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The Large Subunit of Basal Transcription Factor $SNAP_c$ Is a Myb Domain Protein That Interacts with Oct-1

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The human RNA polymerase II and III snRNA promoters have similar enhancers, the distal sequence elements (DSEs), and similar basal promoter elements, the proximal sequence elements (PSEs). The DSE, which contains an octamer motif, binds broadly expressed activator Oct-1. The PSE binds a multiprotein complex referred to as $SNAP_c$ or PTF. On DNAs containing both an octamer site and a PSE, Oct-1 and $SNAP_c$ bind cooperatively. $SNAP_c$ consists of at least four stably associated subunits, SNAP43, SNAP45, SNAP50, and SNAP190. None of the three small subunits, which have all been cloned, can bind to the PSE on their own. Here we report the isolation of cDNAs corresponding to the largest subunit of $SNAP_c$, SNAP190. SNAP190 contains an unusual Myb DNA binding domain consisting of four complete repeats (Ra to Rd) and a half repeat (Rh). A truncated protein consisting of the last two SNAP190 Myb repeats, Rc and Rd, can bind to the PSE, suggesting that the SNAP190 Myb domain contributes to recognition of the PSE by the SNAP complex. SNAP190 is required for snRNA gene transcription by both RNA polymerases II and III and interacts with SNAP45. In addition, SNAP190 interacts with Oct-1. Together, these results suggest that the largest subunit of the SNAP complex is involved in direct recognition of the PSE and is a target for the Oct-1 activator. They also provide an example of a basal transcription factor containing a Myb DNA binding domain.

The regulation of transcription initiation is mediated by the interplay between two classes of promoter elements: the basal promoter elements, which can be defined as those promoter elements sufficient to direct basal levels of transcription in vitro, and the regulatory elements, which modulate the levels of transcription. The basal elements are recognized by basal transcription factors, whereas the regulatory elements are recognized by either transcriptional activators or repressors. Eucaryotic activators are often modular, consisting of a DNA binding domain, which targets the activator to the correct promoter, and of activation domains, whose role is to enhance transcription (see references 21–23, 32, and 33 for reviews).

The human snRNA gene family contains both RNA polymerase II and RNA polymerase III genes. The RNA polymerase II snRNA promoters consist of a proximal sequence element (PSE), which is sufficient to direct basal levels of transcription in vitro, and a distal sequence element, which activates basal transcription. The RNA polymerase III snRNA promoters are similar, except that basal transcription is directed by the combination of a PSE and a TATA box (reviewed in reference 9). The PSE is recognized by a multisubunit complex called the SNAP complex ($SNAP_c$) (7) or PTF (34). Since $SNAP_c$ can bind to the PSE on its own, it corresponds to a sequence-specific DNA binding basal transcription factor. $SNAP_c$ contains at least four subunits, SNAP43, SNAP45, SNAP50, and SNAP190, and cDNAs encoding the SNAP43 (7) or PTF γ (35), SNAP45 (24) or PTF δ (35), and SNAP50 (6) or PTF β (2) subunits have been isolated. Cross-linking studies suggest that SNAP50 (6) and the largest subunit of the complex, SNAP190 (PTF α) (34), are in close contact with DNA. Recombinant SNAP50, however, does not bind to the PSE (6); thus, how $SNAP_c$ recognizes its target sequence is not yet understood.

Although $SNAP_c$ is capable of binding to the PSE on its own, its binding is strongly enhanced by the concomitant binding of at least two factors. On a basal RNA polymerase III promoter, containing both a PSE and a TATA box, $SNAP_c$ binds cooperatively with TBP, and this effect is dependent on the amino-terminal domain of TBP (16). And on DNAs containing an octamer site and a PSE, $SNAP_c$ binds cooperatively with the Oct-1 or Oct-2 POU domain (17) but not with the Pit-1 POU domain (15). Of all the amino acid differences between the Oct-1 and Pit-1 POU domains, a single one is the key determinant for the differential abilities of these two proteins to recruit $SNAP_c$ to the PSE (15). This effect contributes to efficient transcription in vitro and is largely independent of the Oct-1 activation domains, indicating that a function that is the hallmark of activation domains, namely, recruitment of a basal transcription complex resulting in activation of transcription, can be performed by a DNA-binding domain (3, 15). However, it remains to be determined whether cooperative binding results from a direct Oct-1 POU-$SNAP_c$ interaction and which subunit in $SNAP_c$ is contacted by the Oct-1 POU domain.

Here, we report the isolation of a cDNA encoding a 1,469-amino-acid protein that corresponds to full-length SNAP190. SNAP190 is an unusual Myb domain protein. Unlike most Myb domains from animal cells and plant cells, which contain three and two repeats, respectively, (see references 11 and 14 for reviews), SNAP190 contains a half repeat (Rh) followed by four complete repeats, Ra, Rb, Rc, and Rd. We show that just the Rc and Rd repeats are capable of recognizing the PSE, suggesting that the SNAP190 Myb domain contributes to spe-

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cific binding of the SNAP complex to the PSE. SNAP190 interacts with the SNAP45 subunit of SNAP$_c$ and with activator Oct-1. Our observations suggest that the SNAP complexes required for RNA polymerase II and III snRNA gene transcription contain at least four common subunits, provide further insight into the architecture of the complex, and identify SNAP190 as a target for activation by Oct-1.

MATERIALS AND METHODS

Purification of SNAP190 and isolation of corresponding cDNAs. The biochemical purification of SNAP$_c$ has been described previously (7). We also purified SNAP$_c$ by immunoprecipitation. An S100 extract from HeLa cells was first fractionated by ammonium sulfate precipitation. The material precipitating between 18 and 32% ammonium sulfate was resuspended in buffer D (20 mM HEPES [pH 7.9], 0.2 mM EDTA, 15% glycerol, 3 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) containing 100 mM KCl (D_{100}), 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 3 column volumes of buffer D_{100} and then step eluted with 5 column volumes of buffer D_{350}, followed by five column volumes of buffer D_{500}. Then, 80 ml of the 350 to 500 mM KCl elution step mixture (P11-C fraction) was directly used in nondenaturing immunoprecipitations with 4 ml of protein A-agarose beads coupled to an anti-SNAP43 antibody (α-CSH375 antibody) (7). The beads were washed extensively with $HTMG_{100}$ (20 mM HEPES [pH 7.9], 15% glycerol, 0.1% Tween 20, 5 mM $MgCl_2$, 100 mM KCl), and the bound material was then eluted twice in 12 ml of $HTMG_{60}$ containing 0.5-mg/ml specific peptide, CSH375, against which the antibody was raised. The eluate was further fractionated on a 200-μl Q-Sepharose column, and the fractions were tested in an electrophoretic mobility shift assay (EMSA) with a probe containing the mouse U6 PSE. The proteins in the fractions containing DNA binding activity were precipitated with trichloroacetic acid, redissolved in Laemmli buffer, and fractionated on a 5% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The protein band migrating with an apparent molecular mass of 190 kDa was excised from the gel and subjected to amino acid sequencing as described previously (7). Amino acid sequence information obtained both from protein purified by immunoaffinity and from protein purified by conventional chromatography (7) was used to design degenerate oligonucleotide primers for use in PCRs, with cDNA prepared from total HeLa cell RNA as the template. A p190-specific 156-nucleotide (nt) fragment (encoding amino acids [aa] E242 to K294) was obtained.

A partial cDNA encoding the carboxy-terminal half of SNAP190 and containing 3′ noncoding sequences, including a polyadenylation site and a run of A bases, was obtained in a screen related to the one-hybrid screen described in reference 29; the cDNA encoded aa I800 to V1469. This clone was used to screen a Namalwa cell cDNA library, and two more clones were obtained, K35#2 and K35#8, which extended the cDNA sequence upstream to the codon for aa Q422. To obtain 5′ missing sequences upstream of the codon for Q422, we performed PCR with a reverse primer corresponding to a region near the 5′ end of the longest cDNA clone, K35#8, and a forward primer corresponding to a region near the 3′ end of the 156-nt PCR fragment, with total HeLa cell cDNA as the template. We obtained a 500-bp fragment that was used to prepare an [α-^{32}P]dCTP (300 Ci/mmol; New England Nuclear)-radiolabled DNA probe by the random priming method. The probe was used to screen >750,000 phage recombinants of a λgt10 human stomach cDNA library (Clontech). Two positive clones (1711 and 1011) with insert sizes of about 1.1 kb were isolated; this extended the cDNA sequence up to the codon for aa E84.

We then designed nested oligonucleotide primers corresponding to regions near the 5′ end of the 1011 cDNA clone. These reverse primers were used in PCRs together with forward primers derived from vector arms from a variety of libraries. From a Namalwa cDNA library (29), a specific DNA fragment of approximately 500 bp was generated. The amplified PCR product was sequenced; this extended the sequence of the cDNA to the codon for a putative initiating methionine and a further 22 nt upstream. To ensure that this product was not due to some PCR artifact, we designed a forward primer corresponding to the 5′ end of the fragment and used it in a PCR with either of the primers corresponding to regions near the 5′ end of the 1011 cDNA, with cDNA made from total HeLa cell RNA as the template. Fragments with the expected sequences were obtained.

Because the 22 nt upstream of the codon for the putative initiating methionine corresponded to an open reading frame, we designed a second set of nested PCR primers for the region near the codon for the putative initiating methionine and repeated the PCR with forward primers derived from the vector arms from a variety of libraries. We obtained a specific fragment of about 300 nt (Nter fragment). The Nter fragment was sequenced; it was devoid of ATGs upstream of the putative initiating ATG but contained an in-frame stop codon starting 189 nt upstream of the putative initiating ATG. As before, PCR performed with a forward primer corresponding to the 5′ end of the Nter fragment and reverse primers derived from the region near the codon for the putative initiating methionine, with cDNA made from total HeLa cell RNA as the template, gave fragments with the expected sequences. A complete open reading frame encoding SNAP190 was reconstituted from the various overlapping cDNAs by a combination of PCR and conventional cloning techniques. All fragments derived from PCRs were resequenced to ensure that no errors had been introduced during the PCR.

Generation of antipeptide antibodies. Synthetic peptides derived from the predicted amino acid sequence for SNAP190 (see Fig. 1) were coupled to keyhole limpet hemocyanin (Pierce) and injected into rabbits to generate polyclonal antipeptide antibodies. Rabbit antisera were tested in an EMSA as previously described (25).

Immunodepletions and in vitro transcription assays. Rabbit preimmune or anti-SNAP190 antibodies were covalently cross-linked to protein A-agarose beads (5). All immunodepletions were performed for 30 min at room temperature in 1.5-ml Eppendorf tubes. For U6 snRNA, VAI, and AdML transcription experiments, immunodepletions were done with 100 μl of whole-cell extracts (13) depleted with an equal volume of preimmune antibody beads, preimmune and anti-SNAP190 antibody beads mixed at ratios of 2:1 and 1:2, or only anti-SNAP190 antibody beads. Depletions were also performed with the largest amount of anti-SNAP190 antibody beads, which had been preincubated with 10 μg of a nonspecific peptide (CSH483) (6) or a specific peptide (190-3 peptide). For U1 snRNA transcription experiments, 40 μl of whole-cell extract was depleted with 20 μl of antibody beads while maintaining the same bead ratios as those described above. Immunodepletion reaction mixtures were centrifuged for 1 min at room temperature, and the supernatants were transferred to fresh 1.5-ml Eppendorf tubes. The supernatants were immediately tested in in vitro transcription experiments. Eight, 4, 7, and 18 μl of extract were used for U6 snRNA, VAI, AdML, and U1 snRNA transcription reactions, respectively. To test for the effects of dilution of the extract by antibody beads, whole-cell extract or whole-cell extract diluted two- and fourfold in buffer D was tested. Reconstitution of transcription was tested by the addition of 2, 4, and 8 μl of a SNAP$_c$-enriched fraction (mono-Q fraction; approximately 0.3 mg of protein/ml) (7) to transcription reaction mixtures; the reactions were performed with extract treated with the highest level of anti-SNAP190 antibody beads.

Expression of recombinant proteins and coimmunoprecipitations. The SNAP190 coding sequence was subcloned into the pCITE-2a(+) vector (Novagen) to generate construct pCITE-SNAP190. Three micrograms of pCITE-SNAP190 and 1 μg of pCITE-SNAP45 (25) were used as the template for coupled in vitro transcription and translation (Promega) in a final volume of 50 μl containing 4 μl of L-[^{35}S]methionine (1,233 Ci/mmol; New England Nuclear), either in separate reactions or in cotranslation reactions. For immunoprecipitation control experiments, 10 μl of each individually labeled protein was combined with 190 μl of 20 mM HEPES (pH 7.9)–15% glycerol–0.1% Tween 20–5 mM $MgCl_2$–1 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride containing 100 mM KCl ($HMGT_{100}$) and incubated at 4°C for 2 h. Then, 10 μl of protein A-Sepharose beads coupled to either anti-SNAP190 antibody (antibody 402) or anti-SNAP45 antibody (α-CSH467; rabbit 234) was added, and the reaction mixtures were incubated at 4°C for 1 h. The antibody beads were then washed extensively with $HMGT_{100}$, and the bound proteins were eluted by boiling the beads in Laemmli buffer. For coimmunoprecipitation experiments, 3, 10, or 30 μl of cotranslated labeled proteins was used and the reaction mixtures were adjusted to a 200-μl final volume with $HMGT_{100}$. The samples were then processed exactly as described above. The eluted proteins were fractionated by SDS–12.5% polyacrylamide gel electrophoresis and visualized by autoradiography. Similar experiments were performed with in vitro-translated SNAP43 (7) and SNAP50 (6), but no interactions with SNAP190 could be detected.

Expression of SNAP190 Myb repeats in *E. coli*. Fragments corresponding to aa 390 to 518 (RcRd), 283 to 414 (RaRb), 283 to 518 (RaRbRcRd), 283 to 572 (RaRbRcRd and Arg-rich and Ser-rich regions), 330 to 518 (RbRcRd), 238 to 518 (RhRaRbRcRd), and 238 to 572 (RhRaRbRcRd and Arg-rich and Ser-rich regions) of SNAP190 were generated by PCR amplification with *Pfu* polymerase (Stratagene). PCR fragments corresponding to aa 390 to 518, 283 to 414, 283 to 518, and 283 to 572 were ligated into a pET-GST vector (8) to generate constructs pET-GST-190RcRd, pET-GST-190RaRb, pET-GST-190RaRbRcRd, and pET-GST-190RaRbRcRdSer, respectively. PCR fragments corresponding to aa 330 to 518, 238 to 518, and 238 to 572 were ligated into a modified pSBET vector (27) containing a glutathione *S*-transferase (GST)-coding sequence to generate constructs pSBET-GST-190RbRcRd, pSBET-GST-190RhRaRbRcRd, and pSBET-GST-190RhRaRbRcRdSer, respectively. GST fusion proteins were expressed in *Escherichia coli* BL21-DE3 (30), and lysates were prepared as described previously (31). Fusion proteins were bound to glutathione-agarose (Sigma), the beads were washed with phosphate-buffered saline containing 0.1% Nonidet P-40 and 10% glycerol, and the bound proteins were eluted with 10 mM glutathione in 50 mM Tris, pH 8.8. Where indicated, the GST moiety was cleaved by treatment with thrombin. All the proteins were analyzed by SDS-polyacrylamide gel electrophoresis. The various proteins were then tested for binding to wild-type and mutated PSEs in an EMSA as described previously (25). The EMSA whose results are shown in in Fig. 7C was performed as described in reference 29.

Nucleotide sequence accession number. The nucleotide sequence obtained in this study has been assigned GenBank accession no. AF032387.

RESULTS

Isolation of cDNAs encoding p190. In our original purified $SNAP_c$ preparation, three prominent polypeptides with apparent molecular masses of 43, 45, and 50 kDa were readily visible. These polypeptides have all been cloned and are indeed part of the SNAP complex (6, 7, 24). In addition, however, a polypeptide with an apparent molecular mass of about 190 kDa was clearly visible (7). We used an antibody raised against SNAP43 (7) to confirm by coimmunoprecipitation assays that the 190-kDa polypeptide (p190) is associated with $SNAP_c$ (data not shown) and to purify the protein. We obtained p190 peptide sequences from both $SNAP_c$ purified by conventional chromatography (7) and immunopurified $SNAP_c$. With this information, we designed degenerate oligonucleotides for PCR from cDNA prepared from total cellular RNA and obtained a p190-specific PCR fragment. We also identified a partial p190-encoding cDNA in a one-hybrid screen performed with *Saccharomyces cerevisiae* (29) (see below).

We used both the cDNA isolated through the yeast one hybrid screen and the PCR probe to assemble, by a combination of library screens and direct reverse transcription-PCRs (see Materials and Methods for details), an open reading frame that encodes the protein shown in Fig. 1A. The sequence contains all twelve of the peptide sequences we obtained from p190 (shaded in Fig. 1A) and constitutes a novel 1,469-aa protein with a calculated molecular mass of 159.2 kDa and an isoelectric point of 8.3. The open reading frame is probably full length, because it is preceded by an in-frame termination codon 189 nt upstream of the first AUG codon and terminates with a UGA opal codon.

p190 contains a Myb DNA binding domain. As shown in Fig. 1A and B, the p190 sequence contains several striking features. The protein contains an unusual Myb domain, immediately followed by an arginine-rich region and a serine-rich region, and in the carboxy-terminal region of the protein is a leucine zipper-like motif. The Myb domain similarity extends from the tryptophan at position 263 to the glutamine at position 503.

Proteins of the c-Myb family include c-Myb and the related A-Myb and B-Myb proteins (19). The Myb domains of these proteins are each composed of three imperfect tandem repeats, R1, R2, and R3. Plant Myb proteins usually have only two imperfect tandem repeats, R2 and R3. Each repeat contains three diagnostic tryptophan residues (Fig. 1C) (11, 14). The minimal DNA binding domains of Myb proteins consist of just the R2 and R3 repeats, and the solution structure of the mouse c-Myb R2-R3 repeats bound to DNA has been solved (20). Each repeat contains three helices, the second and third of which form a variation of the helix-turn-helix motif with the third helix corresponding to the DNA recognition helix. The recognition helices of both repeats are placed in tandem on the DNA and recognize a continuous sequence within the major groove (20).

Figure 1C shows an alignment of SNAP190 sequences from amino acid 263 to amino acid 503 with the R1, R2, and R3 repeats of the human A-Myb, B-Myb, and c-Myb proteins. Unlike other Myb DNA binding domain proteins, p190 contains a half repeat (Rh) followed by four repeats (Ra, Rb, Rc, and Rd). In Ra, the third tryptophan is replaced by a phenylalanine, while in Rb and Rc, the second and third tryptophans, respectively, are replaced by tyrosines. The p190 repeats that are the most similar to the R2 repeats of the A-, B-, and c-Myb proteins are the Rc and Rd repeats, each with 38% identity with R2, and the p190 repeat that is the most similar to the R3 repeats is the Rd repeat, with 30% identity. All four p190 repeats, however, are most similar to the R2 repeats. Interestingly, repeats Ra, Rc, and Rd all contain a cysteine corresponding to Cys131 in the c-Myb R2 repeat, which in c-Myb has the potential to mediate redox regulation of DNA binding (18).

The presence of a Myb domain-like sequence in p190 suggests that p190 is a DNA-binding subunit of SNAP190, consistent with a cross-linking experiment that detected a protein of about 180 kDa specifically cross-linked to the DNA in a highly purifed PTF fraction (34) and with the observation that none of the other $SNAP_c$ subunits isolated so far can bind DNA specifically on their own (6). Hence, p190 is a strong candidate for a DNA binding subunit of the SNAP complex.

p190 corresponds to the largest subunit of $SNAP_c$. To characterize the function of p190, and in particular to determine whether p190 is indeed part of the SNAP complex, we raised antibodies against four peptides (Fig. 1A) and used the antibody raised against amino acids 843 to 865 (antibody 402) for the experiments described in this work. We first performed an immunoblot analysis. Recombinant p190 translated in vitro was fractionated alongside a $SNAP_c$-containing phosphocellulose P11 column fraction on an SDS-polyacrylamide gel, the proteins were transferred to nitrocellulose, and p190 was visualized with the anti-p190 antibody (antibody 402). As shown in Fig. 2, the recombinant protein comigrated with endogenous p190, even though its calculated molecular mass is only 159 kDa. This is consistent with the cDNA encoding a full-length p190 protein and indicates that p190 migrates anomalously on an SDS-polyacrylamide gel.

We then tested the effects of the anti-p190 antibodies in an EMSA. As shown in Fig. 3A, a DNA-protein complex was formed upon incubation of a probe containing the wild-type mouse U6 PSE, a high-affinity binding site for $SNAP_c$, with a fraction highly enriched in $SNAP_c$ (lane 1; complex labeled $SNAP_c$). This complex was not formed on a probe containing a PSE debilitated by point mutations (ABC mutation [25]) (lane 2). The addition of preimmune antibodies had no effect on the mobility of the $SNAP_c$-PSE complex (lane 3), but the addition of the anti-p190 peptide antibody (α-SNAP190) retarded the migration of the $SNAP_c$-DNA complex (lane 4; complex labeled $SNAP_c$+Ab). The effect was abolished by preincubation of the antibody with 1 or 3 μg of the peptide against which the antibody was raised (lanes 5, 6) but not by preincubation with similar amounts of an irrelevant peptide (lanes 7, 8). The addition of the anti-p190 peptide antibody also resulted in the appearance of a faster-migrating complex, which may correspond to a partially disrupted or degraded SNAP complex (lanes 4 to 8). Whatever the case, however, the specific nature of the anti-p190 antibody-retarded complex strongly suggests that p190 is part of the $SNAP_c$-PSE complex.

To determine whether p190 is also part of the SNAP complex when it is not bound to DNA, we performed nondenaturing immunoprecipitations with nuclear extracts and either the anti-p190 antibody or preimmune antibodies and eluted the bound material with a buffer containing either no peptide or an excess of specific peptide. The eluted material was then used in an EMSA. As shown in Fig. 3B, the material eluted from the preimmune beads did not bind to the PSE probe (lanes 3 to 6). In contrast, material eluted from the anti-p190 beads with a specific peptide, but not without a peptide, bound to the PSE probe (compare lanes 9 and 10 with lanes 7 and 8). Together, these data indicate that the cDNA we have isolated encodes a subunit of $SNAP_c$, and we therefore refer to p190 as SNAP190.

SNAP190 is required for RNA polymerase II and III transcription of snRNA genes. Fractions enriched in the SNAP complex are required for RNA polymerase II and III transcription of snRNA genes, suggesting that $SNAP_c$ is part of the

FIG. 1. Amino acid sequence and schematic structure of SNAP190. (A) Amino acid sequence of SNAP190. The shaded regions correspond to peptide sequences obtained from the purified protein. The dashed arrow corresponds to the Myb half repeat, Rh, and the other arrows indicate Myb repeats Ra, Rb, Rc, and Rd, with the conserved tryptophan (or phenylalanine in the Ra repeat and tyrosines in Rb and Rc repeats) indicated in boldface. The arginine-rich and serine-rich regions are boxed. The leucines spaced as in a leucine zipper are indicated by asterisks. The synthetic peptides used to generate antibodies in rabbits are underlined: peptide 190-1 (aa 1452 to 1469) gave antibodies 398 and 399a; peptide 190-2 (aa 1309 to 1328) gave antibodies 400 and 401; peptide 190-3 (aa 843 to 865) gave antibodies 402 and 403; and peptide 190-4 (aa 712 to 735) gave antibodies 439 and 440. (B) Schematic structure of p190. The location of the Myb domain with the half repeat (Rh) and the four complete repeats (Ra, Rb, Rc, and Rd), as well as the locations of the arginine-rich, serine-rich, and leucine zipper-like regions, are indicated. The region of the protein that interacts with Oct-1 in a yeast one-hybrid assay is indicated by a bracket. (C) Alignment of the p190 half Myb repeat, Rh, and repeats Ra, Rb, Rc, and Rd and Myb repeats R1, R2, and R3 from the human A-Myb, B-Myb (19), and c-Myb (12, 28) proteins. The conserved tryptophans (replaced in some cases by tyrosines or phenylalanines) are indicated in boldface. In this alignment, the p190 Ra repeat is 17, 25, and 22% identical to the Myb R1, R2, and R3 repeats, respectively; the p190 Rb repeat is 14, 23, and 23% identical to the Myb R1, R2, and R3 repeats, respectively; the p190 Rc repeat is 27, 38, and 23% identical to the Myb R1, R2, and R3 repeats, respectively; and the p190 Rd repeat is 21, 38, and 30% identical to the Myb R1, R2, and R3 repeats, respectively.

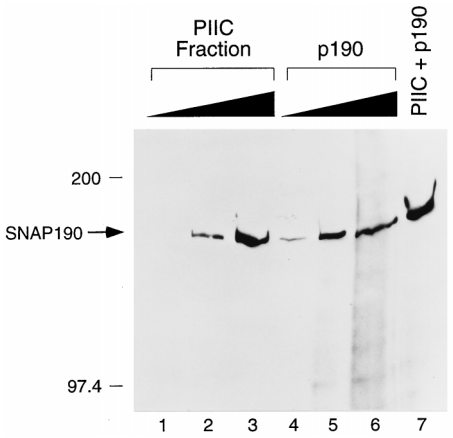


FIG. 2. Recombinant p190 comigrates with SNAP190. Three (lane 1), 10 (lane 2), and 30 (lane 3) μl of a $SNAP_c$-containing P11C fraction (7) and 5 (lane 4), 10 (lane 5), and 30 (lane 6) μl of reticulocyte lysate programmed with the p190 cDNA or a mixture of 10 μl of the P11C fraction and 10 μl of p190 cDNA-programmed reticulocyte lysate (lane 7) were fractionated on an SDS–5% polyacrylamide gel. The proteins were transferred to nitrocellulose, and the filter was immunoblotted with the anti-p190 402 antibody. Unprogrammed reticulocyte lysate gave no signal (data not shown). The positions of molecular weight markers and of SNAP190 in the P11C fraction are indicated.

initiation complexes assembled on both the RNA polymerase II and III snRNA promoters. It is possible, however, that the RNA polymerase II and III snRNA promoters recruit different versions of the SNAP complex containing, for example, different sets of subunits. We tested the involvement of SNAP190 in RNA polymerase II and III transcription from the four types of promoters depicted in Fig. 4A: the adenovirus 2 (Ad2) major-late promoter exemplifies a typical TATA box-containing RNA polymerase II mRNA promoter, the U1 and U6 promoters exemplify RNA polymerase II and III PSE-containing snRNA promoters, respectively, and the Ad2 VAI promoter exemplifies an RNA polymerase III promoter with gene-internal elements.

Transcription from these four promoters was tested in extracts that had been incubated with beads coated either with preimmune antibodies or with anti-SNAP190 antibodies. As shown in Fig. 4B, treatment of a nuclear extract with preimmune-antibody-coated beads had little effect on transcription from any of these promoters (compare lane 4 to lane 2; note that the U1 construct generates two transcripts, only the lower of which results from U1 promoter activity [25]). However, treatment of the extract with increasing amounts of anti-SNAP190 antibody-coated beads and correspondingly decreasing amounts of preimmune-antibody-coated beads resulted in a severe decrease in transcription from the U1 and U6 snRNA promoters (lanes 5 to 7). The inhibitory effect was specific, because it was abolished by preincubation of the beads with the specific peptide against which the anti-SNAP190 antibody was raised (lane 9) but not by incubation with an irrelevant peptide (lane 8). Furthermore, the addition of increasing amounts of a $SNAP_c$ fraction to the immunodepleted extract restored U1 and U6 transcription (compare lanes 10 to 12 with lane 7). In contrast, transcription from the Ad2 major-late and VAI promoters was not affected by depletion of SNAP190, and transcription from the VAI promoter was inhibited rather than increased by addition of the $SNAP_c$ fraction (lanes 10 to 12).

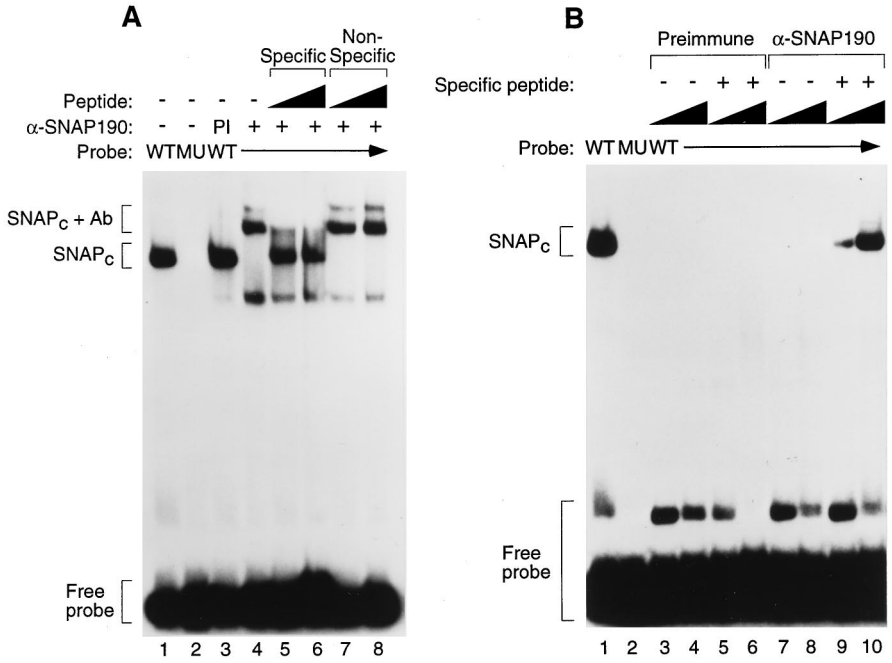


FIG. 3. p190 corresponds to the SNAP190 subunit of $SNAP_c$. (A) EMSA performed with a probe containing the wild-type mouse U6 PSE (lanes 1 and 3 to 8) or a mutated mouse U6 PSE (ABC mutation [25];lane 2), a fraction highly enriched in $SNAP_c$ (mono-Q fraction) (7), and either no antibodies (lanes 1 and 2), 1 μl of preimmune antibodies (lane 3), or 1 μl of anti-p190 (402) antibodies (lanes 4 to 8). Lanes 5 and 6 also contained 1 and 3 μg, respectively, of specific peptide (peptide 190-3), and lanes 7 and 8 contained 1 and 3 μg, respectively, of nonspecific peptide (peptide CSH375). The locations of the free probe and the complexes containing $SNAP_c$ or $SNAP_c$ and anti-p190 antibodies ($SNAP_c$ + Ab) bound to the PSE are indicated. (B) Beads coated with either preimmune (lanes 3 to 6) or anti-p190 (402) (lanes 7 to 10) antibodies were incubated with the $SNAP_c$-enriched mono-Q fraction. The bound material was then eluted either without (lanes 3, 4, 7, and 8) or with (lanes 5, 6, 9, and 10) a specific peptide (peptide 190-3) and used in an EMSA with a probe containing the wild-type mouse U6 PSE. For lanes 1 and 2, the EMSA was performed with a probe containing the wild-type (lane 1) or mutated (lane 2) mouse U6 PSE and the $SNAP_c$-enriched mono-Q fraction.

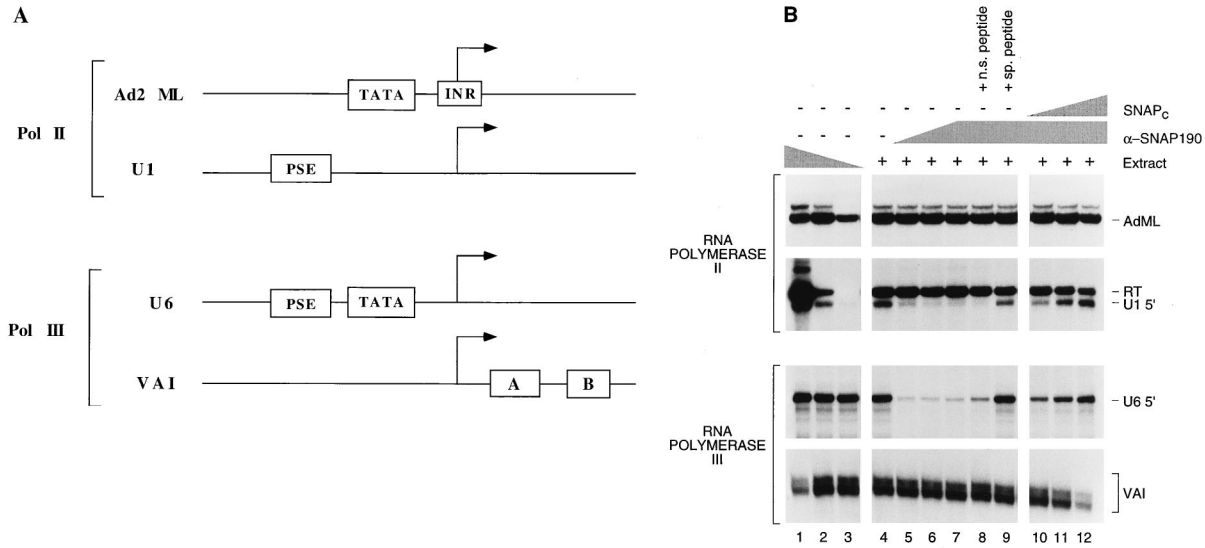


FIG. 4. SNAP190 is required for RNA polymerase II and III transcription from snRNA promoters. (A) Schematic representation of the four promoters used for in vitro transcription. (B) SNAP190 is required for human snRNA gene transcription. For the AdML, VAI, and U6 snRNA gene transcription experiments, 100 μl of HeLa whole-cell extract was depleted with either 100 μl of rabbit preimmune antibody beads (lane 4), 67 μl of preimmune antibody beads plus 33 μl of anti-SNAP190 antibody (antibody 402) beads (lane 5), 33 μl of preimmune antibody beads plus 67 μl of anti-SNAP190 antibody beads (lane 6), or 100 μl of anti-SNAP190 antibody beads (lanes 7 to 12). In lanes 8 and 9, the anti-SNAP190 antibody beads were first incubated with an excess of a nonspecific (CSH483) and a specific (peptide 190-3) peptide, respectively. In lanes 10 to 12, 2, 4, and 8 μl, respectively, of the SNAP$_c$-enriched mono-Q fraction were added to transcription reaction mixtures. Lanes 1 to 3 show transcription performed with undiluted extract (lane 1), extract diluted 1:2 with buffer D (lane 2), and extract diluted 1:4 with buffer D (lane 3). Lane 2 is directly comparable to lanes 4 to 12. The U1 snRNA gene transcription was sensitive to the effects of dilution (lanes 2 and 3); therefore, 40 μl of whole-cell extract was depleted with 20 μl of antibody beads by using the same ratio of preimmune to anti-SNAP190 antibody beads as that described above. The bands corresponding to correctly initiated RNA are labeled AdML for the Ad2 major-late promoter, U1 5′ for the U1 snRNA promoter, U6 5′ for the U6 snRNA promoter, and VAI for the Ad2 VAI promoter. The band labeled RT corresponds to transcripts derived from cryptic promoters located within vector sequences (25).

Similar results were obtained with antibodies directed against different SNAP190 peptide sequences (data not shown). Together, these data indicate that SNAP190 is required for both RNA polymerase II and III transcription of snRNA genes but not for transcription of a typical mRNA promoter or a typical TATA-less RNA polymerase III promoter. Thus, if different forms of SNAP$_c$ are involved in RNA polymerase II and III snRNA gene transcription, they contain at least four common subunits, SNAP43 (7), SNAP45 (24), SNAP50 (6), and SNAP190.

SNAP190 associates with SNAP45. To understand how SNAP190 fits into the architecture of the SNAP complex, we tested whether SNAP190 could associate with any of the previously cloned SNAP$_c$ subunits. SNAP190 was cotranslated in a reticulocyte lysate with either SNAP43, SNAP45, SNAP50, or the three proteins together. We then performed immunoprecipitations with antibodies directed against the various subunits. Figure 5 shows the results for SNAP45. As shown in Fig. 5A, anti-SNAP190 antibodies were able to recognize in vitro-translated SNAP190 (lane 4) but not SNAP45 (lane 8), as expected. However, when increasing amounts of cotranslated SNAP45 and SNAP190 were incubated with the anti-SNAP190 antibody, increasing amounts of SNAP45 were coimmunoprecipitated with SNAP190 (lanes 5 to 7). Reciprocally, as shown in Fig. 5B, anti-SNAP45 antibodies recognized in vitro-translated SNAP45 (lane 4) but not SNAP190 (lane 8). However, when increasing amounts of cotranslated SNAP45 and SNAP190 were incubated with anti-SNAP45 antibodies, increasing amounts of SNAP190 were coimmunoprecipitated with SNAP45 (lanes 5 to 7). Together, these data indicate that SNAP190 and SNAP45 interact strongly with each other. We could not detect, however, any strong interaction between SNAP190 and SNAP43 or SNAP50, and cotranslation of all four subunits did not result in coimmunoprecipitation of SNAP43 or SNAP50 alongside SNAP45 and SNAP190 (data not shown). This raises the question of how the SNAP complex is assembled; this question is discussed below.

The SNAP190 Rc and Rd Myb domain repeats can bind the PSE. The presence of a DNA binding domain sequence in SNAP190 immediately suggested that within the SNAP complex, SNAP190 is involved in recognizing the PSE. To test this hypothesis, we expressed full-length SNAP190 in a baculovirus expression system; we also expressed a number of SNAP190 fragments corresponding to the entire Myb domain or parts of it in *E. coli* (see Materials and Methods). Of these proteins, only one, consisting of the Rc and Rd repeats, bound to DNA, as shown in Fig. 6A. To test DNA binding we used the two probes shown in Fig. 6B. A GST fusion protein containing the Rc and Rd repeats bound to a wild-type mouse U6 PSE probe but not to a mutated probe carrying six point mutations within the PSE (compare lanes 3 to 5 with lanes 6 to 8). Similarly, an RcRd protein lacking the GST moiety bound specifically to the PSE (compare lanes 10 to 13 with lanes 15 to 18). These results suggest that the Rc and Rd repeats of the SNAP190 Myb domain contribute to recognition of the PSE within the SNAP complex.

SNAP190 interacts with Oct-1. SNAP$_c$ and Oct-1 can bind cooperatively to DNA, suggesting protein-protein interactions between these two factors on DNA (15, 17). We therefore checked whether proteins identified in an Oct-1 interaction screen in yeast (29) corresponded to subunits of SNAP$_c$. In this screen, summarized in Fig. 7A, the parental yeast strain carried, as a selectable marker, an integrated copy of a *HIS3* allele with six octamer sites upstream of the TATA element, as well as a plasmid that directs constitutive expression of full-length Oct-1. Because Oct-1 does not activate transcription in yeast, this strain transcribes the *HIS3* gene at basal levels and thus does not grow in the presence of aminotriazole (AT), a com-

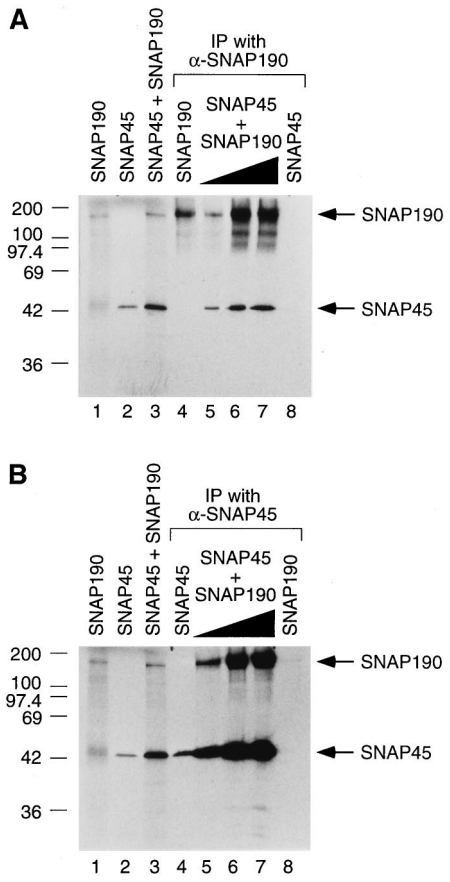


FIG. 5. SNAP190 interacts with SNAP45. (A) A cDNA encoding SNAP190 (lanes 1 and 4), SNAP45 (lanes 2 and 8), or a mixture of cDNAs encoding SNAP190 and SNAP45 (lanes 3 and 5 to 7) were expressed by coupled in vitro transcription and translation in reticulocyte lysates. In lanes 1 to 3, 1 μl of the products was loaded directly on the SDS-polyacrylamide gel. In lanes 4 to 8, 10, 3, 10, 30, and 30 μl, respectively, of the products were first incubated with beads coated with the anti-SNAP190 (402) antibody. The beads were washed, and the bound proteins were eluted by boiling the beads in Laemmli buffer and were fractionated on the SDS-polyacrylamide gel. (B) The same experiment as in panel A was performed, except that in lanes 4 to 8, the products were first incubated with beads coated with an anti-SNAP45 antibody (α-CSH467; rabbit 234).

petitive inhibitor of the *HIS3* gene product. This tester strain was transformed with a library of cDNAs fused to the VP16 transcriptional activation domain, and AT-resistant colonies were selected. Cells that grow under these selective conditions may express VP16 hybrid proteins that stimulate *HIS3* transcription by being recruited to the *HIS3* promoter through their interaction with DNA-bound Oct-1 (29). Indeed, such a screen resulted in the isolation of cDNAs encoding OBF-1 (also called OCA-B [10] and Bob1 [4]), a protein that interacts with octamer-bound Oct-1 (29). As shown below, this type of screen also resulted in the isolation of a cDNA encoding part of SNAP190.

Figure 7B shows the growth of various yeast strains on medium devoid of, or containing, AT. All the strains grew on control medium containing histidine but devoid of AT, showing that expression of the various proteins does not interfere with yeast growth. On the selective medium containing AT, however, the parental strain failed to grow (lane 1). A strain expressing a fusion protein consisting of Oct-1-interacting factor OBF-1 fused to the VP16 activation domain could grow on both the control medium and the AT-containing selective medium, and growth on the selective medium depended on the presence of Oct-1, as expected (lanes 4 and 5). In addition, a strain expressing a SNAP190-VP16 fusion protein also grew on the selective medium in an Oct-1-dependent (lanes 2 and 3) and octamer site-dependent (data not shown) manner. Sequence analysis of the cDNA encoding this SNAP190-VP16 fusion protein revealed that it encodes SNAP190 sequences from isoleucine 800 to the carboxyl terminus. The cDNA also contained 3′ noncoding sequences including a polyadenylation signal and a run of A bases. Thus, the carboxy-terminal half of SNAP190 can interact with DNA-bound Oct-1 in vivo.

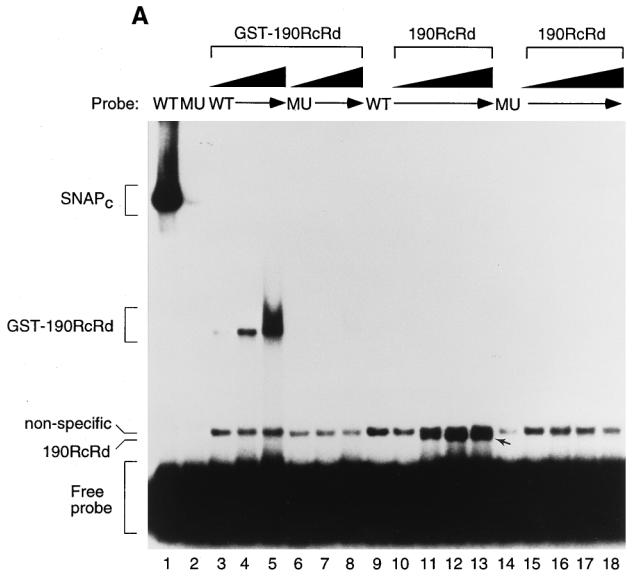
To determine whether the carboxy-terminal half of SNAP190 could also interact with DNA-bound Oct-1 in vitro, we performed an EMSA with a probe containing an octamer motif. As shown in Fig. 7C, The Oct-1 POU domain bound to the octamer probe (lane 2). In the presence of the Oct-1 POU domain and OBF-1, a slower-migrating complex (lane 3), which contains both the Oct-1 POU and OBF-1, was formed (29). Similarly, in the presence of the Oct-1 POU and the carboxy-terminal half of SNAP190 (SNAP190C), a slower-migrating complex (lanes 4 and 5), which was not obtained in the absence of the Oct-1 POU domain, was formed (data not shown). Together, these results show that the largest subunit of $SNAP_c$ can interact with DNA-bound Oct-1 both in vivo and in vitro and thus suggest that SNAP190 is a target of the Oct-1 activator.

DISCUSSION

We describe the characterization of a cDNA encoding the largest subunit of the SNAP complex, SNAP190. That SNAP190 is part of $SNAP_c$ was determined by the observations that antibodies directed against SNAP190 retard the migration of the $SNAP_c$-PSE complex in an EMSA and can immunoprecipitate a protein complex that binds to the PSE and retards the probe indistinguishably from $SNAP_c$. In addition, depletion of extracts with anti-SNAP190 antibodies inhibits both RNA polymerase II and III transcription from snRNA promoters, as expected for a subunit of $SNAP_c$. This result, together with the observations that depletions of transcription extracts with anti-SNAP43 (7), anti-SNAP45 (24), and anti-SNAP50 (6) antibodies also inhibit both RNA polymerase II and III transcription from snRNA promoters, indicates that the SNAP complexes involved in RNA polymerase II and III snRNA gene transcription contain at least four common subunits.

Architecture of the SNAP complex. SNAP190 interacts with SNAP45 in a coimmunoprecipitation assay. We have shown before that SNAP43 and SNAP50 can also be coimmunoprecipitated (6). We interpret these observations as reflecting protein-protein contacts that occur within the SNAP complex. Intriguingly, however, we have been unable to show protein-protein contacts between the SNAP190-SNAP45 pair and the SNAP43-SNAP50 pair of proteins by coimmunoprecipitation experiments in either the presence or absence of TBP (unpublished results). Our recent results indicate that this is due to the lack of a small, previously undetected, subunit. In the presence of this additional subunit, a SNAP complex capable of binding specifically to the PSE can be assembled (6a).

SNAP190 contains a Myb DNA binding domain. SNAP190 contains a region with strong similarity to the c-Myb DNA binding domain. However, unlike the c-Myb DNA binding domain, which contains three repeats, the SNAP190 Myb domain contains four repeats and one half repeat. A number of



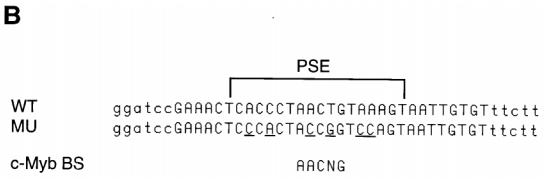


FIG. 6. DNA binding of the SNAP190 Myb repeats. (A) An EMSA was performed with a probe containing a wild-type (lanes 1, 3 to 5, and 9 to 13) or mutated (lanes 2, 6 to 8, and 14 to 18) mouse U6 PSE and either 2 μl of a fraction enriched in SNAP$_c$ (mono-Q fraction; 0.3 mg of protein/ml [7]) (lanes 1 and 2) or 0.1 (lanes 3, 6, 10, and 15), 0.3 (lanes 4, 7, 11, and 16), 1 (lanes 5, 8, 12, and 17) or 3 (lanes 13 and 18) μl of the bacterially expressed proteins indicated above the lanes. In lanes 9 and 14, no proteins were added to the probes. The locations of free probes, complexes containing SNAP$_c$, GST-190RcRd, and 190RcRd are indicated. (B) The sequences of the PSEs present in the wild-type and mutant probes are shown. Uppercase letters correspond to sequences derived from the mouse U6 promoter, with underlined characters corresponding to mutations. Flanking sequences are in lowercase characters. The c-Myb consensus binding site (c-Myb BS) is also indicated.

proteins, including the yeast SWI3 and ADA2 proteins, the transcriptional corepressor N-Cor, and the yeast TFC5 subunit of TFIIIB, contain one or two copies of a domain, which has been called the SANT domain, that resembles a Myb repeat (1). Although the SANT domain has been hypothesized to be involved in DNA binding, this has not been shown experimentally. The SNAP190 repeats are, however, much more related to the Myb R1 to R3 repeats than to the SANT domain, consistent with the ability of the SNAP190 Rc and Rd repeats to bind to the PSE.

The c-Myb DNA binding domain binds to consensus sequence AACNG through the DNA recognition helices of repeats R2 and R3, which contact the DNA in the major groove. In SNAP190, we could observe binding to DNA by repeats Rc and Rd, suggesting that these two repeats might correspond functionally to the c-Myb R2 and R3 repeats. Indeed, the Rc and Rd repeats are the SNAP190 pair of repeats most similar to the R2 and R3 repeats. However, although the mouse U6

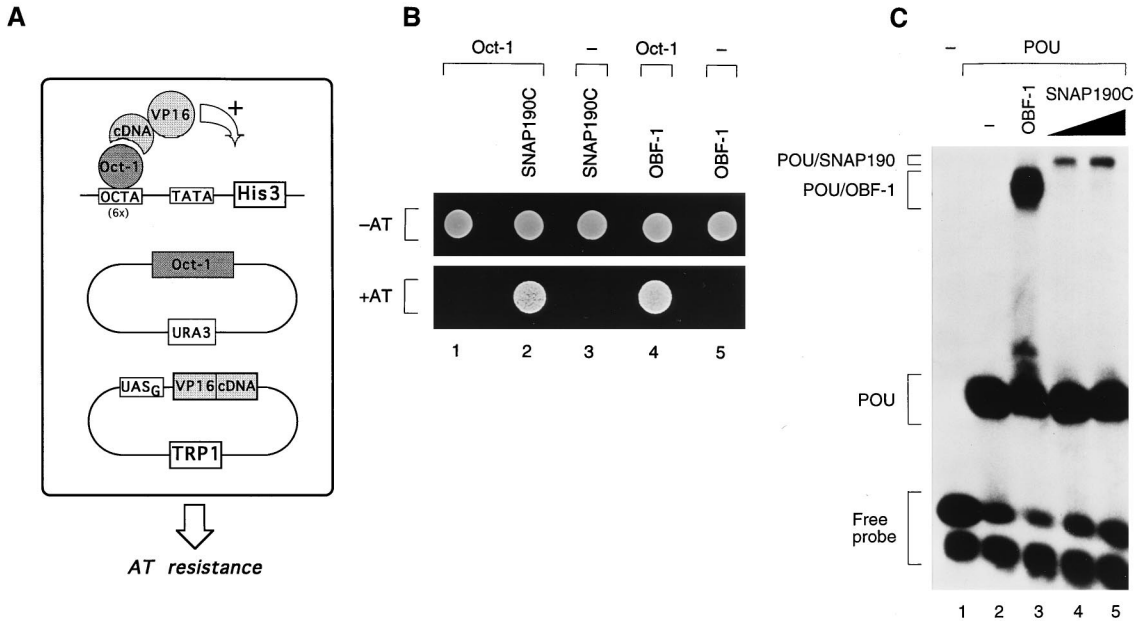


FIG. 7. The carboxy-terminal half of SNAP190 interacts with DNA-bound Oct-1 both in vivo and in vitro. (A) The strategy for isolating proteins interacting with DNA-bound Oct-1 (29) is depicted. An interaction between Oct-1, which is transcriptionally inactive in yeast, and a fusion protein containing the VP16 activation domain results in induction of *HIS3* transcription, which in turn allows cells to grow on AT-containing medium. The Oct-1 protein and the VP16 cDNA library were expressed from single-copy plasmids marked with the *URA3* and *TRP1* genes, respectively. Expression of the VP16 hybrid proteins was placed under control of the galactose-inducible *GAL1*, *10* regulatory sequences (UAS$_G$). (B) Yeast cells expressing Oct-1, SNAP190C, and OBF-1 as indicated above the lanes were plated on medium without AT or containing 40 mM AT. Only cells expressing induced levels of the *HIS3* gene product can grow in the presence of AT. (C) EMSA performed with a probe containing an octamer motif and the in vitro-translated proteins indicated above the lanes. The total amount of reticulocyte lysate was kept constant in all lanes. Lane 1 contains unprogrammed reticulocyte lysate only, lanes 2 to 5 contain 1 μl of Oct-1 POU, lane 3 contains in addition 1 μl of OBF-1, and lanes 4 and 5 contain 1 and 2 μl, respectively, of SNAP190C.

PSE, a high-affinity site for $SNAP_c$, contains an AACTG sequence which matches the AACNG sequence recognized by the R2 and R3 repeats of c-Myb (Fig. 6B), none of the amino acids involved in base pair recognition in the c-Myb R2R3-DNA structure, including the K128, E132, and N136 residues in R2 and the N179, K182, N183, N186, and S187 residues in R3 (20), are conserved within any of the SNAP190 repeats, including repeats Rc and Rd (Fig. 1C). Thus, the Rc and Rd repeats may recognize another sequence within the PSE.

The observation that the full-length SNAP190 protein as well as several SNAP190 truncations did not bind to the PSE is puzzling. This may be due to improper folding of the largest subunit in the absence of the other $SNAP_c$ subunits or perhaps to the presence of negative regulatory elements within SNAP190 itself. Nevertheless, the observation that the SNAP190 Rc and Rd repeats can bind specifically to the PSE strongly suggests that this subunit contacts the DNA directly within the SNAP complex, consistent with the cross-linking results of Yoon et al. (34). It seems unlikely, however, that the Rc and Rd repeats impart to $SNAP_c$ all of its DNA binding specificity. Indeed, the PSE is a large promoter element, and other parts of SNAP190 (within or outside of the Myb domain) or other subunits of $SNAP_c$ probably contribute to its recognition. For example, SNAP50 can also be cross-linked to the PSE (6) and thus may contribute to the specificity of DNA binding of the SNAP complex.

SNAP190 interacts with Oct-1. One of the mechanisms by which transcriptional activators can activate transcription is by recruiting members of the basal machinery to a promoter. For example, the *Drosophila melanogaster* activators Bicoid and Hunchback can activate transcription in vitro by recruitment of basal transcription factor TFIID. Recruitment involves protein-protein contacts with two of the TFIID subunits, $TAF_{II}110$ and $TAF_{II}60$ (26). In the case of snRNA promoters, the DNA binding POU domain of activator Oct-1 can recruit $SNAP_c$ to the PSE (15, 17), and this effect contributes to efficient snRNA gene transcription in vitro (15). We have isolated the region encoding the carboxy-terminal half of SNAP190 in a yeast one-hybrid screen as a DNA-bound Oct-1-interacting protein, and we could show that this region of SNAP190 can form a complex with the Oct-1 POU domain bound to an octamer motif in vitro. Thus, the DNA binding domain of Oct-1 may contribute to transcription activation by contacting the carboxy-terminal half of SNAP190 and so recruiting basal transcription complex $SNAP_c$ to the PSE.

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