A Map of Protein-Protein Contacts within the Small Nuclear RNA-activating Protein Complex SNAPc*

The nucleation of RNA polymerases I–III transcription complexes is usually directed by distinct multisubunit factors. In the case of the human RNA polymerase II and III small nuclear RNA (snRNA) genes, whose core promoters consist of a proximal sequence element (PSE) and a PSE combined with a TATA box, respectively, the same multisubunit complex is involved in the establishment of RNA polymerase II and III initiation complexes. This factor, the snRNA-activating protein complex or SNAPc, binds to the PSE of both types of promoters and contains five types of subunits, SNAP190, SNAP50, SNAP45, SNAP43, and SNAP19. SNAPc binds cooperatively with both Oct-1, an activator of snRNA promoters, and in the RNA polymerase III snRNA promoters, with TATA-binding protein, which binds to the TATA box located downstream of the PSE. Here we have defined subunit domains required for SNAPc subunit-subunit association, and we show that complexes containing little more than the domains mapped here as required for subunit-subunit contacts bind specifically to the PSE. These data provide a detailed map of the subunit-subunit interactions within a multifunctional basal transcription complex.

The basal transcription machineries that recruit RNA polymerases I–III to promoters are all composed of large multisubunit complexes. Such complexes are well suited to combinatorial mechanisms of transcription regulation because they provide for a large amount of flexibility by having many protein surfaces accessible for interactions with transcription activators and repressors. Thus, the same multisubunit complexes are used at many different promoters, but in each case different subsets of protein surfaces are functional. The small nuclear RNA (snRNA)-activating protein complex SNAPc (1, 2), also called PTF (3, 4), is an especially interesting example because it offers a unique system to understand the functions of the various surfaces of a basal transcription multisubunit complex. It also offers a unique opportunity to understand how such a complex is assembled. Toward these goals, we have mapped subunit-subunit contacts within SNAPc. We show that we can assemble a complex missing SNAP19, SNAP45, and large segments of SNAP190 and SNAP43 that is still capable of binding to DNA. Together, these results reveal the detailed architecture of a DNA-binding complex capable of nucleating both RNA polymerase II and III transcription initiation complexes.

EXPERIMENTAL PROCEDURES

Translations in Vitro—The various truncated open reading frames were subcloned into derivatives of the expression vector pCite (Novagen). All SNAP190 truncations were fused to an HA tag at the N terminus of the protein, whereas the C-terminal SNAP43 truncations were fused at their C terminus to the last 14 aa of SNAP43, which are recognized by the anti-SNAP43 antibody anti-CH3375 (1). The pCite constructs were used as templates for in vitro transcription and translation with the TNT T7 coupled reticulocyte lysate system (Promega L4610). Two μg of DNA template were mixed with 25 μl of reaction buffer, (ii) 1 μl of 1 mM amino acids minus methionine mix, and (iii) 1 μl of 40 units/μl RNasin ribonuclease inhibitor, all supplied by the manufacturer, and 2 μl of [35S]methionine at 10 μCi/ml in a total volume of 50 μl, and incubated for 30 min at 30 °C. For proteins that contained few or no methionines,
The regions of protein-protein contacts within SNAPc, which is shown in Fig. 1. From this assay we have established a crude map of protein-protein interactions in SNAPc. Note that the stoichiometry of the various subunits is not known.

The 1 μl of 1 mM amino acids minus methionine mix was replaced by 1 μl of 1 mM amino acids minus cysteine mix, and the 2 μl of [35S]methionine at 10 μCi/ml were replaced by 2 μl of [35S]cysteine at 10 μCi/ml.

**Immunoprecipitations**—Five μl of the relevant in vitro translated proteins were mixed with 10 μl of protein G-agarose beads (packed bed volume) covalently coupled to the 12CA5 monoclonal antibody, which recognizes the HA tag (16), or 10 μl of protein A-agarose beads (packed bed volume) covalently coupled to the anti-SNAP43 anti-CSH3755 antibody (CS48), which recognizes the last 14 aa of SNAP43 (1), or 10 μl of protein A-agarose beads coupled to the anti-SNAP50 antibody (anti-CSH482, CS303 (17)), in 500 μl of HEMGT-150 (25 mM HEPES (pH 7.9), 0.2 mM EDTA, 12.5 mM MgCl2, 10% glycerol, 0.1% Tween 20, 150 mM KCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 0.5 mM bismuthate, and 0.5 mM benzamidine), and the mixture was rotated for 1 h at room temperature. The beads were then washed four times with HEMGT-150, pelleted, and resuspended in 25 μl of 2× Laemmli buffer (125 mM Tris (pH 6.8), 20% glycerol, 4% SDS, 2% 2-mercaptoethanol, 0.01% bromphenol blue). The beads were boiled for 3 min and pelleted, and the supernatant was loaded onto a 15% SDS-polyacrylamide gel. The [35S]methionine-labeled proteins were then detected by autoradiography.

**Assembly of SNAP Subcomplexes**—Open reading frames encoding various SNAP, subunits or truncated subunits were subcloned into derivatives of the bacterial expression vector pSBet (18) and expressed in BL21 bacterial cells according to Studier et al. (19). The SNAP190 derivatives contained six histidines fused to the C terminus. The subcomplexes were formed by mixing the relevant soluble protein lysates for 2 h at 4°C. Each complex was purified by a 20–45% ammonium sulfate precipitation followed by chromatography on Ni2+-nitrilotricarboxylic acid-agarose beads (Qiagen). The various subcomplexes were analyzed for DNA binding in an electromobility shift assay with a probe containing a high affinity mouse U6 PSE or a mutated PSE as described by Mittal and Hernandez (14).

**Results**

By determining the abilities of various SNAPc subunits translated in vitro to coimmunoprecipitate with HA-tagged SNAP190 truncations lacking increasing amounts of C-terminal sequences. The results are shown in Fig. 3A. The HA-SNAP190 truncations as well as SNAP19 could be expressed by in vitro translation (lanes 1–7). SNAP19 was coimmunoprecipitated with HA-SNAP190 truncations lacking C-terminal sequences up to aa 133 but not up to aa 92 (compare lane 13 to lanes 8–12). The anti-HA antibody did not immunoprecipitate SNAP19 in the absence of HA-SNAP190 (lane 14). Fig. 3B shows coimmunoprecipitations of the same HA-SNAP190 C-terminal truncations, SNAP19 and SNAP43. In vitro translated SNAP43 (lane 8) was mixed with cotranslated HA-SNAP190 truncations and SNAP19 (lanes 1–7), and the resulting mixtures of proteins were used for immunoprecipitations with the anti-SNAP43 antibody. HA-SNAP190 truncations lacking C-terminal sequences up to aa 133 but not up to aa 92 were coimmunoprecipitated with SNAP19 and SNAP43 (compare lane 14 to lanes 9–13). In no case did we see coimmunoprecipitation of SNAP43 with either SNAP190 or SNAP19 alone, consistent with our previous observation that in this assay SNAP43 associates efficiently with SNAP190 only in the presence of SNAP19 (2). As expected, the anti-SNAP43 antibody did not immunoprecipitate SNAP19 or HA-SNAP190 in the absence of SNAP43 (lane 15). An HA-SNAP190 truncation extending from aa 63 to 121 (SNAP190-(63–121)) could also associate with SNAP19 and SNAP43 together with...
A SNAP190 region extending from aa 84 to 133 is sufficient for association with SNAP19 and both SNAP43 and SNAP19. A, left panel, 1 μl of the HA-SNAP190 deletions indicated on top cotranslated with SNAP19 (lanes 1–6) or 1 μl of SNAP19 alone (lane 7) was loaded directly on the gel. In the right panel, 5 μl of the cotranslated proteins indicated on top (lanes 8–13) or 5 μl of SNAP19 alone (lane 14) was used for immunoprecipitations with an anti-HA antibody, and the immunoprecipitated material was loaded on the gel. The locations of the HA-SNAP190 deletions and of SNAP19 are indicated. B, left panel, 1 μl of the HA-SNAP19 deletions indicated on top cotranslated with SNAP19 (lanes 1–6) or 1 μl of SNAP19 alone (lane 7) or 1 μl of SNAP43 (lane 8) was loaded directly on the gel. In the right panel, 5 μl of cotranslated SNAP19 and the SNAP190 deletions indicated on top (lanes 8–13) were mixed with 5 μl of SNAP43 (lanes 9–14), incubated at 4 °C for 10 min, and used for immunoprecipitations with an anti-SNAP43 antibody. In lane 15, SNAP19 was absent from the starting material, and in lane 16, the starting material only contained SNAP43. The locations of the HA-SNAP190 deletions, SNAP19, and SNAP43 are indicated. C, lanes 1–4 and 11–14 show 1 μl of the SNAP190 deletions indicated on top cotranslated with SNAP19. Lane 5 shows 1 μl of SNAP19 alone, and lane 15 shows 1 μl of SNAP43 alone. In lanes 6–10, 5 μl of the material shown in lanes 1–5 was used for immunoprecipitation with an anti-HA antibody. In lanes 16–19, 5 μl of the cotranslated SNAP190 deletions and SNAP19 indicated on the top was mixed with 5 μl of SNAP43, incubated at 4 °C for 10 min, and used for immunoprecipitations with an anti-SNAP43 antibody. In lane 20, SNAP43 was absent from the starting material. The locations of the HA-SNAP190 deletions, SNAP19, and SNAP43 are indicated.

SNAPc Subunit-Subunit Interactions

FIG. 3. A SNAP190 region extending from aa 84 to 133 is sufficient for association with SNAP19 and both SNAP43 and SNAP19. A SNAP190 region extending from aa 84 to 133 is sufficient for association with SNAP19 and both SNAP43 and SNAP19. A, left panel, 1 μl of the HA-SNAP190 deletions indicated on top cotranslated with SNAP19 (lanes 1–6) or 1 μl of SNAP19 alone (lane 7) was loaded directly on the gel. In the right panel, 5 μl of the cotranslated proteins indicated on top (lanes 8–13) or 5 μl of SNAP19 alone (lane 14) was used for immunoprecipitations with an anti-HA antibody, and the immunoprecipitated material was loaded on the gel. The locations of the HA-SNAP190 deletions and of SNAP19 are indicated. B, left panel, 1 μl of the HA-SNAP19 deletions indicated on top cotranslated with SNAP19 (lanes 1–6) or 1 μl of SNAP19 alone (lane 7) or 1 μl of SNAP43 (lane 8) was loaded directly on the gel. In the right panel, 5 μl of cotranslated SNAP19 and the SNAP190 deletions indicated on top (lanes 8–13) were mixed with 5 μl of SNAP43 (lanes 9–14), incubated at 4 °C for 10 min, and used for immunoprecipitations with an anti-SNAP43 antibody. In lane 15, SNAP19 was absent from the starting material, and in lane 16, the starting material only contained SNAP43. The locations of the HA-SNAP190 deletions, SNAP19, and SNAP43 are indicated. C, lanes 1–4 and 11–14 show 1 μl of the SNAP190 deletions indicated on top cotranslated with SNAP19. Lane 5 shows 1 μl of SNAP19 alone, and lane 15 shows 1 μl of SNAP43 alone. In lanes 6–10, 5 μl of the material shown in lanes 1–5 was used for immunoprecipitation with an anti-HA antibody. In lanes 16–19, 5 μl of the cotranslated SNAP190 deletions and SNAP19 indicated on the top was mixed with 5 μl of SNAP43, incubated at 4 °C for 10 min, and used for immunoprecipitations with an anti-SNAP43 antibody. In lane 20, SNAP43 was absent from the starting material. The locations of the HA-SNAP190 deletions, SNAP19, and SNAP43 are indicated.
Fig. 4. Amino acid changes on the same face of a putative α-helix in SNAP190-(84–133) disrupt association with SNAP19 and SNAP43 together with SNAP19. A, top, the sequence of SNAP190 from aa 84 to 133 is shown. The leucines and glutamines that are separated by 6 aa and therefore predicted to be on the same side of an α-helix are circled. The brackets above the sequence indicate the aa mutated in double aa changes, and the dots indicate single aa changes. Bottom, the effects of the various mutations on communoprecipitation with SNAP19 or SNAP43 with SNAP19 are summarized. B, left panel, 1 μl of the HA-SNAP190-(84–133) mutations indicated on top cotranslated with SNAP19 (lanes 1–4) or 1 μl of SNAP19 alone (lanes 5) or 1 μl of SNAP43 (lane 6) were loaded directly on the gel. In the middle panel, 5 μl of the material shown in lanes 1–5 was used for communoprecipitation with an anti-HA antibody (lanes 7–11). In the right panel, 5 μl cotranslated SNAP19 and the SNAP190-(84–133) mutations indicated on top were mixed with 5 μl of SNAP43 (lanes 12–15), incubated at 4 °C for 10 min, and used for communoprecipitations with an anti-SNAP43 antibody. In lane 16, SNAP190 was absent from the starting material. The locations of the HA-SNAP190-(84–133) mutations, SNAP19, and SNAP43 are indicated. C, left panel, 1 μl of the HA-SNAP190-(84–133) mutations indicated on top cotranslated with SNAP19 (lanes 1–5) or 1 μl of SNAP19 alone (lane 6) or 1 μl of SNAP43 (lane 7) were loaded directly on the gel. In the middle panel, 5 μl of the material shown in

V96A had only a small effect on association with SNAP19 (lane 9). The L89A/V96A mutation had also a minimal effect on association with both SNAP43 and SNAP19, as did the single amino acid changes Q94A and Q115L (lanes 15, 16 and 18). The Q94L mutation had the most severe effect on association with SNAP43 together with SNAP19, but association was still clearly detectable (lane 17). Together, these data indicate that in general association with SNAP19 alone is more sensitive to mutations than association with both SNAP43 and SNAP19. This is not unexpected and is consistent with the idea that SNAP43 contacts weakly both SNAP19 and SNAP190 and thus stabilizes the SNAP190-SNAP19 interaction. The results also support the idea that SNAP190 adopts an α-helical structure within at least part of the region analyzed, one face of which is involved in protein-protein contacts with SNAP19 and SNAP43. The region is not a typical leucine zipper, however, as replacement of the two glutamines with leucines is deleterious.

Mutations in the N-terminal Half of SNAP19 Disrupt Association with SNAP190 and SNAP19 Together with SNAP43—We then turned our attention to SNAP19. The N-terminal half of SNAP19 contains five leucines separated by 6 aa that could potentially form a leucine zipper (2), and indeed, this region receives very high scores when analyzed with the COILS program (default parameters, 0.907–0.961 between aa 4 and 20 with a window of 14; 0.906 to 0.999 between aa 1 and 36 with a window of 28) (20). We suspected, therefore, that this region may interact with the putative α-helix in SNAP190. To test this possibility, we introduced a number of single and double amino acid changes, whose locations and whose effects on association with SNAP190 or SNAP43 together with SNAP190 are summarized in Fig. 5A. The results are shown in Fig. 4, B and C. The mutations L8A, L15A/L22A, and L29A/L36A all modify leucines that are part of the putative leucine zipper. In contrast, L18A modifies a leucine residue that is out of register with the leucine zipper, and thus on another face of the putative α-helix. All mutated versions of SNAP19 were efficiently expressed by in vitro translation, but some of the mutations significantly retarded the migration of the protein in an SDS-polyacrylamide gel (Fig. 5B, lanes 1–6). When tested for association with HA-SNAP190-(84–133) alone, all mutations had a dramatic negative effect except for the L18A mutation, which is out of register with the leucine zipper and which had little or no effect (Fig. 5B, lanes 7–12). When tested for association with SNAP43 together with HA-SNAP190-(84–133), both double mutations had a strong negative effect, whereas the L8A and L18A mutations still retained some activity (Fig. 5C, lanes 7–12). Together, these results suggest that the N-terminal region of SNAP19 assumes an α-helical structure with one face, encompassing Leu-8 to at least Leu-29 and perhaps Leu-36, important for contacts with SNAP190 and the region from Leu-15 or perhaps Leu-22 to Leu-29 or Leu-36, important for contacts with SNAP43 together with SNAP190.

A SNAP43 Region Extending from aa 164 to 268 Is Sufficient for Interaction with SNAP19 and SNAP190—To determine which region of SNAP43 is required for interaction with SNAP19 and SNAP190, we constructed a number of SNAP43 truncations. Fig. 6A shows the location of these truncations and a summary of their ability to interact with SNAP19 and
SNAP19 (1st column). Some of the results for association with SNAP19 and SNAP190 are shown in Fig. 6B. The SNAP43 deletions were in general difficult to translate in vitro, and several constructs gave rise to a number of bands (see for example in Fig. 6B, lanes 2, 3, 5, and 6). We mixed the various SNAP43 deletions with SNAP190-(1–261) and SNAP19, and we used an antibody directed against the C terminus of SNAP190, and SNAP190 together with SNAP43. A, top, the sequence of SNAP19 from aa 1 to 40 is shown. The leucines that are separated by 6 aa and therefore predicted to be on the same side of an α-helix are circled. The brackets above the sequence indicate the aa mutated in double aa changes, and the dots indicate single aa changes. Bottom, the effects of the various SNAP19 mutations on co-immunoprecipitation with HA-SNAP190-(84–133), or SNAP43 with SNAP190-(84–133), are summarized. B, left panel, 1 μl of the SNAP19 mutations indicated on top cotranslated with HA-SNAP190-(84–133) (lanes 1–5) or 1 μl of HA-SNAP190-(84–133) alone (lane 6) were loaded directly on the gel. In the right panel, 5 μl of the material shown in lanes 1–6 were used for immunoprecipitation with an anti-HA antibody (lanes 7–12). The locations of the SNAP19 mutations and HA-SNAP190-(84–133) are indicated. C, left panel, 1 μl of the SNAP19 mutations indicated on top cotranslated with HA-SNAP190-(84–133) mixed with 1 μl of SNAP43 (lanes 1–5) or 1 μl of SNAP19 cotranslated with HA-SNAP190-(84–133) alone (lane 6) were loaded on the gel. In the right panel, 5 μl of the material shown in lanes 1–6 were used for immunoprecipitation with an anti-SNAP43 antibody (lanes 7–12). The locations of the SNAP19 mutations, HA-SNAP190-(84–133), and SNAP43 are indicated.

FIG. 5. Mutations in the N-terminal half of SNAP19 disrupt association with SNAP190 and SNAP190 together with SNAP43. A, top, the sequence of SNAP19 from aa 1 to 40 is shown. The leucines that are separated by 6 aa and therefore predicted to be on the same side of an α-helix are circled. The brackets above the sequence indicate the aa mutated in double aa changes, and the dots indicate single aa changes. Bottom, the effects of the various SNAP19 mutations on co-immunoprecipitation with HA-SNAP190-(84–133), or SNAP43 with SNAP190-(84–133), are summarized. B, left panel, 1 μl of the SNAP19 mutations indicated on top cotranslated with HA-SNAP190-(84–133) (lanes 1–5) or 1 μl of HA-SNAP190-(84–133) alone (lane 6) were loaded directly on the gel. In the right panel, 5 μl of the material shown in lanes 1–6 were used for immunoprecipitation with an anti-HA antibody (lanes 7–12). The locations of the SNAP19 mutations and HA-SNAP190-(84–133) are indicated. C, left panel, 1 μl of the SNAP19 mutations indicated on top cotranslated with HA-SNAP190-(84–133) mixed with 1 μl of SNAP43 (lanes 1–5) or 1 μl of SNAP19 cotranslated with HA-SNAP190-(84–133) alone (lane 6) were loaded on the gel. In the right panel, 5 μl of the material shown in lanes 1–6 were used for immunoprecipitation with an anti-SNAP43 antibody (lanes 7–12). The locations of the SNAP19 mutations, HA-SNAP190-(84–133), and SNAP43 are indicated.
SNAP43 for immunoprecipitation. SNAP190-(1–261) migrated well above the SNAP43 bands and was therefore easily visible on the gels, but SNAP19 (marked with white arrowheads in Fig. 6B) migrated close to some of the SNAP43 bands and was therefore more difficult to see (see Fig. 6B). Nevertheless, by comparing the immunoprecipitated bands (lanes 8–14) with the bands present in the starting materials (lanes 1–7), it was possible to obtain unambiguous results. Full-length SNAP43 coimmunoprecipitated SNAP19 and SNAP190-(1–261) (Fig. 3B, lane 10), but neither the N-terminal (SNAP43-(1–185)) nor the C-terminal (SNAP43-(187–368)) half of SNAP43 did (Fig. 6B, lanes 10 and 11). We therefore truncated increasing amounts of N-terminal sequences from full-length SNAP43, and we found that a truncation missing the first 163 aa (SNAP43-(164–368)) was still active (not shown, see Fig. 6A). Thus, the N-terminal border of the region required for association with SNAP19 and SNAP190-(1–261) is located between aa 164 and 187. C-terminal truncations showed that a SNAP43 protein ending at position 268 coimmunoprecipitated SNAP19 and SNAP190-(1–261) (Fig. 6B, lanes 9), but one ending at position 227 did not (Fig. 6B, lane 13, and data not shown; see Fig. 6A). We then constructed a SNAP43 truncation extending from 164 to 268 and, as shown in lane 12 of Fig. 6B, this truncation efficiently coimmunoprecipitated SNAP190-(1–261) and SNAP19. Thus, this SNAP43 region is sufficient for association with SNAP190-(1–261) and SNAP19.

The First 168 aa of SNAP43 Are Sufficient for Association with SNAP50—SNAP43 associates directly with SNAP50 (17), and we therefore checked the abilities of the various SNAP43 truncations to associate with SNAP50. The results are summarized in Fig. 6A (2nd column), and some of the results are shown in Fig. 6C. When full-length SNAP50 was mixed with full-length SNAP43 and immunoprecipitated with an anti-SNAP50 antibody, SNAP43 was coimmunoprecipitated (Fig. 6C, lane 10). Similarly, the N-terminal half of SNAP43 extending from aa 1 to 185, but not the C-terminal half extending from aa 187 to 368, was coimmunoprecipitated with SNAP50 (lanes 11 and 12). A further truncation containing aa 1–163 could also associate with SNAP50 (not shown). Truncations removing aa from the N terminus showed that deletions of the first 32 aa of SNAP43 did not prevent association with SNAP50 (lanes 14 and 15), but deletion of the first 57 aa did (not shown, see Fig. 6A). Thus, the N-terminal border of the SNAP43 region required for association with SNAP50 lies between aa 33 and 58. These data also show that SNAP43 sequences between aa 33 and 163 are required for association with SNAP50 although, because we did not test whether a SNAP43 truncation extending from aa 33 to 163 can still associate with SNAP50, we do not know that these sequences are sufficient. Nevertheless, these results delimit sequences required for association with SNAP50 and show that they are completely separate from the sequences required for association with SNAP190 and SNAP19.

A Small SNAP190 Region Extending from aa 1281 to 1393 Is Sufficient for Efficient Association with SNAP45—We have shown before that SNAP45 associates directly with the C-terminal half of SNAP190 (10). To map more precisely the SNAP190 region required for this association, we generated the HA-tagged SNAP190 truncations shown in Fig. 7A, and we tested them for association with SNAP45 in immunoprecipitations with the anti-HA 12CA5 monoclonal antibody. The results are summarized in Fig. 7A and some of them are shown in Fig. 7B.

As we observed previously (10), a SNAP190 truncation encompassing aa 800 to 1469 could associate with SNAP45 (not shown, Fig. 7A). We then tested separately the N-terminal half and C-terminal half of this SNAP190 truncation; the N-terminal half extending from aa 800 to 1176 did not coimmunoprecipitate SNAP45, whereas the C-terminal half extending from aa 1177 to 1469 did (not shown, Fig. 7A). When this C-terminal truncation was further divided into two halves, extending from aa 1177 to 1338 and from 1338 to 1469, the ability to coimmunoprecipitate SNAP45 was lost (Fig. 7B, lanes 10 and 11). We then generated N- and C-terminal truncations of the HA-SNAP190-(1177–1393) fragment. The N-terminal border of the region required for association with SNAP45 is between aa 1281 and 1308 because the HA-SNAP190-(1281–1393) truncation coimmunoprecipitated SNAP45, whereas the HA-SNAP190-(1281–1308) truncation coimmunoprecipitated SNAP45 less efficiently (Fig. 7B, compare lanes 10 and 12). The C-terminal border is between aa 1364 and 1393, because the HA-SNAP190-(1281–1393) truncation coimmunoprecipitated SNAP45, whereas the HA-SNAP190-(1281–1364) truncation did not (Fig. 7B, lanes 9 and 13). Thus, the smallest SNAP190 fragment we tested that was still capable of associating fully efficiently with SNAP45 extends from aa 1281 to 1393.

Single and Double aa Changes within the SNAP190 1281–1393 Region Debilitate Association with SNAP45—We further defined the SNAP190 sequences required for association with SNAP45 by introducing the leucine to alanine changes shown...
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Fig. 8. Single and double aa changes within the SNAP190-(1281–1393) region debilitate association with SNAP45. A, top, the sequence of SNAP190 from aa 1281 to 1393 is shown. The leucines that are separated by 6 aa and therefore predicted to be on the same side of an α-helix are circled. The brackets above the sequence indicate the aa mutated in double aa changes, and the dots indicate single aa changes. Bottom, the effects of the various HA-SNAP190-(1281–1393) mutations on coimmunoprecipitation with SNAP45 are summarized. B, left panel, 1 µl of the HA-SNAP190-(1281–1393) mutants indicated on top cotranslated with SNAP45 (lanes 2–7) or 1 µl of SNAP45 alone (lane 8) were loaded directly on the gel. Lane 1 shows molecular weight markers. In the right panel, 5 µl of the material shown in lanes 2–8 were used for immunoprecipitation with an anti-HA antibody (lanes 9–15). The locations of SNAP45 and the HA-SNAP190-(1281–1393) mutations are indicated. C, left panel, 1 µl of the HA-SNAP190-(1281–1393) mutants indicated on top cotranslated with SNAP45 (lanes 2–4) were loaded on the gel. Lane 1 shows molecular weight markers. In the right panel, 5 µl of the material shown in lanes 2–4 were used for immunoprecipitation with an anti-HA antibody (lanes 5–7). In lane 8, the starting material for the immunoprecipitation was SNAP45 alone. The locations of SNAP45 and the HA-SNAP190-(1281–1393) mutations are indicated.

In Fig. 8A in SNAP190-(1281–1393) and testing their effect on association with SNAP45. The results are summarized in Fig. 8A and shown in Fig. 8 B and C. Changing leucine 1297 and glutamine 1304 had no effect, and changing leucines 1301 and 1308 had little effect on association with SNAP45 (Fig. 8B, compare lanes 10 and 11 with lane 9). However, changing leucine 1314 to alanine severely debilitated association with SNAP45 (lane 14). This is consistent with the analysis of N-terminal SNAP190 truncations above (Fig. 7B, lanes 9 and 12), which indicates that aa 1281–1308 contribute to efficient association with SNAP45 but are not absolutely required.

The C-terminal part of the 1281–1393 SNAP190 region contains 4 leucines (circled in Fig. 8A) separated by 6 aa. However, it is unlikely to form a leucine zipper structure because of the presence of a proline (9), and indeed, the region does not score well when analyzed with the COILS program (20). We mutated three of the leucines that are in register, namely leucine 1355 together with leucine 1362 as well as leucine 1369 as alanines. We also mutated leucine 1364, which is out of register, to alanine. Mutation of leucines 1355 together with 1362 as well as mutation of the out of register leucine 1364 reduced association with SNAP45 to undetectable levels (Fig. 8C, compare lanes 6 and 7 with lane 5). In contrast, mutation of leucine 1369 weakened but did not abolish association with SNAP45 (Fig. 8B, lane 13). These data further argue against this region forming a leucine zipper and are consistent with the deletion analysis above (Fig. 7, A and B, lanes 9 and 13) which indicates that the C-terminal border of the SNAP190 region required for interaction with SNAP45 is between aa 1364 and 1393. The mutation analysis suggests that the C-terminal border lies C-terminal of Leu-1369.

SNAPc Consisting of Little More Than Protein Sequences Mapped as Required for Subunit-Subunit Interactions Can Be Assembled and Bind to the PSE—We have shown before that we can assemble a subcomplex of SNAPc, which we call mini-SNAPc, and which consists of SNAP190 sequences extending from aa 1 to 514, full-length SNAP43, and full-length SNAP50. Although in coimmunoprecipitations of in vitro translated proteins, SNAP19 is required for detectable association of SNAP190 and SNAP43, SNAP19 is dispensable for the association of mini-SNAPc in insect cells, perhaps because a high SNAPc subunit concentration can be achieved in baculovirus-infected cells (10). Mini-SNAPc is capable of binding to the PSE and of directing basal RNA polymerase II and III transcription of smRNA genes (10). The analysis above identifies several regions within the first 514 aa of SNAP190 and within SNAP43 that are dispensable for subunit-subunit interactions in the coimmunoprecipitation assay. We therefore asked whether complexes capable of binding to the PSE could be assembled from Escherichia coli overexpressed subunits lacking these regions. The compositions of the various subcomplexes we tested are shown in Fig. 9A and an electromobility shift assay (EMSA) performed with probes containing either the high affinity wild type PSE derived from the mouse U6 gene (7) or a mutant PSE is shown in Fig. 9B.

Mini-SNAPc assembled in baculovirus-infected insect cells bound specifically to the PSE, as observed before (10) (Fig. 9B, lanes 2, 3, and 17). Furthermore, complex 1, which is nearly identical to mini-SNAPc except for a slightly shorter SNAP190 truncation (ending at aa 505 instead of 514 in mini-SNAPc), also bound specifically to the PSE (lanes 4, 5, and 18). Complex 1 formed a slightly faster migrating DNA-protein complex in the EMSA than mini-SNAPc expressed in insect cells. This may due to the small difference in size of the SNAP190 truncation or to differences in post-translational protein modifications. Complex 2 contains a truncated SNAP43 lacking the region (aa 269–368) dispensable for association with both SNAP190 and SNAP50 in coimmunoprecipitations (see Fig. 6A). It also bound efficiently to the PSE, although surprisingly, it formed a slower migrating DNA-protein complex than complex 1, even though
it is smaller. Perhaps deletion of part of SNAP43 changes the conformation of the complex.

Complexes 4, 6, and 8 contain the same truncated SNAP43 as complex 2 and SNAP190 truncations lacking increasing amounts of N-terminal sequences up to aa 84. These SNAP190 N-terminal sequences are not required for association with SNAP43 and SNAP19 in the coimmunoprecipitation assay (see Fig. 2). All these complexes could bind specifically to the PSE (lanes 6–13 and 19–22). Complex 15 contains full-length SNAP50, SNAP43, SNAP19, and a SNAP190 truncation lacking aa 1–84 as well as the region from aa 134 to 262, which separates the domain required for association with SNAP43 and SNAP19 (aa 84–133, see Fig. 2) from the Myb repeat domain (aa 263–503). This complex could bind specifically to the PSE, albeit not as efficiently as the other subcomplexes (lanes 14, 15, and 23). The same complex assembled in the absence of SNAP19 did not generate a DNA-protein complex in the EMSA (data not shown), suggesting that, with this particular SNAP190 truncation, either association with SNAP43 is not efficient enough in the absence of SNAP19 for assembly of the complex or SNAP19 is required for DNA binding. Importantly, however, these results show that DNA-binding complexes can be assembled from truncated subunits lacking sequences dispensable for coimmunoprecipitation of individual subunits. This observation strongly suggests that the protein-protein contacts mapped by the coimmunoprecipitation assay do indeed occur within SNAPc.

DISCUSSION

We have mapped protein regions required for subunit-subunit association within SNAPc. The assay we have used, coimmunoprecipitation of polypeptides translated in vitro, is a stringent assay that probably does not detect weak interactions. Thus the interactions we have mapped are probably the strongest but not necessarily the only protein-protein interactions within SNAPc.

Fig. 10 summarizes the functional domains mapped in this and previous work in the various SNAPc subunits. Within SNAP190, aa 84 to 133 are sufficient for association with SNAP19 alone and with SNAP43 together with SNAP19. This SNAP190 region, and the N-terminal part of SNAP19, are likely to form α-helices and may be involved in a coiled-coil type of interaction with each other. Indeed, SNAP19 contains five leucines spaced by 6 aa, and mutations of these leucines have a strong negative effect, whereas mutation of a leucine out of register has little effect (Fig. 5). Similarly, SNAP190 contains six leucines and glutamines that are separated by 6 aa and are, therefore, predicted to reside on the same face of an α-helix. Mutations that change subsets of these ααααααa delemitate association with SNAP19 and SNAP43 together with SNAP19, whereas a double mutation that changes 2 aa predicted to reside on another face of the helix, one of which is a leucine, has a much weaker negative effect on these associations (Fig. 4).

Interestingly, changing in register glutamines to leucines (mutations Q94L and Q115L) was as debilitating as changing them to alanines (see Fig. 4A). Thus, although this SNAP190 region is likely to form an α-helix involved in a coiled-coil type of interaction, it does not correspond to a classical leucine zipper. In SNAP43, aa 164–268 are sufficient for association with SNAP19 and SNAP19 and aa 1–163 are sufficient for association with SNAP50. Thus, these two association domains in SNAP43 are completely separable.

C-terminal to the SNAP19/SNAP43 association domain, SNAP190 contains an unusual Myb domain extending from aa 263 to 503, with four and a half Myb repeats designated the Rh (for R half), Ra, Rb, Rc, and Rd repeats (9). The last two Myb
repeats (Rc and Rd), but not the first two and a half (Rh, Ra, and Rb), are required for binding to the PSE (10). Within the C-terminal half of SNAP190 are two additional regions involved in protein-protein contacts. The Oct-1 interaction domain (OIR) lies between aa 869 and 912 (13), and the region required for interaction with SNAP45 lies between aa 1281 and 1393. Thus, the SNAP190 regions required for association with SNAP19/SNAP43 and with SNAP45 lie at opposite ends of the linear molecule. In our original description of SNAP190, we pointed out that the region defined here as required for association with SNAP45 contains leucine residues spaced by 6 aa, but we stressed that the leucines were unlikely to form a leucine zipper because of the presence of a proline (9). Indeed, our mutagenesis of this SNAP190 region does not support a model in which this region would form an α-helix with one face of the helix involved in protein-protein contacts with SNAP45 (Fig. 8).

The protein-protein contacts described above were defined in an assay in which we tested the abilities of two or three SNAP subunits to coimmunoprecipitate. The observation that we can assemble subcomplexes of SNAPc with subunits lacking many of the regions dispensable in the coimmunoprecipitation assay (see Fig. 9) strongly suggests, however, that the protein-protein contacts mapped in the coimmunoprecipitation assay reflect protein-protein contacts that indeed occur within SNAPc. The assembly of subcomplexes also provides some additional information. In our previous work, we assembled mini-SNAPc, a complex missing SNAP19, SNAP45, and the last two-thirds of SNAP190 (10). However, because in the coimmunoprecipitation assay, SNAP43 does not associate efficiently with SNAP190 in the absence of SNAP19, and because mini-SNAPc was assembled from recombinant subunits overexpressed in insect cells, we could not exclude the possibility that a SNAP19 endogenous to insect cells was getting incorporated into mini-SNAPc. Here we show that subcomplexes assembled from subunits expressed in E. coli and missing SNAP19 can also be assembled and bind DNA. This confirms that SNAP19 is not absolutely required for assembly of SNAPc. SNAP19 probably has a stabilizing role, however, because when we used a SNAP190 subunit consisting only of aa 84–133 and 263–518, we observed DNA binding only when SNAP19 was present in addition to SNAP43 and SNAP50.

Neither the C-terminal two-thirds of SNAP190 nor SNAP45 are required for basal RNA polymerase II and III transcription of snRNA genes (10), suggesting that this region of the complex has a regulatory role. Indeed, this region is involved in downregulation of SNAPc binding to DNA because deletion of this entire region results in a complex that binds DNA much more efficiently than complete SNAPc (10). This region is also required for cooperative binding with the Oct-1 POU domain and contains the OIR, a small SNAP190 region sufficient for association with Oct-1 in an electrophoretic mobility shift assay (13). It is possible that a region within the C-terminal two-thirds of SNAP190 and/or SNAP45 normally mask the DNA binding domain of SNAPc but undergo a conformational change upon binding of the OIR to the Oct-1 POU domain. It will be interesting to determine whether the C-terminal two-thirds of SNAP190 and/or SNAP45 are capable of associating with mini-SNAPc or derivatives thereof in trans. This work provides a detailed map of subunit-subunit contacts within a multisubunit complex involved in both RNA polymerase II and III basal transcription and opens the way to determining the functions of SNAPc subunit domains not required for subunit-subunit interactions.

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REFERENCES
1. Henry, R. W., Sadowski, C. L., Kobayashi, R., and Hernandez, N. (1995) Nature 374, 653–657.
2. Henry, R. W., Mittal, V., Ma, B., Kobayashi, R., and Hernandez, N. (1998) Genes Dev. 12, 2664–2672.
3. Murphy, S., Yoon, J.-B., Gerster, T., and Roeder, R. G. (1992) Mol. Cell. Biol. 12, 3247–3261.
4. Yoon, J.-B., Murphy, S., Bai, L., Wang, Z., and Roeder, R. G. (1995) Mol. Cell. Biol. 15, 2019–2027.
5. Henry, R. W., Ford, E., Mittal, R., Mittal, V., and Hernandez, N. (1998) Cold Spring Harb. Symp. Quant. Biol. 63, 111–120.
6. Lobo, S. M., Lister, J., Sullivan, M. L., and Hernandez, N. (1991) Genes Dev. 5, 1477–1489.
7. Sadowski, C. L., Henry, R. W., Lobo, S. M., and Hernandez, N. (1993) Genes Dev. 7, 1535–1548.
8. Yoon, J.-B., and Roeder, R. G. (1996) Mol. Cell. Biol. 16, 1–9.
9. Wong, M. W., Henry, R. W., Ma, B., Kobayashi, R., Klages, N., Matthias, P., Strubin, M., and Hernandez, N. (1999) Mol. Cell. Biol. 19, 368–377.
10. Mittal, V., Ma, B., and N., H. (1999) Genes Dev. 13, 1807–1821.
11. Mittal, V., Cleary, M. A., Herr, W., and Hernandez, N. (1996) Mol. Cell. Biol. 16, 1955–1965.
12. Ford, E., and Hernandez, N. (1997) J. Biol. Chem. 272, 16048–16055.
13. Ford, E., Strubin, M., and Hernandez, N. (1998) Genes Dev. 12, 3528–3540.
14. Mittal, V., and Hernandez, N. (1997) Science 275, 1136–1140.
15. Schramm, L., Pendergrast, P. S., Sun, Y., and Hernandez, N. (2000) Genes Dev., 14, 2650–2663.
16. Niman, H. L., Houghten, R. A., Walker, L. E., Reisfeld, R. A., Wilson, I. A., Hogle, J. M., and Lerner, R. A. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 4949–4953.
17. Henry, R. W., Ma, B., Sadowski, C. L., Kobayashi, R., and Hernandez, N. (1996) EMBO J. 15, 7129–7136.
18. Schenk, P. M., Baumann, S., Mattes, R., and Steinbiss, H.-H. (1995) BioTechniques 18, 196–200.
19. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dobendorff, J. W. (1990) Methods Enzymol. 185, 60–89.
20. Lupas, A., Van Dyke, M., and Stock, J. (1991) Science 252, 1162–1164.