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Co-expression of multiple subunits enables recombinant $SNAP_C$ assembly and function for transcription by human RNA polymerases II and III

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Abstract

Human small nuclear (sn) RNA genes are transcribed by either RNA polymerase II or III depending upon the arrangement of their core promoter elements. Regardless of polymerase specificity, these genes share a requirement for a general transcription factor called the snRNA activating protein complex or $SNAP_C$. This multi-subunit complex recognizes the proximal sequence element (PSE) commonly found in the upstream promoters of human snRNA genes. $SNAP_C$ consists of five subunits: SNAP190, SNAP50, SNAP45, SNAP43, and SNAP19. Previous studies have shown that a partial $SNAP_C$ composed of SNAP190 (1–514), SNAP50, and SNAP43 expressed in baculovirus is capable of PSE-specific DNA binding and transcription of human snRNA genes by RNA polymerases II and III. Expression in a baculovirus system yields active complex but the concentration of such material is insufficient for many bio-analytical methods. Herein, we describe the co-expression in *Escherichia coli* of a partial $SNAP_C$ containing SNAP190 (1–505), SNAP50, SNAP43, and SNAP19. The co-expressed complex binds DNA specifically and recruits TBP to U6 promoter DNA. Importantly, this partial complex functions in reconstituted transcription of both human U1 and U6 snRNA genes by RNA polymerases II and III, respectively. This co-expression system will facilitate the functional characterization of this unusual multi-protein transcription factor that plays an important early role for transcription by two different polymerases.

Keywords

Transcription; $SNAP_C$; Human snRNA; Co-expression; *Escherichia coli*

In eukaryotic organisms, transcription occurs by three different RNA polymerases I, II, and III that are unable to directly recognize promoter elements but instead require distinct assemblies of general transcription factors for efficient promoter recruitment. The initial step of promoter recognition by the general transcription machinery is a key step in transcription and is frequently targeted for intervention during gene regulation [1]. Interestingly, those components of the general transcription machinery that function directly in core promoter DNA binding

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are often composed of large multi-subunit complexes. For example, the TFIID complex is involved in pre-initiation complex assembly for RNA polymerase II transcription [reviewed in 2] and is composed of at least 13 subunits in humans [3]. While somewhat less complicated, other promoter recognition factors share this multi-subunit nature including SL1 for RNA polymerase I transcription, as well as TFIIIB and TFIIIC for RNA polymerase III transcription [4,5]. Potentially, multi-subunit complexes provide flexibility for modulating transcriptional responses to changing environmental conditions, in part, by providing many exposed surfaces for interactions with regulatory factors.

Typically, promoter recognition complexes are also specialized for transcription by a single class of polymerase; however, the $SNAP_C$ general transcription factor [6], also known as PTF [7], provides an interesting example of functional versatility through its role in snRNA gene transcription by both RNA polymerases II and III. Regardless of polymerase specificity, the promoters of human snRNA genes are very similar containing a proximal sequence element (PSE)[1] within the core promoter region [reviewed in 8,9]. $SNAP_C$ binds to the PSE and depending upon the presence or absence of an adjacent TATA box participates in different pathways of pre-initiation complex assembly. The presence of a TATA box, such as in U6 snRNA genes, dictates a role for $SNAP_C$ in pre-initiation complex assembly for RNA polymerase III transcription along with TBP, as a component of TFIIIB [10,11]. In contrast, the absence of a TATA box, such as in U1 snRNA genes, prescribes $SNAP_C$ participation in pre-initiation complex assembly with other general transcription factors including TBP, TFIIA, TFIIB, TFIIE, and TFIIF, commonly used for RNA polymerase II transcription [12]. $SNAP_C$ participation in either pathway is stimulated by direct interactions with the transcriptional activator protein Oct-1 when bound to the distal sequence element (DSE) located upstream of most human snRNA genes [13–16]. Thus, the early steps of promoter recognition by $SNAP_C$ are common to both pathways, but subsequent steps involving unique interactions with other general transcription factors are specialized for transcription by a single polymerase. Presumably, components of $SNAP_C$ are differentially important for RNA polymerase II versus III transcription, although no evidence for subunit specialization has yet been uncovered.

$SNAP_C$ is composed of at least five subunits, SNAP19 [17], SNAP43 [18,19], SNAP45 [19, 20], SNAP50 [21,22], and SNAP190 [23]. SNAP190, the largest of the subunits, provides the fundamental scaffold for assembly of the complex and directly interacts with all other $SNAP_C$ subunits [24] and with TBP [25,26]. SNAP190 also contains an unusual DNA binding domain in its N-terminal region consisting of four and a half myb domain repeats that is required for $SNAP_C$ DNA binding to the PSE [23] and for cooperative promoter recruitment of TBP to the TATA box during U6 transcription [26]. The central region of SNAP190 may negatively regulate $SNAP_C$ DNA binding, but interestingly, auto-inhibition of DNA binding can be overcome by interactions between the central region of SNAP190 and Oct-1 during activated transcription by RNA polymerases II and III [15]. The C-terminal region of SNAP190 interacts with SNAP45 [25], which may stabilize DNA binding of $SNAP_C$ in the context of the natural complex [15]. However, DNA binding by $SNAP_C$ does not require SNAP45 or SNAP19 as DNA binding activity can be reconstituted using a partial recombinant $SNAP_C$ (hereafter referred to as mini-$SNAP_C$) that contains SNAP43, SNAP50, and the N-terminal region of SNAP190 encompassing amino acids 1–505 [15]. DNA binding by mini-$SNAP_C$ requires all three subunits [26] with SNAP50 possibly involved in direct DNA contacts, and SNAP43 acting as a bridge to coordinate DNA recognition by SNAP190 and SNAP50.

[1]*Abbreviations used*: sn, small nuclear RNA genes; PSE, proximal sequence element; DSE, distal sequence element; IPTG, isopropyl-β-D-thiogalactopyranoside; TGEM, 5% Tris–glycine–EDTA–Mg^{2+}; EMSA, electrophoretic mobility shift assay; wt, wild-type.

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Recently, much has been learned about the composition and function of $SNAP_C$. For example, both $SNAP_C$ and mini-$SNAP_C$ can be reconstituted from recombinant proteins produced in a baculovirus expression system or by in vitro translation [15,17], but the amounts of pure material are limiting. In bacterial expression systems, the recovery of individual subunits is problematic, resulting in complexes capable of DNA binding but not capable of transcription initiation. To better understand the function of $SNAP_C$ a co-expression system in *Escherichia coli* was employed, enabling the production of fully functional partial $SNAP_C$ at significantly increased amounts and purity. The partial complex is capable of specific DNA binding, promoter recruitment of TBP, and transcription initiation for both RNA polymerases II and III.

Materials and methods

Cloning of $SNAP_C$ expression plasmids

To generate an expression plasmid that permits co-expression in E. *coli* of the SNAP50 and SNAP43 subunits of human $SNAP_C$, the full-length SNAP50 open reading frame was first amplified by polymerase chain reaction using Turbo *Pfu* polymerase with the primers 5′-TCAGCCATGGCTGAAGGAAGC-3′ and 5′-AGAGCTCTTAAT TAAAGGTTCCAGG-3′. The resultant amplified DNA fragment was inserted into the first multiple cloning site of the pCDFDuet-1 polycistronic expression vector (Novagen) via the *Nco*I and *Sac*I recognition sites to generate the expression vector pCDF50-1. Next, the full-length SNAP43 open reading frame was amplified using the primers 5′-GGAATTCCATATGGGGACTCCTCCCGGCCTGCA-3′ and 5′-GAGGATCCTCAGTGTTTTCTCCTCTTCTTG GATGC-3′. The resultant PCR product was inserted into the pCDF50-1 expression vector via the *Nde*I and *Bgl*II recognition sites to generate the expression vector pCDF43/50-1 that permits co-expression of SNAP50 with SNAP43, and provides resistance to streptomycin. The full-length SNAP19 open reading frame was also amplified by PCR using the primers 5′-CTCACCATGGTGAGCCGGCTT C-3′ and 5′-AAGGATCCTTAGGAATCTGATTCTTC-3′. The resultant PCR product was inserted into pRSFDuet-1 (Novagen) via the *Nco*I and *Bam*HI recognition sites to generate the expression vector pRSF19-1 that permits expression of full-length SNAP19, and provides resistance to kanamycin. Schematic representations of these plasmids are shown in Fig. 2.

Expression of $mSNAP_C\gamma3$ and $mSNAP_C\gamma4$

To generate partial $SNAP_C$ complexes, the expression plasmid pGST190 (1–505) [26], which expresses SNAP190 (1–505) with a N-terminal GST-tag, was first transformed into the *E. coli* strain BL21(DE3) CodonPlus RIL (Strat-agene) and plated on LB plates supplemented with ampicillin (100 μg/mL) and chloramphenicol (50 μg/mL). Single colonies were then transferred to LB broth containing the same antibiotics and cells were made competent by $CaCl_2$ treatment for subsequent transformation with pCDF43/50-1. Transformed cells harboring the pGST190 (1–505) and pCDF43/50-1 expression plasmids were incubated on LB plates supplemented with ampicillin (50 μg/ mL), chloramphenicol (50μg/mL), and streptomycin (20 μg/mL). These cells were then used for co-expression of GST-SNAP190 (1–505), SNAP50, and SNAP43 to generate the recombinant complex $mSNAP_C\gamma3$ as described below. To co-express a partial $SNAP_C$ that additionally contains SNAP19, the previous strain was made competent for subsequent transformation with the expression plasmid pRSF19-1. Transformed cells harboring the three $SNAP_C$-specific expression plasmids were plated on LB plates supplemented with ampicillin (50 μg/mL), chloramphenicol (50 g/mL), streptomycin (20 μg/mL), and kana-mycin (20 μg/mL). For these experiments, serial transformation of each expression plasmid was necessary as attempts to simultaneously transform all expression plasmids were unsuccessful. These cells were used for co-expression of GST-SNAP190 (1–505), SNAP50, SNAP43, and SNAP19 to generate the recombinant complex $mSNAP_C\gamma4$, as described below.

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Purification of $mSNAP_C$ and $mSNAP_C\gamma4$

To generate the recombinant complexes $mSNAP_C\gamma3$ and $mSNAP_C\gamma4$, bacteria harboring the appropriate plasmids were grown at 37 °C in 1 L of LB broth containing ampicillin (50 μg/mL), chloramphenicol (50 g/mL), streptomycin (20 μg/mL), and if necessary, kanamycin (20 μg/mL) until a cell density of 0.8–1 OD_{600} was reached. Cultures were induced with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 14–16 h at 16 °C. Typically, 6 × 1 L cultures were used for expression and purification of either $mSNAP_C\gamma3$ or $mSNAP_C\gamma4$. The concentration of recombinant proteins during purification was estimated by Bradford assay or by comparison to BSA standards after SDS–PAGE and Coomassie blue staining. To purify $mSNAP_C\gamma3$ and $mSNAP_C\gamma4$, cells were collected from 6 L cultures by centrifugation using a Komp spin KA-9 rotor (5000 rpm, 20min, 4 °C). The supernatant was decanted and the cell pellets were resuspended to a total volume of 160mL using HEMGT-250 (25mM Hepes, pH 7.9, 2mM EDTA, 12.5mM $MgCl_2$, 10% glycerol, 0.1% Tween 20, 250mM KCl, and 3mM DTT) plus protease inhibitors (complete mini EDTA-free protease inhibitor cocktail tablets, Roche). The cells were lysed by ultrasonication using a Branson sonifier (3/8" tip, 3 × 1min pulses, 80% duty, power level 7), and the lysate was clarified by centrifugation using a GSA rotor (5000 rpm, 1 h, 4 °C). Recombinant $mSNAP_C\gamma3$ or $mSNAP_C\gamma4$ were affinity purified by mixing the clarified lysates with glutathione agarose beads (Amersham) at a ratio of 4:1 (v/v) for 2 h at 4 °C. After binding, the resin was collected by centrifugation and the beads were washed five times with 40mL HEMGT-250 buffer. The washed beads (~40mL) were transferred to a Kontes Flex-Column (2.5 cm ID 20 cm length 98mL maximum volume) and an additional 20mL of HEMGT-250 was added. The recombinant complexes were released from the beads by digestion with thrombin (50U, 12 h, 4 °C). Subsequently, digested proteins were eluted from the column with HEMGT-250 and 15-mL fractions were collected. Those fractions enriched for the recombinant complexes were then pooled. Approximately 9.6mg of $mSNAP_C\gamma3$ (1.6 mg/L culture; average of six runs) and 13.5mg of $mSNAP_C\gamma4$ (2.2mg/L culture; average of 17 runs) were obtained at this stage of purification. The partially purified complexes were then subjected to ion exchange column chromatography using 2mL of Source-S resin (Amersham). Bound proteins were eluted from the Source-S column using a 50mL linear elution gradient from buffer A (10mM Tris, pH 7.5, 50mM NaCl, 15.5mM $MgCl_2$, 10% glycerol, 0.05% Tween 20, and 3mM DTT) to buffer A-500 (buffer A containing 500mM NaCl). Both $mSNAP_C\gamma3$ and $mSNAP_C\gamma4$ eluted from this column at salt concentrations between 150 and 250mM NaCl in a volume of ~15mL. The average recovery was 5mg for $mSNAP_C\gamma3$ (52% average yield) and 10mg for $mSNAP_C\gamma4$ (74% average yield). The recombinant complexes were diluted to an appropriate concentration in HEMGT-80 for functional studies, as described below.

Electrophoretic mobility shift assay

EMSA was performed using either recombinant $mSNAP_C\gamma4$ or mini-$SNAP_C$ containing SNAP190 (1–505), SNAP43, and SNAP50 that were expressed individually in *E. coli* and assembled into a complex, as previously described [26]. The individually expressed mini-$SNAP_C$ used for the experiments shown in Fig. 1 was assembled and purified by binding each subunit to glutathione aga-rose beads followed by thrombin digestion. Briefly, mini-$SNAP_C$ was assembled by mixing approximately 60 ng of each purified subunit together for 2 h. The complex was then further purified by mono-Q HR 5/5 column chromatography (~5 ng mini-$SNAP_C$/μL). The amounts of mini-$SNAP_C$ and $mSNAP_C\gamma4$ used for the DNA binding reactions are indicated in the figure legends. To test $mSNAP_C\gamma4$ for ability to recruit TBP, an additional 50 ng of full-length human recombinant TBP was included in DNA binding reactions, as indicated. The radiolabeled DNA probes used contained a high affinity wild-type mouse U6 PSE and human U6 TATA box. Additional reactions were performed with probes that contain debilitating mutations in each element. Resultant protein–DNA complexes were

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separated on a 5% Tris–glycine–EDTA–Mg^{2+} (TGEM) polyacrylamide gel at 150V and protein–DNA complexes were visualized by autoradiography.

In vitro transcription assay

Antibody immunodepletion of HeLa cell nuclear extract was performed with either rabbit α-SNAP43 antiserum or preimmune sera as described previously [17,23]. In vitro transcription assays of human U1 and U6 snRNA genes were performed for 1 h at 30 °C using the depleted extracts as described previously [6,27–29]. For the U6 transcription experiment shown in Fig. 1, approximately 1, 2.5, and 7.5 μL of recombinant mini-$SNAP_C$ (~1ng/μL) and 7.5 μL of a mono-Q fraction enriched for endogenous $SNAP_C$ (~0.3ng $SNAP_C$/μL) was used to reconstitute transcription. For the experiment shown in Fig. 4, approximately 0.08, 0.25, 0.75, 2.5, 7.5, 25, and 75 ng of $mSNAP_C\gamma4$ was used for the reconstitution of both U1 and U6 transcription.

Results and discussion

As a first step towards dissecting the function of $SNAP_C$, a partial complex containing SNAP190 (1–505), SNAP50, and SNAP43 was assembled using recombinant factors individually expressed in *E. coli* as previously described [26]. This bacterially expressed mini-$SNAP_C$ was compared to partially purified endogenous $SNAP_C$ [18] for its ability to bind to DNA in an electrophoretic mobility shift assay (EMSA) using radiolabeled probes that contain either a wild-type (wt) PSE or a mutant (mu) PSE sequence. As shown in Fig. 1A, the endogenous $SNAP_C$ binds specifically to the wtPSE but not a muPSE, as expected, and the bacterially expressed mini-$SNAP_C$ exhibited similar DNA binding properties (compare lanes 5–7 with lane 3). Thus, recombinant mini-$SNAP_C$ assembled from individually expressed subunits is functional for DNA binding. This result is consistent with those previously described for this complex when it was expressed and assembled in a similar manner [26] or by co-expression of the recombinant $SNAP_C$ subunits using a baculovirus expression system [30].

Next, these same amounts of endogenous $SNAP_C$ and recombinant mini-$SNAP_C$ were tested for ability to reconstitute U6 snRNA gene transcription in vitro using a HeLa cell whole cell extract that was depleted of endogenous $SNAP_C$. As shown in Fig. 1B, addition of recombinant mini-$SNAP_C$ to the extract did not substantially influenced RNA polymerase III transcription from this reporter plasmid (lane 2) nor did mock depletion of the extract using an irrelevant preimmune serum (lane 3). However, transcription was debilitated by removal of endogenous $SNAP_C$ by anti-SNAP43 immunodepletion (lane 4). As expected, transcription was efficiently restored by addition of the bio-chemically purified $SNAP_C$ (lane 5), whereas recombinant mini-$SNAP_C$ was essentially inactive in these assays (lanes 6–8). Thus, the recombinant mini-$SNAP_C$ assembled from individually expressed subunits is crippled for U6 transcription even though it is capable of efficient DNA binding and U6 promoter recognition.

One explanation for the inability of the recombinant mini-$SNAP_C$ to function for U6 transcription is that a post-translational modification of $SNAP_C$ is crucial for its activity and that this event is not successfully recapitulated in *E. coli* cells. Indeed, the SNAP190 (1–505) subunit is a substrate for phosphorylation by the protein kinase CK2 [31]. Another possibility is that mini-$SNAP_C$ does not obtain its fully active, native conformation when its subunits are expressed separately and reassembled in vitro. Both possibilities are consistent with the observation that a functional mini-$SNAP_C$ can be obtained by co-expression of these mini-$SNAP_C$ subunits in a baculovirus expression system [15].

To address the latter hypothesis, a co-expression system was devised wherein the individual subunits of mini-$SNAP_C$ were simultaneously expressed in *E. coli*. This approach has been

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used in other systems for high level expression soluble protein complexes [reviewed in 32]. The open reading frames encoding full-length SNAP50 and SNAP43 were sub-cloned into a pCDFDuet-1 plasmid for co-expression from the same promoter, while full-length SNAP19 was sub-cloned into a pRSFDuet-1 plasmid. Both pCDFDuet-1 and pRSFDuet-1 are polycistronic expression plasmids, and although in the current example SNAP19 was expressed from its own plasmid, the use of the pRSFDuet-1 plasmid allows, if necessary, for the co-expression of SNAP19 and SNAP190 (1–505) that together form a tight complex. In the expression system presented herein, GST-SNAP190 (1–505) was also expressed from its own plasmid. Schematic representations of the newly generated pCDF43/50-1 and pRSF19-1 plasmids are shown in Fig. 2. As different antibiotic selection can be used for each vector, we were able to sequentially transform pGST190 (1–505) and pCDF43/50-1 into *E. coli* cells thus enabling the expression of the three subunits SNAP190 (1–505), SNAP50, and SNAP43 to generate the recombinant complex hereafter referred to as $mSNAP_C\gamma3$. The co-expression of an additional fourth subunit SNAP19 from the pRSF19-1 plasmid was also performed to generate the recombinant complex hereafter referred to as $mSNAP_C\gamma4$.

The purification properties of $mSNAP_C\gamma4$ were next compared to the properties of the same $SNAP_C$ components that were expressed individually in *E. coli* (Fig. 3). This analysis revealed two important advantages for the co-expression system. First, the recovery of full-length SNAP43 is typically very low (10–30%) when it is expressed individually even though expression of the parent GST-SNAP43 protein is relatively robust (Fig. 3C). After thrombin cleavage of GST-SNAP43 the majority of the cleaved SNAP43 typically remains irreversibly bound to the glutathione agarose beads despite the lack of a covalently attached GST-tag. In contrast, Fig. 3E shows that the proportion of SNAP43 recovered is improved when it is expressed together with the rest of the $SNAP_C$ proteins. Presumably, the recovery is improved because SNAP43 can form a tight complex with SNAP50 or SNAP190 (1–505) to prevent inappropriate aggregation on the glutathione agarose beads. Second, when SNAP50 is expressed individually, stoichiometric amounts of a 60kDa contaminating protein associate with SNAP50 (Fig. 3B). Although the identity of this contaminating protein from *E. coli* is unknown, the association between this factor and SNAP50 is stable to a least 1M KCl (data not shown). Furthermore, after assembly of mini-$SNAP_C$ using individually expressed subunits, this factor also remains associated during a variety of affinity purification and column chromatography steps making the purification of mini-$SNAP_C$ problematic (data not shown). In contrast, no trace of this contaminating factor was observed during the one-step affinity purification of the $mSNAP_C\gamma4$ complex. Therefore, the overall purity of the complex is markedly improved by the co-expression of the SNAPC components.

Previous experiments demonstrated that even though SNAP19 is not essential for transcription [15], it facilitates the association between SNAP43 and SNAP190 [17], and the inclusion of SNAP19 in assembly reactions of mini- $SNAP_C$ dramatically improved the DNA binding performance of mini-$SNAP_C$ [26]. Therefore, whether SNAP19 was necessary to facilitate the assembly of a complex in this co-expression system was examined by comparing the partial complexes with and without SNAP19 (Fig. 4A). Samples of the individually expressed subunits are shown in lanes 2–5, and served to identify these factors within the co-expressed complex. In the presence of co-expressed SNAP19, there is a substantial increase in the amounts of SNAP43 and SNAP50 recovered during affinity purification of the complex through GST-SNAP190 (1–505) as compared to the complex lacking SNAP19 (compare lane 6 to lane 7). Nonetheless, the partially purified $mSNAP_C\gamma3$ and $mSNAP_C\gamma4$ obtained by binding complexes to glutathione agarose beads subsequently eluted as single complexes of apparent molecular weight of 150kDa during size exclusion chromatography (data not shown), suggesting that differences in SNAP43 and SNAP50 recovery after ion exchange chromatography are due to differences in complex stability rather than complexes with altered subunit stoichiometry during complex expression and assembly. This result suggests that co-expression of SNAP19

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stimulates complex assembly and is consistent with the previously described role for SNAP19 in stimulating association between SNAP43 and SNAP190. Furthermore, while SNAP19 is not essential for function of recombinant SNAPC, the amount of complex recovered was increased using the co-expression system that includes SNAP19 and therefore $mSNAP_C\gamma4$ was chosen for further study.

To determine whether the $mSNAP_C\gamma4$ obtained using this co-expression system was functional, the ability of $mSNAP_C\gamma4$ to bind DNA in a PSE-specific fashion and recruit TBP was tested using EMSA. As shown in Fig. 4B, $mSNAP_C\gamma4$ bound efficiently to both the wtPSE/wtTATA (lanes 2 and 3) and wtPSE/muTATA probes (lanes 10 and 11), but failed to bind to any probes in which the PSE was mutated (lanes 6 and 7, and lanes 14 and 15). The $mSNAP_C\gamma4$ also functioned to recruit TBP to the wtPSE/wtTATA DNA probe (lane 5) but not to a wtPSE/muTATA probe lacking a functional TATA box (lane 12). These results show that the $mSNAP_C\gamma4$ obtained using the co-expression system does indeed behave as previously described for both endogenous SNAPC and all other previously described versions of recombinant $SNAP_C$ for DNA binding and TBP recruitment.

Next, the ability of $mSNAP_C\gamma4$ to function for transcription by both RNA polymerases II and III was examined using reconstituted in vitro transcription initiated from plasmids containing either a human U1 snRNA promoter (Fig. 4C) or human U6 snRNA promoter (Fig. 4D). In these experiments, HeLa cell nuclear extract was either mock depleted with rabbit preimmune sera or with α-SNAP43 antiserum to remove endogenous $SNAP_C$. In the absence of endogenous $SNAP_C$, production of the U1-specific transcript is markedly reduced relative to the mock depleted extract (compare lanes 1 and 2 with lane 3), whereas the non-specific read-through transcript (labeled RT) is unaffected by $SNAP_C$ depletion. U1-speciWc transcription was restored by addition of increasing amounts of $mSNAP_C\gamma4$ (lanes 4–10), again with no obvious effect on read-through transcription. Thus, $mSNAP_C\gamma4$ functions specifically to reconstitute RNA polymerase II transcription from the U1 promoter with no effect on RNA polymerase II transcription driven by a cryptic mRNA-like promoter located upstream from the U1 start site. Similarly, U6 snRNA transcription by RNA polymerase III was diminished by removal of endogenous $SNAP_C$ (Fig. 4D, lane 2), and transcription was restored by increasing amounts of $mSNAP_C\gamma4$ (lanes 3–9), whereas transcription was not restored by addition of a non-specific protein GST (lane 10). For both U1 and U6 transcription, the amounts of recombinant $mSNAP_C\gamma4$ required to restore transcription levels equivalent to the untreated extract was similar (~2.5ng), although U1 transcription continued to be stimulated by further increasing the amounts of $mSNAP_C\gamma4$ (Fig. 4C, lanes 8–10), perhaps indicating that $SNAP_C$ activity is limiting for U1 transcription in this in vitro system. Importantly, robust U6 transcriptional activity was achieved with $mSNAP_C\gamma4$ using the amounts of recombinant protein that were completely ineffective when these factors were combined from individually expressed factors (see Fig. 1B, lane 7). These results show that $mSNAP_C\gamma4$ is indeed functional for both RNA polymerases II and III transcription. In these experiments, reconstitution was achieved using a similar mass of recombinant complex as that achieved with endogenous $SNAP_C$, indicating that a priori modification of the complex, such as by phosphorylation by CK2, is not essential for activity. This study does not examine whether the complex could be targeted for modification during transcription. Overall, this report describes an important advance in the production of a functional multi-protein complex by co-expression of its recombinant subunits, which will accelerate further structure and functional studies of this interesting general transcription factor.

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References

1. Roeder RG. Transcriptional regulation and the role of diverse coactivators in animal cells. FEBS Lett 2005;579:909–915. [PubMed: 15680973]
2. Albright SR, Tjian R. TAFs revisited: more data reveal new twists and conWrm old ideas. Gene 2000;242:1–13. [PubMed: 10721692]
3. Tora L. A uniWed nomenclature for TATA box binding protein (TBP)-associated factors (TAFs) involved in RNA polymerase II transcription. Genes Dev 2002;16:673–675. [PubMed: 11963920]
4. Geiduschek EP, Kassavetis GA. Comparing transcriptional initiation by RNA polymerases I and III. Curr. Opin. Cell Biol 1995;7:344–351. [PubMed: 7662364]
5. Paule MR, White RJ. Survey and summary: transcription by RNA polymerases I and III. Nucleic Acids Res 2000;28:1283–1298. [PubMed: 10684922]
6. Sadowski CL, Henry RW, Lobo SM, Hernandez N. Targeting TBP to a non-TATA box *cis*-regulatory element: a TBP-containing complex activates transcription from snRNA promoters through the PSE. Genes Dev 1993;7:1535–1548. [PubMed: 8339931]
7. Murphy S, Yoon JB, Gerster T, Roeder RG. Oct-1 and Oct-2 potentiate functional interactions of a transcription factor with the proximal sequence element of small nuclear RNA genes. Mol. Cell. Biol 1992;12:3247–3261. [PubMed: 1535687]
8. Henry RW, Ford E, Mital R, Mittal V, Hernandez N. Crossing the line between RNA polymerases: transcription of human snRNA genes by RNA polymerases II and III. Cold Spring Harb. Symp. Quant. Biol 1998;63:111–120. [PubMed: 10384275]
9. Hernandez N. Small nuclear RNA genes: a model system to study fundamental mechanisms of transcription. J. Biol. Chem 2001;276:26733–26736. [PubMed: 11390411]
10. Mittal V, Hernandez N. Role for the amino-terminal region of human TBP in U6 snRNA transcription. Science 1997;275:1136–1140. [PubMed: 9027316]
11. Schramm L, Pendergrast PS, Sun Y, Hernandez N. DiVerent human TFIIIB activities direct RNA polymerase III transcription from TATA-containing and TATA-less promoters. Genes Dev 2000;14:2650–2663. [PubMed: 11040218]
12. Kuhlman TC, Cho H, Reinberg D, Hernandez N. The general transcription factors IIA, IIB, IIF, and IIE are required for RNA polymerase II transcription from the human U1 small nuclear RNA promoter. Mol. Cell. Biol 1999;19:2130–2141. [PubMed: 10022900]
13. Ford E, Hernandez N. Characterization of a trimeric complex containing Oct-1, $SNAP_C$, and DNA. J. Biol. Chem 1997;272:16048–16055. [PubMed: 9188510]
14. Ford E, Strubin M, Hernandez N. The Oct-1 POU domain activates snRNA gene transcription by contacting a region in the $SNAP_C$ largest subunit that bears sequence similarities to the Oct-1 coactivator OBF-1. Genes Dev 1998;12:3528–3540. [PubMed: 9832505]
15. Mittal V, Ma B, Hernandez N. SNAP(c): a core promoter factor with a built-in DNA-binding damper that is deactivated by the Oct-1 POU domain. Genes Dev 1999;13:1807–1821. [PubMed: 10421633]
16. Hovde S, Hinkley CS, Strong K, Brooks A, Gu L, Henry RW, Geiger J. Activator recruitment by the general transcription machinery: X-ray structural analysis of the Oct-1 POU domain/human U1 octamer/SNAP190 peptide ternary complex. Genes Dev 2002;16:2772–2777. [PubMed: 12414730]
17. Henry RW, Mittal V, Ma B, Kobayashi R, Hernandez N. SNAP19 mediates the assembly of a functional core promoter complex ($SNAP_C$) shared by RNA polymerases II and III. Genes Dev 1998;12:2664–2672. [PubMed: 9732265]
18. Henry RW, Sadowski CL, Kobayashi R, Hernandez N. A TBPTAF complex required for transcription of human snRNA genes by RNA polymerase II and III. Nature 1995;374:653–656. [PubMed: 7715707]
19. Yoon JB, Roeder RG. Cloning of two proximal sequence element-binding transcription factor subunits (gamma and delta) that are required for transcription of small nuclear RNA genes by RNA polymerases II and III and interact with the TATA-binding protein. Mol. Cell. Biol 1996;16:1–9. [PubMed: 8524284]
20. Sadowski CL, Henry RW, Kobayashi R, Hernandez N. The SNAP45 subunit of the small nuclear RNA (snRNA) activating protein complex is required for RNA polymerase II and III snRNA gene

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transcription and interacts with the TATA box binding protein. Proc. Natl. Acad. Sci.USA 1996;93:4289–4293. [PubMed: 8633057]

21. Henry RW, Ma B, Sadowski CL, Kobayashi R, Hernandez N. Cloning and characterization of SNAP50, a subunit of the snRNA-activating protein complex $SNAP_C$. EMBO J 1996;15:7129–7136. [PubMed: 9003788]
22. Bai L, Wang Z, Yoon JB, Roeder RG. Cloning and characterization of the beta subunit of human proximal sequence element-binding transcription factor and its involvement in transcription of small nuclear RNA genes by RNA polymerases II and III. Mol. Cell. Biol 1996;16:5419–5426. [PubMed: 8816454]
23. Wong MW, Henry RW, Ma B, Kobayashi R, Klages N, Matthias P, Strubin M, Hernandez N. The large subunit of basal transcription factor $SNAP_C$ is a Myb domain protein that interacts with Oct-1. Mol. Cell. Biol 1998;18:368–377. [PubMed: 9418884]
24. Ma B, Hernandez N. A map of protein-protein contacts within the small nuclear RNA-activating protein complex $SNAP_C$. J. Biol. Chem 2001;276:5027–5035. [PubMed: 11056176]
25. Ma B, Hernandez N. Redundant cooperative interactions for assembly of a human U6 transcription initiation complex. Mol. Cell. Biol 2002;22:8067–8078. [PubMed: 12391172]
26. Hinkley CS, Hirsch HA, Gu L, LaMere B, Henry RW. The SNAP190 Myb DNA binding domain stimulates TBP-TATA box recognition. J. Biol. Chem 2003;278:18649–18657. [PubMed: 12621023]
27. Hirsch HA, Gu L, Henry RW. The retinoblastoma tumor suppressor protein targets distinct general transcription factors to regulate RNA polymerase III gene expression. Mol. Cell. Biol 2000;20:9182–9191. [PubMed: 11094070]
28. Hirsch HA, Jawdekar GW, Lee KA, Gu L, Henry RW. Distinct mechanisms for repression of RNA polymerase III transcription by the Retinoblastoma tumor suppressor protein. Mol. Cell. Biol 2004;24:5989–5999. [PubMed: 15199152]
29. Gridasova AA, Henry RW. The p53 tumor suppressor protein represses human snRNA gene transcription by RNA polymerases II and III independently of sequence-speciWc DNA binding. Mol. Cell. Biol 2005;25:3247–3260. [PubMed: 15798209]
30. Mittal V, Cleary MA, Herr W, Hernandez N. The Oct-1 POU-spe-ciWc domain can stimulate small nuclear RNA gene transcription by stabilizing the basal transcription complex $SNAP_C$. Mol. Cell. Biol 1996;16:1955–1965. [PubMed: 8628262]
31. Gu L, Esselman WJ, Henry RW. Cooperation between small nuclear RNA-activating protein complex (SNAPC) and TATA-box-binding protein antagonizes protein kinase CK2 inhibition of DNA binding by $SNAP_C$. J. Biol. Chem 2005;280:27697–27704. [PubMed: 15955816]
32. Tolia NH, Joshua-Tor L. Strategies for protein coexpression in *Escherichia coli*. Nat. Methods 2006;3:55–64. [PubMed: 16369554]

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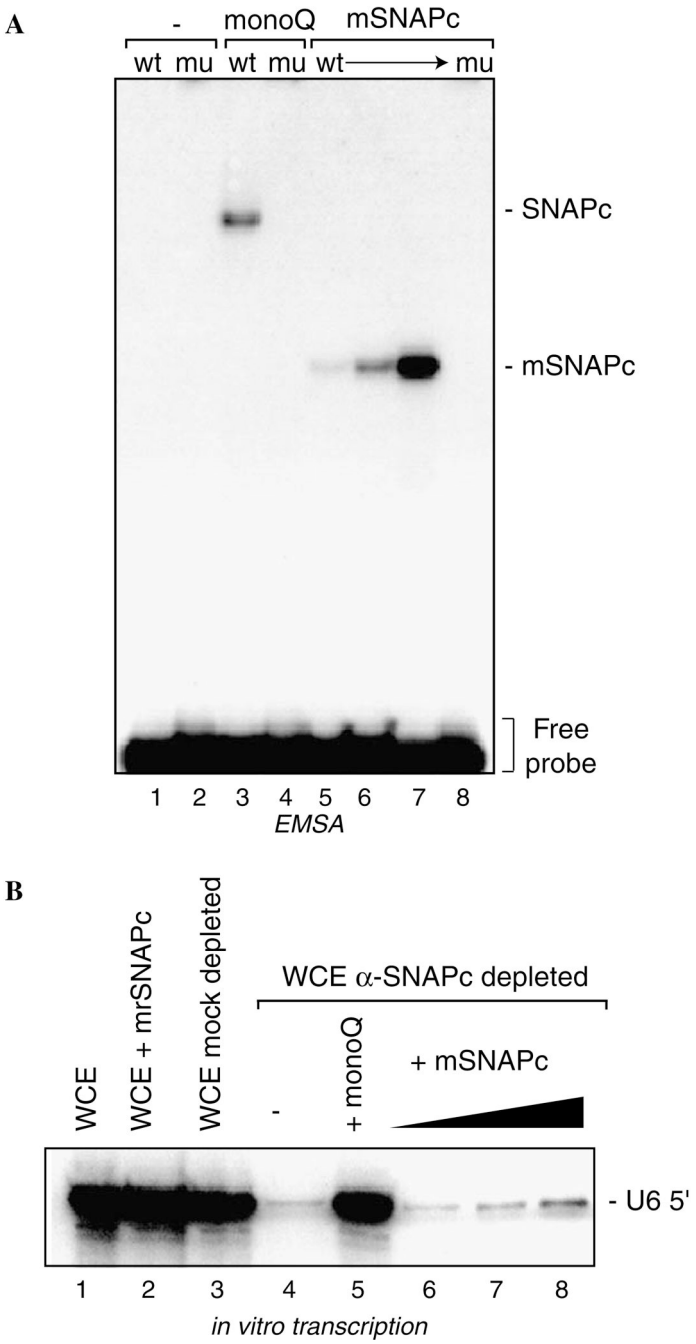

Fig. 1.

A partial $SNAP_C$ assembled from individually expressed subunits is functional for DNA binding but not for transcription of a human U6 snRNA gene by RNA polymerase III. (A) Increasing amounts of mini-$SNAP_C$ that was assembled from subunits individually expressed in *E. coli* (~1, 2.5, and 7.5 ng; lanes 5–7, respectively) were added to EMSA reactions containing dsDNA probes containing a wild-type (wt) or mutant (mu) PSE, as indicated. Reactions loaded in lanes 3 and 4 contained 7.5 μL of partially purified endogenous $SNAP_C$ (~0.3 ng $SNAP_C$/mL). Reactions containing only the probe DNAs are shown in lanes 1 and 2. (B) Increasing amounts of mini-$SNAP_C$ were added to human U6 in vitro transcription reactions for which the HeLa whole cell extract (WCE) was treated with α-SNAP43 antisera

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to remove endogenous $SNAP_C$, as shown in lanes 6–8. Lane 4 shows the decreased signal for the correctly initiated U6 transcription upon removal of endogenous $SNAP_C$. Lane 5 shows the U6 signal dependent upon addition of endogenous $SNAP_C$ obtained from biochemical fractionation of a HeLa cell nuclear extract. Note, this signal is comparable to the mock depleted WCE (lane 3) or WCE alone (lane 1).

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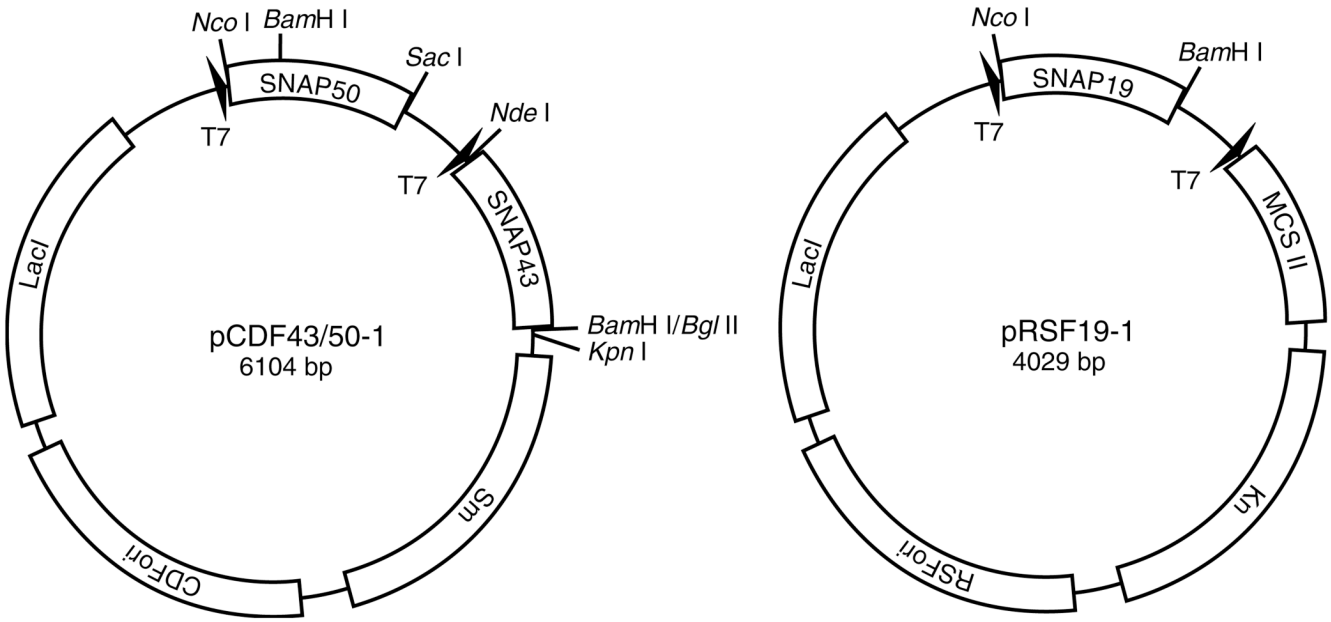

Fig. 2.

The polycistronic plasmids used for co-expression of mSNAP$_{C}\gamma$4. The pCDF43/50-1 expression plasmid contains the full-length SNAP50 ORF in the first multiple cloning site and the full-length SNAP43 ORF in the second multiple cloning site. This plasmid carries the CDF origin of replication and provides streptomycin resistance. The pRSF19-1 expression plasmid contains the SNAP19 ORF in the first multiple cloning site. The second multiple cloning site was not used in this strategy. This plasmid carries the RSF origin of replication and provides kanamycin resistance.

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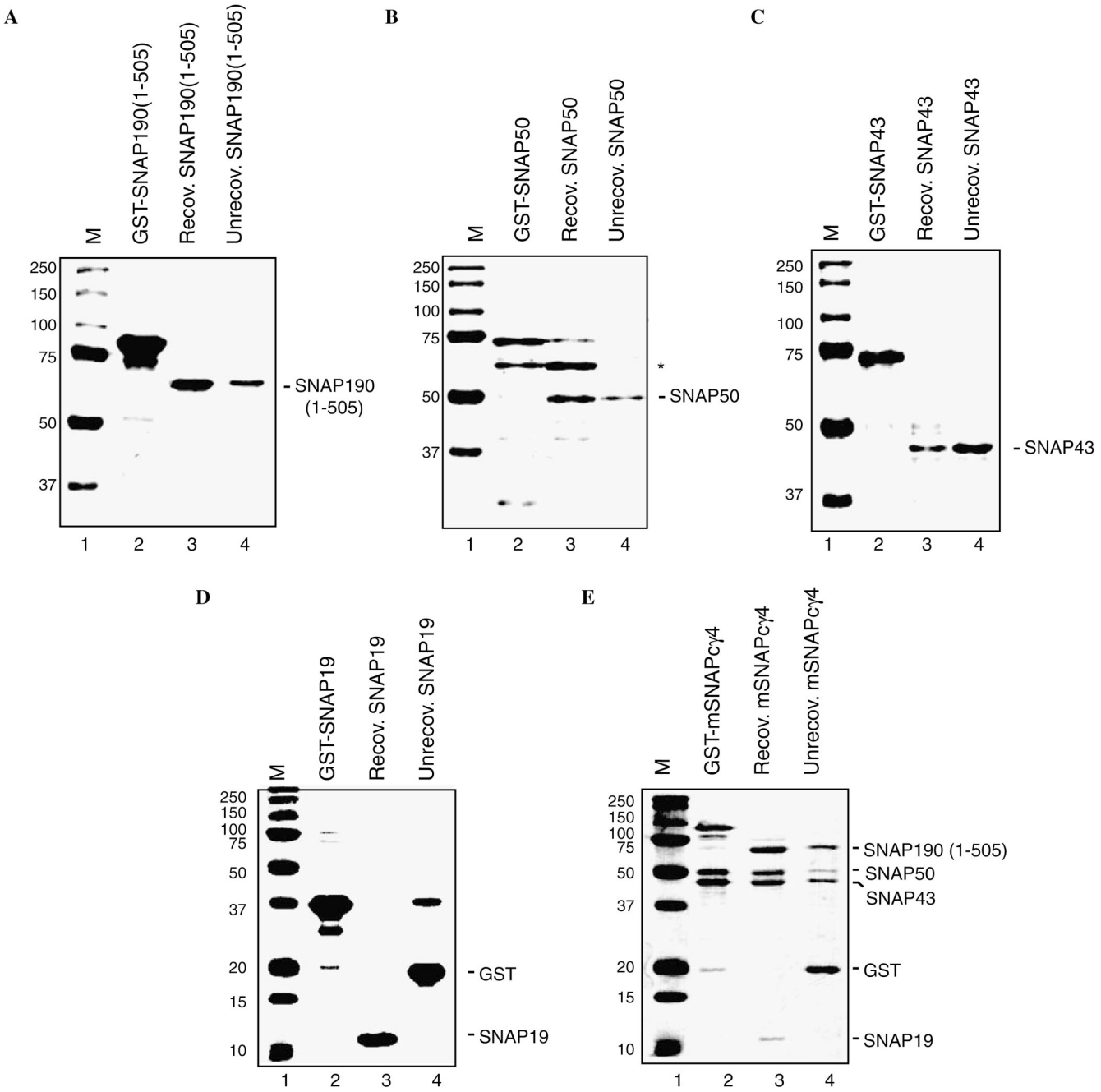

Fig. 3.

Co-expression of $mSNAP_C\gamma4$ improves subunit recovery and purity. (A–D) Each $SNAP_C$ subunit was individually expressed in *E. coli* as a GST-fusion protein for subsequent affinity purification using glutathione agarose beads. The amounts of each GST-fusion protein originally bound to the beads are shown in lane 2. After binding, proteins were released by digestion with thrombin, which cuts between the GST-tag and each $SNAP_C$ protein to release the soluble untagged protein (lane 3). Laemmli buffer was then added to the washed beads and any proteins that remained associated with the beads were released by boiling (lane 4). Equivalent amounts of the recovered and unrecovered samples were loaded to estimate the proportion of each recombinant protein that remains associated with the beads after digestion.

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Proteins were separated by SDS–PAGE and were visualized by staining with Coomassie blue. Note the association of stoichiometric amounts of an *E. coli* protein with SNAP50 [(B), labeled *] and the significant proportion of SNAP43 that remains bound to the beads after thrombin digestion (C) when these proteins are expressed individually. (E) The multi-subunit complex $mSNAP_{C}\gamma4$ was co-expressed in *E. coli* from the expression plasmids pCDF43/50-1, pRSF19-1, and pGST-190 (1–505). In this case, the complex was affinity purified through GST-SNAP190 (1–505) and the recovery of each component was estimated as above.

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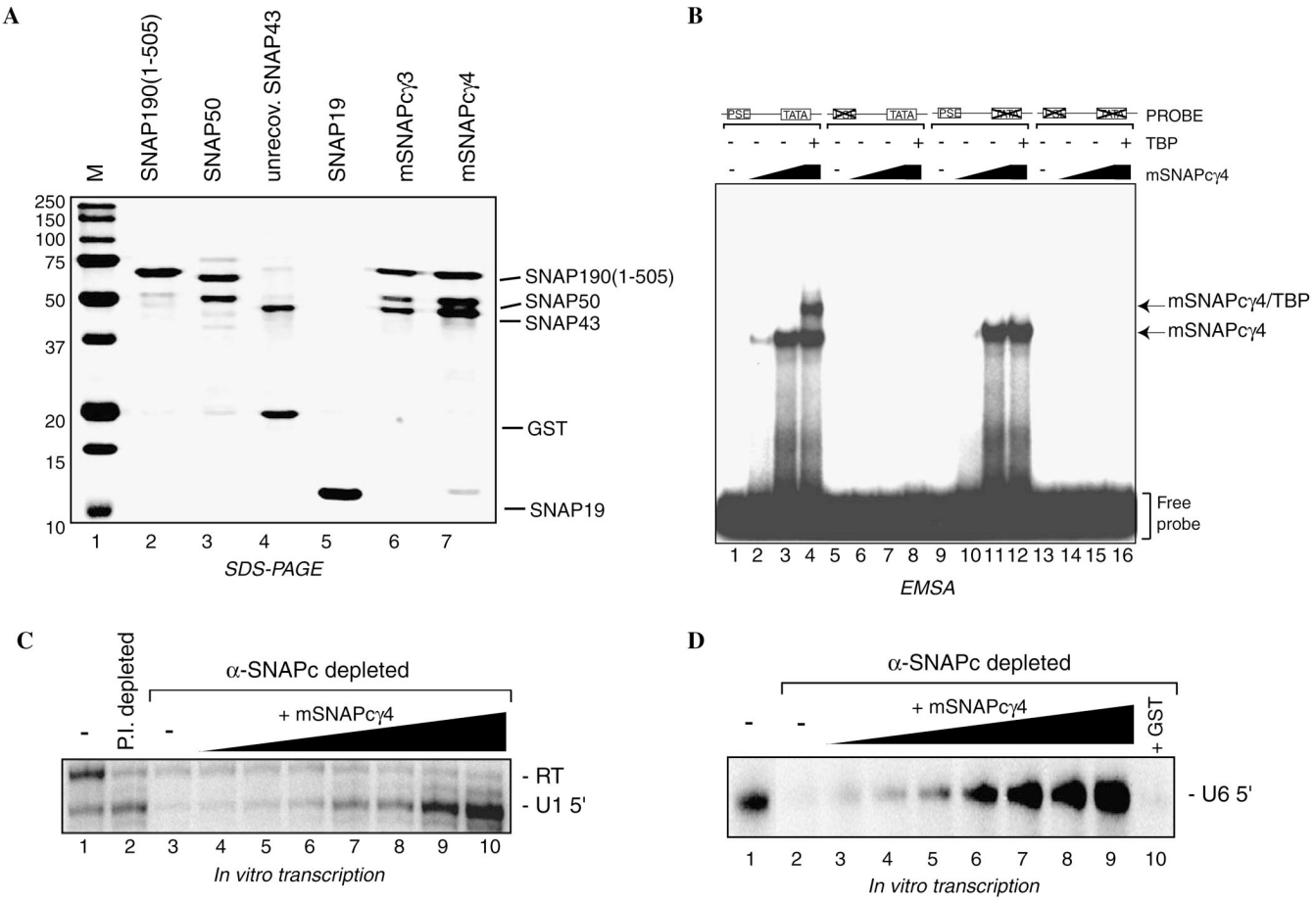

Fig. 4.

mSNAP$_C$γ4 functions for DNA binding, TBP recruitment, and in vitro transcription by RNA polymerases II and III. (A) SNAP19 improves complex stability and subunit recovery. Subunit recovery for partial SNAP$_C$ assembled without and with co-expressed SNAP19 (mSNAP$_C$γ3 and mSNAP$_C$γ4, respectively) was estimated by SDS–PAGE and Coomassie blue staining. Note the increased recovery of SNAP43 and SNAP50 in mSNAP$_C$γ4 (lane 7) compared to mSNAP$_C$γ3 (lane 6). Lanes 2–5 contain aliquots of the individually expressed SNAP$_C$ subunits for reference. (B) mSNAP$_C$γ4 facilitates TBP promoter recruitment. Increasing amounts of mSNAP$_C$γ4 (3 and 10 ng) were added to DNA binding reactions containing radiolabeled probes that harbor a wt PSE and wt TATA box (lanes 1–4), mu PSE and wt TATA box (lanes 5–8), wt PSE and mu TATA box (lanes 9–12), or mu PSE and mu TATA box (lanes 13–16). Lanes 4, 8, 12, and 16 contain 50 ng of recombinant full-length human TBP in addition to 10 ng of mSNAP$_C$γ4. Reactions containing only the DNA probes are shown in lanes 1, 5, 9, and 13. Positions of the mSNAP$_C$γ4 and mSNAP$_C$γ4 plus TBP complexes are indicated. (C) mSNAP$_C$γ4 functions for U1 snRNA transcription by RNA polymerase II. HeLa cell nuclear extract was either mock depleted with a preimmune rabbit sera (lane 2) or α-SNAP43 antisera (lanes 3–10) to deplete endogenous SNAP$_C$. Extracts were then used for human U1 in vitro transcription assays. The U1-specific signal was diminished upon removal of endogenous SNAP$_C$, as shown in lane 3. Increasing amounts of mSNAP$_C$γ4 (0.08, 0.25, 0.75, 2.5, 7.5, 25, and 75 ng) reconstituted the correctly initiated transcription from a human U1 reporter, as shown in lanes 4–10. Lanes 1 and 2 show the U1 signal obtained from either untreated or mock depleted reactions. (D) mSNAP$_C$γ4 functions for U6 snRNA transcription by RNA polymerase III. In vitro transcription of human U6 snRNA was carried out using HeLa cell nuclear extract

that was treated as in (C). Lane 2 shows the reduced U6 signal upon removal of endogenous $SNAP_C$. Increasing amounts of $mSNAP_C\gamma4$ (0.08, 0.25, 0.75, 2.5, 7.5, 25, and 75 ng) reconstituted the correctly initiated transcription from a human U6 reporter as shown in lanes 3–9. Approximately 75 ng of GST was added to the transcription reaction shown in lane 10.

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