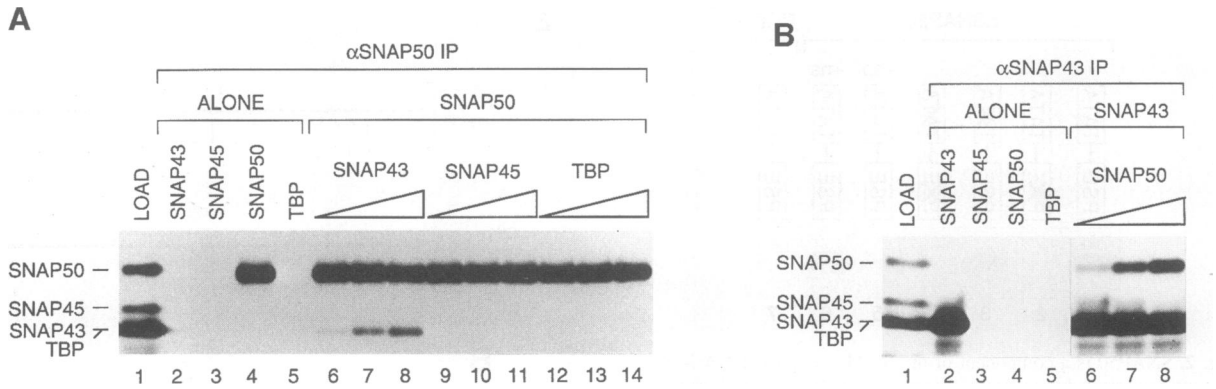

Fig. 4. SNAP50 associates with SNAP43 *in vitro*. (**A**) SNAP43 is co-immunoprecipitated with SNAP50. Antibodies directed against SNAP50 were tested for their ability to co-immunoprecipitate *in vitro* translated SNAP43, SNAP45 and TBP in the presence of *in vitro* translated SNAP50. Lane 1 shows a mixture of SNAP43, SNAP45, SNAP50 and TBP (3 μl each) to indicate the relative mobilities of these proteins. Ten μl of SNAP43 (lane 2), SNAP45 (lane 3), SNAP50 (lane 4) and TBP (lane 5) alone, or 3, 10 or 30 μl of each protein plus 10 μl of SNAP50 (lanes 6–14) were adjusted to 200 μl with HEMGT/100 mM KCl buffer and incubated at 4°C for 2 h to allow for possible protein association. The reactions were then mixed with 30 μl of anti-SNAP50 antibody beads and incubated at 4°C for an additional 1 h. Antibody beads were then washed extensively with HEMGT/100 mM KCl buffer. Bound proteins were eluted in 1× Laemmli buffer and separated by 12.5% SDS–PAGE, and the results detected by autoradiography. (**B**) SNAP50 is co-immunoprecipitated with SNAP43. Antibodies directed against SNAP43 were tested for their ability to co-immunoprecipitate *in vitro* translated SNAP50 in the presence of *in vitro* translated SNAP43. Lane 1 is as in (A). Ten μl of SNAP43 (lane 2), SNAP45 (lane 3), SNAP50 (lane 4) and TBP (lane 5) alone, or 3, 10 or 30 μl of SNAP50 plus 10 μl of SNAP43 (lanes 6–8) were adjusted to 200 μl with HEMGT/100 mM KCl buffer and incubated at 4°C for 2 h to allow possible protein association. Reactions were then mixed with 30 μl of anti-SNAP43 antibody beads and processed as above.

that some of the U1 sample was lost in lane 2). As we decreased the amounts of pre-immune beads and proportionally increased the amounts of anti-SNAP50 beads such that the total bead volume was kept constant, we observed progressive inhibition of both U6 and U1 transcription (compare lanes 3–6 with lane 2). Although the effect was small, it was reproducible, and it is specific because (i) it is blocked by pre-incubation of the anti-SNAP50 beads with specific peptide (CSH482, lane 8), but not with non-specific peptide (CSH483, lane 7), and (ii) transcription could be restored to levels similar to those observed in extracts treated with pre-immune beads by addition of biochemically purified $SNAP_c$ (compare lane 9 with lanes 2 and 6). These results suggest that, as observed previously for SNAP43/PTFγ and SNAP45/PTFδ (Henry *et al.*, 1995; Sadowski *et al.*, 1996; Yoon *et al.*, 1996), SNAP50 is present in the SNAP complex required for snRNA gene transcription by RNA polymerases II and III. Thus, the SNAP complexes required for RNA polymerase II and III transcription of snRNA genes contain at least three common subunits.

SNAP50 associates with SNAP43

To begin to elucidate the architecture of the SNAP complex, we examined the ability of SNAP50 to interact with other members of $SNAP_c$ by co-immunoprecipitation. SNAP43, SNAP45, TBP and SNAP50 were expressed by *in vitro* translation in a reticulocyte lysate, and mixed in different pairs. As shown in Figure 4A, the anti-SNAP50 antibodies immunoprecipitated SNAP50 (lane 4), as expected, but they did not cross-react with either SNAP43 (lane 2), SNAP45 (lane 3) or TBP (lane 5). When SNAP50 was mixed with increasing amounts of SNAP43 before immunoprecipitation, increasing amounts of SNAP43 were co-immunoprecipitated with SNAP50 (lanes 6–8). In contrast, when it was mixed with increasing amounts of SNAP45 or TBP, no detectable SNAP45 or TBP was co-immunoprecipitated with SNAP50 (lanes 9–14). This suggested that SNAP50 associates with SNAP43. To confirm this, we performed a similar experiment, but with anti-SNAP43 antibodies directed against the C-terminus of the protein (Henry *et al.*, 1995). As expected and shown in Figure 4B, these antibodies immunoprecipitated SNAP43 (lane 2), but not SNAP45, SNAP50 or TBP (lanes 3–5). However, when increasing amounts of SNAP50 were mixed with SNAP43 before immunoprecipitation, increasing amounts of SNAP50 were co-immunoprecipitated with SNAP43 by the anti-SNAP43 antibodies (lanes 6–8). Together, these results strongly suggest that SNAP43 and SNAP50 associate directly *in vitro* and may, therefore, be in direct contact within the SNAP complex.

A 53 kDa band can be cross-linked specifically to the PSE

To examine whether SNAP50 might contact DNA when $SNAP_c$ is bound to the PSE, we performed a binding reaction with wild-type or mutant PSE radiolabeled probes substituted with bromodeoxyuridine, and exposed the binding reactions to UV light, as described previously (Sadowski *et al.*, 1993). The DNA was then digested extensively with DNase I and micrococcal nuclease, and the DNA-cross-linked proteins were separated by SDS–PAGE and visualized by autoradiography. As shown in Figure 5A, we observed efficient cross-linking of a 53 kDa polypeptide with a probe containing the wild-type PSE (lane 1), but not a mutant PSE (lane 2). In addition, cross-linking was strongly reduced when the binding reaction was performed in the presence of an excess of unlabeled wild-type PSE competitor DNA (lane 3), but was unaffected in the presence of an excess of unlabeled mutant PSE competitor DNA (lane 4).

To determine whether the 53 kDa polypeptide might correspond to SNAP50, we performed the following experiment. We first bound $SNAP_c$ to the PSE and UV cross-linked the complex to the DNA as above. The cross-linked complexes were then used as the starting material

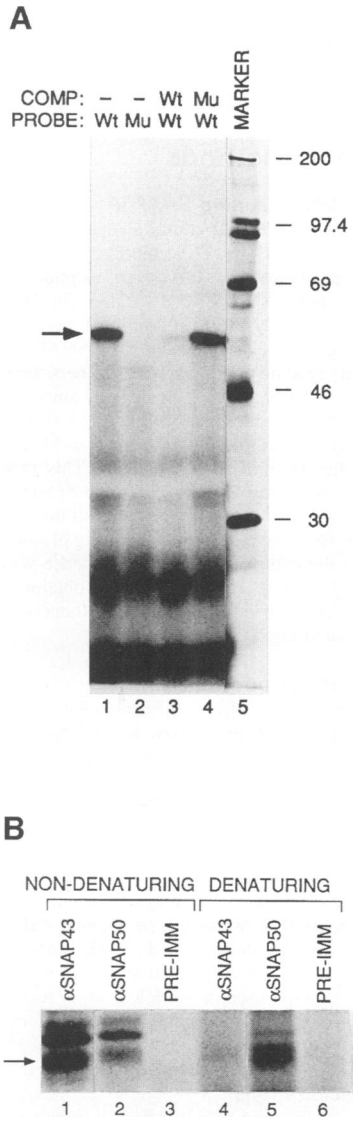

Fig. 5. SNAP50 contacts DNA when $SNAP_c$ is bound to the PSE. (**A**) A polypeptide of ~53 kDa can be UV cross-linked specifically to the PSE. Homogeneously labeled probes containing either the wild-type mouse U6 PSE (WT) or a mutated PSE (MU) were substituted with bromodeoxyuridine. Forty μl of DNA binding reactions were performed using fractions enriched in $SNAP_c$ (mono-Q, 15 μl) and WT probe (lane 1) or MU probe (lane 2), or WT probe in the presence of excess wild-type (WT, lane 3) or mutant (MU, lane 4) competitor DNAs. Proteins were cross-linked to DNA with UV light (230 nm, 200 mJ), digested with DNase I (20 U) and micrococcal nuclease (2 U), and size fractionated by 12.5% SDS–PAGE. The results were visualized by autoradiography. Lane 5 contains protein size markers. The 53 kDa protein specifically cross-linked to the PSE is indicated by an arrow. (**B**) The 53 kDa polypeptide is specifically immunoprecipitated by anti-SNAP50 antibodies. Eighty μl DNA binding and UV cross-linking reactions were performed with the wild-type U6 PSE probes as above. Non-denaturing (lanes 1–3) or denaturing (lanes 4–6) immunoprecipitation reactions were performed using anti-SNAP43 (lanes 1 and 4), anti-SNAP50 (lanes 2 and 5) or pre-immune (lanes 3 and 6) antibody beads. Beads were washed extensively with buffer D and resuspended in 40 μl of buffer D containing DNase I (2.5 U) and micrococcal nuclease (1 U). After incubating the samples at 37°C for 1 h, antibody beads were washed extensively with buffer D and bound proteins were eluted by boiling the beads in 30 μl of 1× Laemmli buffer. The eluates were separated by 12.5% SDS–PAGE and the results observed by autoradiography. A complex pattern of eluted polypeptides was observed and only the region surrounding the 53 kDa size range is shown. The 53 kDa polypeptide is indicated with an arrow.

for immunoprecipitations with either anti-SNAP43, anti-SNAP50 or pre-immune antibody beads, under both native and strongly denaturing conditions. The samples were then treated with DNase I and micrococcal nuclease as in a regular cross-linking experiment, the beads were washed extensively and the proteins bound to the beads were eluted by boiling in Laemmli buffer. The eluates were fractionated by SDS–PAGE, and the proteins cross-linked to DNA (and therefore radioactive) were visualized by autoradiography. We observed a complex pattern of polypeptides, and only the region of the gel surrounding the 53 kDa protein is shown in Figure 5B. Under non-denaturing conditions, the 53 kDa protein was immunoprecipitated by both anti-SNAP43 and anti-SNAP50 antibodies (lanes 1 and 2, respectively; band indicated by an arrow) but not by pre-immune antibodies (lane 3), as expected if this polypeptide is part of the SNAP complex. Significantly, however, when the immunoprecipitations were performed under strongly denaturing conditions that disrupt the SNAP complex (65°C, 1% SDS), the 53 kDa polypeptide was immunoprecipitated by the anti-SNAP50 antibody (lane 5) but not by the anti-SNAP43 antibody (lane 4), nor the pre-immune antibodies (lane 6). This result strongly suggests that the cross-linked 53 kDa polypeptide is recognized directly by the anti-SNAP50 antibody, and therefore indeed corresponds to SNAP50. Together, these results suggest that SNAP50 is in close proximity to the DNA when $SNAP_c$ is bound to the PSE.

Discussion

We have isolated a cDNA encoding the SNAP50 subunit of the SNAP complex. SNAP50 has little homology to other proteins, but it contains a potential zinc finger domain at the carboxy-terminus, which may be involved in protein–DNA and/or protein–protein interactions.

We find by co-immunoprecipitation experiments using recombinant proteins that SNAP50 can interact directly with SNAP43 (Figure 4A and B). SNAP50 is very negatively charged at physiological pH (charge of –22.3 at pH 7.4), whereas SNAP43 is very positively charged (+17.0 at pH 7.4), raising the possibility that the interaction is not specific. We do not consider this likely, however, because TBP is also very positively charged (+16 at pH 7.4) and yet does not interact with SNAP50 in the same assay. Thus, it is likely that the interaction we observe is specific and reflects the architecture of SNAP50 and SNAP43 within the SNAP complex.

By UV cross-linking experiments combined with immunoprecipitations, we show that SNAP50 is in proximity to the DNA when $SNAP_c$ is bound to the PSE. This result is consistent with our previous UV cross-linking experiments (Sadowski *et al.*, 1993), in which we observed three major bands, the smallest of which migrated with an apparent mol. wt of ~53 kDa. In the cross-linking experiment shown here (Figure 5A), we obtain a single major 53 kDa band. Thus, although the exact pattern differs, perhaps due to incomplete DNase I digestion in our previous experiment, we consistently obtain a 53 kDa band. This band is likely to consist, at least in part, of SNAP50, because, after UV cross-linking, we can immunoprecipitate a 53 kDa polypeptide with anti-

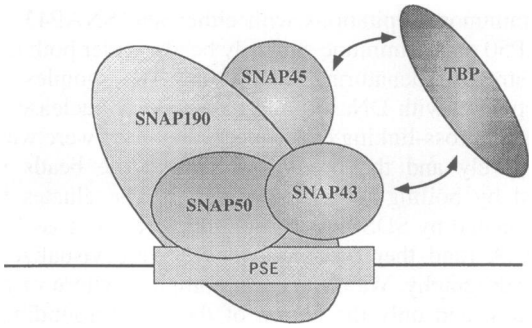

Fig. 6. Model of the SNAP complex bound to the PSE. Both the TATA-containing and TATA-less snRNA genes contain a PSE to which $SNAP_c$ binds. Presumably, the binding of $SNAP_c$ to the PSE is a crucial early step in the assembly of the pre-initiation complex and allows recruitment of RNA polymerase II to, for example, the U1 and U2 snRNA promoters, and RNA polymerase III to the U6 snRNA promoter. SNAP50 can be cross-linked to the DNA; however, SNAP190 probably is also required for specific DNA binding (Yoon *et al.*, 1995; our unpublished results). SNAP50 interacts strongly with SNAP43. Both SNAP43 and SNAP45 can interact with TBP (Henry *et al.*, 1995; Sadowski *et al.*, 1996; Yoon and Roeder, 1996). These interactions with TBP may be important for stabilizing TBP as part of the SNAP complex. Alternatively, they may be involved in interacting with TBP bound to the TATA box (V.Mittal and N.Hernandez, unpublished results).

SNAP50 antibodies under strongly denaturing conditions that disrupt the SNAP complex (Figure 5B).

The observation that SNAP50 can be cross-linked to the PSE differs from the results of Yoon *et al.* (1995), who detected strong cross-linking of a single protein of ~180 kDa, suggesting that PTFα/SNAP190 is binding to DNA. This possibility is consistent with the failure of recombinant SNAP50 to bind independently to DNA in a PSE-specific manner, either alone or in combination with SNAP43, SNAP45 or TBP (data not shown). Thus, we suspect that both SNAP50 and SNAP190 contact DNA. Our failure to detect cross-linking of SNAP190 to the DNA may reflect protein degradation; indeed, although the 53 kDa band clearly contains SNAP50, we cannot exclude that it contains, in addition, a proteolytic fragment derived from SNAP190. Another possibility, however, is that this difference reflects different UV cross-linking conditions. Yoon *et al.* (1995) performed their UV cross-linking experiments with a probe containing a PSE and an octamer site, in the presence of both PTF and the transcriptional activator Oct-1. In contrast, Oct-1 was not present in our experiments. Perhaps $PTF/SNAP_c$ assumes different conformations on the DNA in the presence or absence of Oct-1.

Our present view of the architecture of the SNAP complex bound to the PSE is shown in Figure 6. In this model, SNAP190 is contacting the promoter. Additional DNA contacts are provided by SNAP50, which also interacts with SNAP43, possibly stabilizing the complex on the PSE. A small fraction of $SNAP_c$ also contains TBP, and TBP interacts with both SNAP43/PTFγ and SNAP45/PTFδ in GST pulldown (Henry *et al.*, 1995; Sadowski *et al.*, 1996) and immunoprecipitation (Yoon and Roeder, 1996) assays, suggesting that TBP can associate with the complex. However, because the RNA polymerase II and III snRNA promoters differ by the presence of a TATA box, the configuration of TBP in the RNA polymerase II and III snRNA initiation complexes is most probably different. This may, in turn, determine RNA polymerase specificity.

Materials and methods

Isolation of cDNAs encoding SNAP50

The purification of $SNAP_c$ and amino acid sequencing was described previously (Henry *et al.*, 1995). The amino acid sequence obtained from the 50 kDa protein revealed a mixture of peptides derived from an unrelated protein and SNAP50. The cDNAs for both proteins were obtained and that for SNAP50 is described here. A partial cDNA sequence encoding two of the peptides from SNAP50 was detected in database searches. Specific oligonucleotide primers were designed based on this sequence and were used in a PCR to amplify a 371 bp DNA fragment from cDNA prepared from total RNA from HeLa cells as a template. This fragment was used to prepare a [α-^{32}P]dCTP-radiolabeled DNA probe by the random primer method. This probe was used to screen ~750 000 phage recombinants of a λgt10 human cDNA library prepared from a human fetal teratocarcinoma cell line (Skowronski *et al.*, 1988). The inserts from six positively hybridizing plaques were subcloned into pUC118 and the complete nucleotide sequence was determined for both strands of two clones. The cDNA clones obtained differed only in the amount of N-terminal sequence of SNAP50 encoded and the length of the 3′-untranslated region.

Generation of anti-peptide antibodies

Synthetic peptides derived from the predicted amino sequence for SNAP50 (see Figure 1) were coupled to keyhole limpet hemocyanin (Pierce) and injected into rabbits to generate polyclonal anti-peptide antibodies. Rabbit antisera were tested in an EMSA as previously described (Sadowski *et al.*, 1993, data not shown) and also in a McKay assay (see below and Figure 2).

McKay assay

Fractions enriched for $SNAP_c$ (mono-Q peak) were bound to γ-^{32}P-end-labeled probes containing wild-type or mutant PSE and TATA box sequences as described (Sadowski *et al.*, 1993). Rabbit anti-SNAP50 or pre-immune antibodies were conjugated to protein A–Sepharose (Harlow and Lane, 1988) and were used in an McKay assay reaction as previously described (McKay, 1981).

Expression of recombinant proteins and co-immunoprecipitations

TBP, SNAP43, SNAP45 and SNAP50 coding sequences were amplified by PCR with *Pfu* polymerase (Stratagene). The amplification products were ligated into the pCITE-2a(+) vector (Novagen) to generate the constructs pCITE-TBP, pCITE-SNAP43, pCITE-SNAP45 and pCITE-SNAP50, respectively. Supercoiled plasmid (1 μg) for each construct was used as template for coupled *in vitro* transcription and translation reactions (TnT, Promega) in a final volume of 100 μl containing 8 μl of L-[^{35}S]methionine (1233 Ci/mmol; NEN). For immunoprecipitation control experiments, 10 μl of labeled proteins were added to 190 μl of HEMGT/100 mM KCl buffer (Sadowski *et al.*, 1996) and were incubated at 4°C for 2 h. Ten μl of anti-SNAP50 (rabbit CS303) or anti-SNAP43 (rabbit CS49) antibodies coupled to protein A–Sepharose beads were then added and the reactions were incubated at 4°C for 1 h. Antibody beads were then washed extensively with HEMGT/100 mM KCl and bound proteins were eluted by boiling in 1× Laemmli buffer. For co-immunoprecipitation experiments, 10 μl of labeled primary target protein were mixed with 3, 10 or 30 μl of labeled secondary target protein. Reactions were adjusted to 200 μl final volume with HEMGT/100 mM KCl, incubated at 4°C for 2 h and the samples were processed as described above. Eluted proteins were fractionated by 12.5% SDS–PAGE and visualized by autoradiography.

Immunodepletions and in vitro transcription assays

Rabbit pre-immune or anti-SNAP50 antibodies were covalently cross-linked to protein A–Sepharose beads as described. Whole cell extracts were mixed with an equal volume of pre-immune beads or anti-SNAP50 beads. Alternatively, pre-immune beads were mixed with anti-SNAP50 beads at ratios of 3:1, 1:1 or 1:3, respectively and these were used to deplete whole cell extracts. Depletions using the highest amount of anti-SNAP50 beads were also performed in the presence of a specific blocking peptide (CSH482) or an irrelevant peptide (CSH483). For U6

transcription, depletions were repeated a second time. Extracts were then tested for their ability to support U6 transcription from the U6 (pU6/Hae/RA.2) and U1 (pU1/S1 G$^-$) promoters (Lobo *et al.*, 1992; Sadowski *et al.*, 1993).

UV cross-linking

Fractions of 15 µl enriched for $SNAP_c$ (mono-Q peak, Henry *et al.*, 1995) were incubated with bromodeoxyuridine triphosphate-substituted probes homogeneously labeled with [^{32}P]dATP and [^{32}P]dGTP. Probe and unlabeled competitor DNAs contained either a wild-type mouse U6 PSE or mutant mouse U6 PSE. Cross-linking was performed using 200 mJ of UV light (230 nm) in a Stratalinker (Stratagene). The experiment was performed as previously described (Sadowski *et al.*, 1993) except that fractions enriched in $SNAP_c$ were prepared from HeLa cell nuclear extracts.

For immunoprecipitation experiments, 80 µl DNA binding reactions were performed using wild-type mouse U6 PSE probes as above. After cross-linking with UV light, 8 µl of buffer D was added to reactions to be used for non-denaturing immunoprecipitations and samples were incubated at room temperature for 15 min. Alternatively, for denaturing immunoprecipitation reactions, 8 µl of 10% SDS was added and the reactions were incubated at 65°C for 15 min. All reactions were then diluted to 1 ml with buffer D and 30 µl of anti-SNAP50, anti-SNAP43 or pre-immune antibody beads were added and the reactions incubated at room temperature for 1 h. The beads were washed extensively with buffer D and then resuspended in 60 µl of buffer D containing DNase I (2.5 U) and micrococcal nuclease (0.1 U) and were then incubated at 37°C for 1 h. The beads were washed extensively with buffer D, and the bound proteins were eluted by boiling the beads in 1× Laemmli buffer. The eluates were fractionated by 12.5% SDS–PAGE and the results were visualized by autoradiography.

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