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Leucine Periodicity of U2 Small Nuclear Ribonucleoprotein Particle (snRNP) A′ Protein Is Implicated in snRNP Assembly via Protein-Protein Interactions

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Recombinant A′ protein could be reconstituted into U2 small nuclear ribonucleoprotein particles (snRNPs) upon addition to HeLa cell extracts as determined by coimmunoprecipitation and particle density; however, direct binding to U2 RNA could not be demonstrated except in the presence of the U2 snRNP B″ protein. Mutational analysis indicated that a central core region of A′ was required for particle reconstitution. This region consists of five tandem repeats of approximately 24 amino acids each that exhibit a periodicity of leucine and asparagine residues that is distinct from the leucine zipper. Similar leucine-rich (Leu-Leu motif) repeats are characteristic of a diverse array of soluble and membrane-associated proteins from yeasts to humans but have not been reported previously to reside in nuclear proteins. Several of these proteins, including Toll, chaoptin, RNase/angiogenin inhibitors, lutropin-choriogonadotropin receptor, carboxypeptidase N, adenylyl cyclase, CD14, and human immunodeficiency virus type 1 Rev, may be involved in protein-protein interactions. Our findings suggest that in cell extracts the Leu-Leu motif of A′ is required for reconstitution with U2 snRNPs and perhaps with other components involved in splicing through protein-protein interactions.

The assembly of small nuclear ribonucleoprotein particles (snRNPs) involves three modes of macromolecular recognition: RNA-RNA, RNA-protein, and protein-protein interactions. Each U snRNP particle is composed of at least one distinct, short, highly structured and modified uridylate-rich RNA (snRNA) that is associated with a distinct set of 6 to 10 polypeptides. A core sextet of proteins, designated the Sm complex, is common to U snRNPs, but another set of accompanying proteins is unique to each snRNP. For example, U2 snRNP is composed of the 188-nucleotide U2 snRNA, the Sm sextet designated B′, B, D, E, F, and G, and two unique proteins, denoted A′ (28.4 kDa) and B″ (25.5 kDa) (17, 60; for a review, see reference 67).

While the participation of particular U snRNPs at specific points along the pre-mRNA splicing pathway has been established, the precise functions and modes of interaction of the snRNPs have only begun to be elucidated. Definition of the molecular interactions among snRNP components ultimately requires structural analysis and reconstitution of snRNP particles. For example, U snRNP particles have been reconstituted in vitro by the addition of cellular or synthetic snRNAs to cell extracts enriched for snRNP components (9, 18, 19, 44, 49–51). Progress toward reconstitution of snRNP complexes by using in vitro-synthesized proteins has been achieved by incubation of in vitro translation products of total HeLa cell poly(A)$^+$ RNA with a mixture of U snRNAs (65, 66) and by using the *Saccharomyces cerevisiae* RNA11 protein in vitro to complement yeast temperature-sensitive splicing mutants (5).

Evolutionarily conserved domains in snRNAs and snRNP proteins that mediate specific intra-snRNP intermolecular interactions have been identified by studies of native snRNP particles and of reconstituted snRNP components. For example, the conserved site on the U snRNAs to which the Sm protein complex binds (4, 33) as well as other protein binding sites on U1 and U2 snRNAs have been elucidated (6, 19, 37, 41, 42, 52, 57). In addition, the RNA binding domains on three snRNP proteins that recognize U1 and U2 snRNAs have been delineated also (1a, 38, 53, 57). Direct evidence for protein-protein interactions in snRNPs has not been reported, however. The presence of 10 or more proteins on a single snRNP improves the likelihood of such interactions. Recent evidence from our laboratory has suggested that an interaction between U2 snRNP A′ and B″ proteins is required for efficient U2 snRNA binding in vitro (1a), but A′ protein alone cannot bind to U2 RNA (13a).

To elucidate features of the U2 snRNP-A′ protein that are required for assembly with snRNP components and to study the nature of its interaction with U2 snRNA, we used snRNP reconstitution assays. A′ protein, whether synthesized in vitro or produced in *Escherichia coli*, was shown to reconstitute into U2 snRNPs in HeLa cell extracts. In addition, A′-derived polypeptides were assayed to define regions that mediated reconstitution. The region of A′ protein required for reconstitution encompassed tandem repeats that display a periodicity of leucines and an asparagine residue. This leucine-rich sequence, which we have termed the Leu-Leu motif, is distinct from the leucine zipper class of coiled coils. However, this motif is similar to that found in a number of eukaryotic proteins with diverse physiological roles and has been proposed to participate in intermolecular protein-protein interactions.

MATERIALS AND METHODS

Immunoprecipitation. Immunoprecipitation of radiolabeled cell extracts with autoantibodies was performed as described previously (14, 24, 32).

Preparation of HeLa cell extracts. Nuclear and S100 ex-

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tracts were prepared from HeLa cells by the method of Dignam et al. (8).

A′ constructs. Standard protocols were used for all recombinant techniques (40). Preparation of wild-type and mutant constructs with C-terminal deletions that terminated at amino acids 255, 249, 212, 207, 195, 175, 143, and 125 were created by restriction of the A′PL5 cDNA in pGEM-3zf(−) with *Nae*I, *Tth*III1, *Apa*I, *Bsm*I, *Hae*III, *Bst*NI, *Sca*I, and *Bst*EII, respectively. The N-terminal A′ deletion constructs used for in vitro translation, (39-255) and (22-255), in which the A′ open reading frame was fused in frame with the AUG initiation codon and the following 12 amino acids of the herpesvirus *tk* gene, were produced by cloning of A′ *Eco*RI fragments into the *Eco*RI site of in vitro transcription-translation vector pTK3.010 (55). The *Eco*RI A′ fragment of the N-terminal deletion mutant (39-255) was created by introducing an *Eco*RI restriction site by oligonucleotide-directed mutagenesis immediately 5′ to the beginning of the first leucine repeat of the full-length cDNA. Other N- and C-terminal deletions were constructed by using standard oligonucleotide-directed mutagenesis and the polymerase chain reaction. Site-directed mutants were generated with synthetic oligonucleotides by the oligonucleotide-directed in vitro mutagenesis system version 2 (Amersham). Capped runoff transcripts were produced in the presence of the cap analog diguanosine triphosphate (m^7G-5′ppp5′-G). RNAs were transcribed with SP6 (Promega Biotec) or T7 (USB) RNA polymerase as described by Promega. Transcripts were treated with DNase, phenol extracted, ethanol precipitated, and resuspended and one-fifth of the transcription product was translated in a rabbit reticulocyte lysate (Promega). In vitro translation products were immunoprecipitated directly from the A′ mRNA-driven reticulocyte lysate following a 10-fold dilution and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% gels. For in vivo synthesis of recombinant proteins with immunoreactive tags, deletion constructs were subcloned into the pET vector and extracts were prepared as described in reference 38.

RNase treatment of nuclear extract. For pretreatment with RNase, 13 μl of nuclear extract was incubated with RNase T_1 (Calbiochem) at a final concentration of 1 mg/ml or with 0.04 volume of 0.05 M $CaCl_2$ and 0.04 volume of micrococcal nuclease (MN) (Sigma) at 5 U/μl in a total volume of 17.5 μl for 30 min at 30°C. A sample of 0.08 volume of 0.1 M EGTA was added to the MN-treated mixture, and following a brief incubation, the mixture was adjusted to approximately 3.2 mM $MgCl_2$ and 40 μg of *E. coli* or yeast RNA (Sigma) was added prior to incubation with the mRNA-driven reticulocyte lysate as described above. Pretreatment of the extract by oligonucleotide-directed degradation was performed by incubation of 13 μl of nuclear extract, 1.3 U of RNase H (Boehringer Mannheim Biochemicals), 3.2 mM $MgCl_2$, 20 mM creatine phosphate, 0.5 mM ATP, and 10 μM complementary oligonucleotide in a total volume of 24 μl for 45 min at 30°C. Complementary oligonucleotides U2-L15 and U1-16 are identical to those previously demonstrated to degrade the respective snRNAs (2). To assess the extent of RNase digestion in each case, one-half of each pretreated extract mixture was aliquoted, incubated at 65°C with proteinase K (Bethesda Research Laboratories), and phenol-chloroform extracted. Deproteinized RNA was ethanol precipitated, resuspended, and analyzed by PAGE on an 7 M urea–8% acrylamide gel that was subsequently silver stained (Bio-Rad).

CsCl gradient analysis. The reaction mixture described above was scaled up 17-fold, incubated under conditions identical to those of the smaller-scale reaction, and then fractionated by isopycnic centrifugation in cesium chloride as described previously (31) in 3.2 mM Mg^{2+}. Fractions were dialyzed against buffer D (see below) and aliquoted. For protein analysis, fractions were immunoprecipitated with either Sm antiserum (AW) or A′-specific antiserum (EW) described previously (14). For RNA analysis, fractions were phenol-chloroform extracted, ethanol precipitated, and analyzed by PAGE on a denaturing 10% polyacrylamide gel.

RNP reconstitution assays. cDNA-driven reticulocyte lysate (3 μl) was added to 7.5 μl of buffer D (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 20% [wt/vol] glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol) or to HeLa cell nuclear or S100 extract in buffer D at approximately 8 mg/ml, and the mix was then adjusted to 3.2 mM $MgCl_2$ by the addition of 2 μl of 20 mM $MgCl_2$. Following incubation for 30 min at 30 or 42°C, the mixture was diluted by the addition of buffer A (10 mM Tris [pH 7.5], 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% Nonidet P-40) and adjusted to 10 mg of bovine serum albumin per ml to minimize nonspecific sticking of the translation product to the *Staphlyococcus* A protein. Optimization experiments at various temperatures indicated that incubation at 42°C was approximately twofold more efficient than incubation at 30°C, and thus the higher temperature was used. The mixture was precleared by successive incubations with Pansorbin (Calbiochem) and normal human serum prior to immunoprecipitation with 3 μl of the indicated antiserum as described previously (24, 32). Immunoprecipitates were washed five times in $NET2^+$ (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate), once in $NET2^+$–1 M urea, and once in NET2 (150 mM NaCl, 50 mM Tris [pH 7.4], 0.05% Nonidet P-40), resuspended in Laemmli buffer, and analyzed by PAGE on an SDS-15% polyacrylamide gel. Reconstitutions with ^{32}P-labeled HeLa cell extracts were performed as described above except that following the final wash, the immunoprecipitate was phenol extracted and ethanol precipitated, and the RNA was electrophoresed on a 5% acrylamide-urea gel. Reconstitutions with HeLa cell [^{32}P]RNA were performed as described previously (1a, 38, 53).

RESULTS

Association of A′ translation product with snRNPs. The derivation of cDNA clones used in this study is described elsewhere (13a), and they are similar to an A′ cDNA clone described previously (60). RNA transcripts containing either complete or partial coding sequences of A′ were synthesized in vitro by using either the SP6 or the T7 promoter in pGEM-3zf(−) plasmid vectors containing cDNA inserts. When the full-length A′ in vitro transcript was used to program rabbit reticulocyte lysates employing [^{35}S]methionine, [^{3}H]leucine, and [^{3}H]serine, an in vitro translation product was synthesized that comigrated with HeLa cell A′ protein at an apparent molecular mass of approximately 29 kDa (data not shown). The translation product was immunoprecipitable with numerous A′-specific antisera but not with other autoimmune or normal human sera (see Fig. 3B).

To determine whether the in vitro-translated polypeptide encoded by the A′ cDNA was functional, we assessed its association with ribonucleoprotein complexes. In vitro-radiolabeled A′ cDNA-programmed reticulocyte lysate was incubated with HeLa cell nuclear or S100 extracts or with

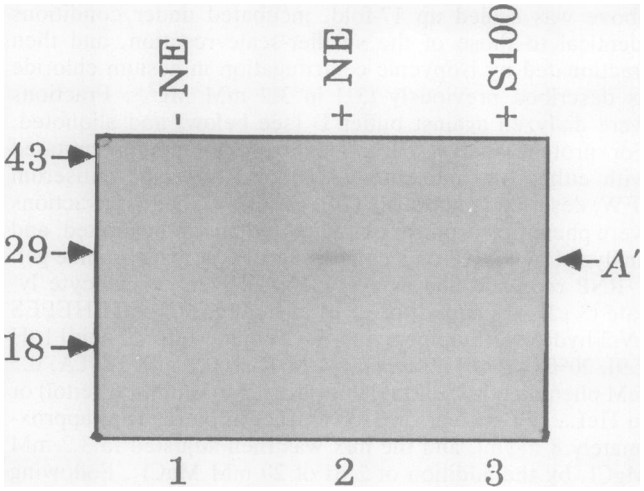


FIG. 1. Reconstitution of A′ translation product into an snRNP as demonstrated by immunoprecipitation with Sm antiserum. Radioactively labeled in vitro-synthesized A′ was incubated with buffer D (lane 1), HeLa cell nuclear extract (lane 2), or HeLa cell S100 extract (lane 3) and immunoprecipitated with an antiserum reactive with the Sm protein complex on U snRNPs. Immunoprecipitates were analyzed on an SDS–10% polyacrylamide gel. Numbers at left indicate molecular mass (in kilodaltons).

buffer alone for 30 min at 42°C and immunoprecipitated with an Sm antiserum specific for the Sm protein complex on U snRNPs (24). Incubation at 42°C allowed more efficient reconstitution than incubation at 30°C as noted in Materials and Methods. Following incubation, synthetic A′ protein was coimmunoprecipitated in the presence of HeLa cell nuclear extract (Fig. 1, lane 2) or S100 (Fig. 1, lane 3) but failed to be coimmunoprecipitated in the absence of HeLa cell extract (Fig. 1, lane 1). Association of synthetic A′ protein with the complex was shown to be independent of the presence of ATP and creatine phosphate by prior incubation of the extract at 30°C to deplete endogenous ATP (data not shown). This finding demonstrates that exogenous A′ protein can associate with the Sm complex in the presence of cell extracts and suggests that it reconstituted into snRNPs.

The association of synthetic A′ protein with snRNPs was studied further by subjecting the A′ cDNA-programmed reticulocyte lysate combined with nuclear extract to cesium chloride (CsCl) fractionation under conditions in which snRNPs band at a characteristic density. Fractions were collected and assayed for RNA and protein species. Immunoprecipitation of gradient fractions with an A′-specific antiserum (serum EW) revealed the presence of the labeled A′ translation product in fractions 1 to 7 (Fig. 2, anti-A′). Immunoprecipitation of gradient fractions with an Sm antiserum (serum AW) showed that labeled A′ peaked in fractions 3 to 5 and was detected as far as fraction 8 from the top (Fig. 2, anti-Sm). Free A′ protein representing the material that did not enter the gradient is shown in lane 1 at the left of Fig. 2, middle. RNA was detected by ethidium bromide staining of acrylamide gels representing each CsCl gradient fraction. U2 RNA was detected in fractions 4 to 13 (Fig. 2). Thus, A′ protein banded at a buoyant density characteristic of RNA-protein particles, which migrate at an intermediate position between free protein and RNA. This demonstrates that association of the exogenously added A′ protein with snRNPs was stable to CsCl gradient fractionation. Furthermore, these results demonstrate that upon incubation in HeLa cell nuclear extracts, synthetic A′ protein could migrate in a region that overlaps with U2 snRNPs. However, A′ protein peaked in fractions 3 to 5, whereas U2 RNA peaked in fractions 9 to 11. There are several possible explanations for this discrepancy in migration. It may reflect a difference in composition among U2 snRNPs, only a subset of which contain A′ protein or, perhaps, results from partial dissociation of U2 snRNPs due to the high salt or low Mg^{2+} concentration of the gradient. Alternatively, it could result from assembly of more than one A′ protein per U2 snRNP. Other possible explanations include association of in vitro-synthesized A′ protein with snRNPs other than U2 or interactions with unknown components in the extracts. A similar discrepancy in which U1 snRNP activity was displaced from the peak of U1 snRNA on CsCl gradients has been observed previously (56). The distribution of snRNPs and their associated components in CsCl density gradients is under investigation.

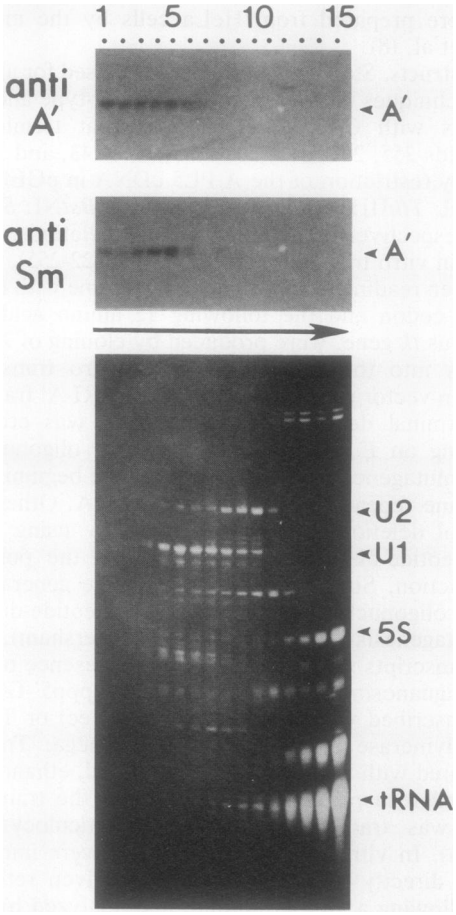


FIG. 2. Reconstituted A′-snRNP is stable in CsCl gradients. Radioactively labeled in vitro-translated A′ and HeLa cell nuclear extract were incubated and fractionated on a CsCl density gradient. Fraction 1 represents the top of the gradient. Fractions were immunoprecipitated with A′-specific antiserum (EW) or Sm-specific antiserum (AW) and electrophoresed on an SDS-10% polyacrylamide gel. The gel obtained with AW serum was exposed 10 times longer than the gel obtained with EW serum. RNA was isolated from each fraction and electrophoresed on a 10% denaturing polyacrylamide gel, and RNAs were visualized by ethidium bromide staining (bottom). The identities of various RNA species are indicated.

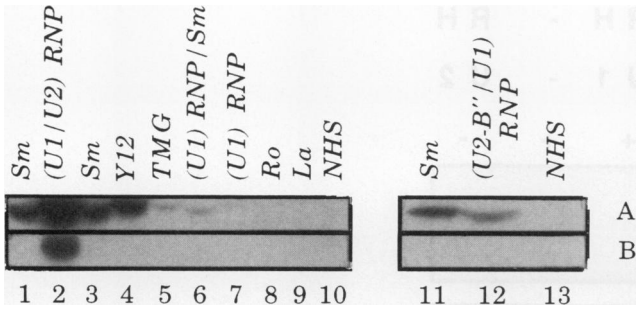


FIG. 3. Reconstitution of A′ translation product into snRNPs demonstrated by immunoprecipitation. Radioactively labeled in vitro-synthesized A′ protein was incubated with HeLa cell nuclear extract (A) or buffer D (B) and immunoprecipitated. The specificity of each antiserum is indicated above each lane. Y12 is an Sm monoclonal antibody, TMG is a trimethyl cap-specific monoclonal antibody, Ro and La are unrelated autoimmune sera, and NHS is normal human serum.

Reconstitution of A′ protein into U2 snRNPs in nuclear extracts. To determine whether the association of synthetic A′ protein involved specific snRNP components, as suggested by CsCl gradient fractionation, we subjected the complexes to additional analysis by immunoprecipitation. The radioactively labeled in vitro-translated A′ protein was incubated with a HeLa cell nuclear extract, and the mixture was immunoprecipitated with autoimmune sera of various specificities. All sera of the Sm specificity (Fig. 3A, lanes 1 to 4, 6 and 11), including the monoclonal Sm antibody, Y12 (lane 4), coimmunoprecipitated synthetic A′ protein. A unique feature of many U snRNAs is their 5′ cap structure, m^2_3,2,2,7G, for which a monoclonal antibody (TMG or m_3G) has been generated (36). TMG antibodies also coimmunoprecipitated the in vitro-translated A′ following incubation in the nuclear extract (Fig. 3A, lane 5). As a control for cross-reactivity, parallel immunoprecipitations were performed on radiolabeled synthetic A′ added to buffer lacking nuclear extract (Fig. 3B). As expected, only the (U1/U2) RNP antiserum EW (14) immunoprecipitated synthetic A′ protein from both nuclear extract and buffer reaction mixtures due to the presence of α-A′ antibodies (Fig. 3B, lane 2). (U1) RNP (lane 7), Ro (lane 8), La (lane 9), and normal human (lane 10) sera all failed to immunoprecipitate synthetic A′ protein regardless of prior incubation in the nuclear extract.

The association of synthetic A′ protein with U2 snRNPs was evidenced further by coimmunoprecipitation of labeled A′ protein with (U2/U1) RNP antiserum, which recognizes B″ protein and lacks antibody reactive with A′ protein (Fig. 3A, lane 12). Due to the inherent weakness of most B″ antisera, fourfold more antiserum was required for immunoprecipitation. In a control experiment, A′ protein was not coimmunoprecipitated by B″ antiserum in the absence of added nuclear extract (Fig. 3B, lane 12).

The findings that synthetic A′ protein was coimmunoprecipitated by Sm antiserum, B″ antiserum, and TMG antibodies but not by (U1) RNP antiserum indicated that in vitro-translated A′ protein is physically associated with a U snRNA-containing complex that harbors SM and B″ antigenic determinants, distinct from those on U1 snRNPs. Thus, the results of CsCl density centrifugation and immunoprecipitation experiments suggest that A′ protein can be reconstituted into a form of U2 snRNPs, either by exchange or by de novo assembly. However, these data do not rule out an association of A′ protein with additional RNP components in these extracts.

Reconstitution of A′ protein into snRNPs is RNA dependent. To determine whether RNA was required for the A′ protein-snRNP association, we treated nuclear extracts with various RNases prior to incubation with the in vitro translation product. Silver-stained gels of residual RNAs in the pretreated extracts confirmed the extent of nuclease digestion in each case. Extracts were pretreated with MN (Fig. 4, lane 1), followed by the addition of *E. coli* tRNA or yeast RNA subsequent to inactivation of MN with EGTA or with RNase T_1 (Fig. 4, lane 2). When incubated with in vitro-translated A′ protein, subsequent immunoprecipitation with Sm antiserum failed to coimmunoprecipitate in vitro-translated A′ protein (Fig. 4, upper panel, lanes 1 and 2). In control reactions in which MN was omitted but to which CA^{2+}, EGTA, MG^{2+}, and *E. coli* or yeast RNA were added, A′ protein was still coimmunoprecipitated with Sm antiserum (data not shown). T_1 and MN sensitivity of the reconstitution reaction demonstrates that an RNA is required for A′ protein to be coimmunoprecipitated by Sm antisera. To rule out the possibility that interference with snRNP reconstitution occurred from the liberation of nonspecific RNA binding proteins, we added an excess of carrier RNA to the MN-pretreated, EGTA-inactivated extract prior to the addition of synthetic A′ protein.

To test whether the association of A′ protein was specifically with U2 snRNPs, we performed targeted degradation of U1 and U2 snRNAs in the nuclear extract. Pretreatment of nuclear extracts by targeted RNase H digestion with oligonucleotides that are complementary to the 5′ end and first loop of the U2 snRNA (Fig. 4, lane 3) and the 5′ end of U1 RNA (lane 4) had no detectable effect on reconstitution as synthetic A′ protein was still coimmunoprecipitated by Sm antiserum. In comparison with the results of Black et al. (2), who achieved complete degradation of U2 snRNA using the complementary oligonucleotide U2-L15, under our conditions the 5′ end of U2 snRNA was degraded but the residual portion of the molecule 3′ to the first stem-loop that contains the binding sites for the Sm complex (33, 41) and A′ protein (6, 13a, 42) remained intact. Thus, the failure of U2-L15 oligonucleotide-directed cleavage by RNase H to abolish the specific association may be explained by the fact that A′ protein interacts directly or indirectly with U2 snRNA near the 3′ end and that removal of the 5′ end is inconsequential to the continued association of A′ protein and the Sm complex with U2 RNA. Thus, although the reconstitution was clearly RNA dependent, the involvement of U2 snRNA in the reconstituted complex could not be definitively assessed by this approach.

Regions of A′ protein critical for Sm-precipitable reconstitution. To further examine the specificity of the incorporation of in vitro-synthesized A′ protein into Sm-precipitable snRNPs and to delimit the required region(s), we examined in vitro translation products corresponding to C-terminal and N-terminal deletions of A′ protein (Fig. 5a). Terminal truncation products of in vitro-translated A′ protein were created by cleavage of the cDNA template with a variety of restriction enzymes as well as by fusion of N-terminal A′ deletion mutants in frame with a functional heterologous ATG. 3′ truncation products encompassing amino acids 1 to 249, 1 to 236, 1 to 207, 1 to 195, and 1 to 175 retained the property to reconstitute (Fig. 5a and 6). However, 1 to 143 and 1 to 125 A′ truncated polypeptides were unable to reconstitute into U2 snRNPs as detected by immunoprecipitation with an Sm

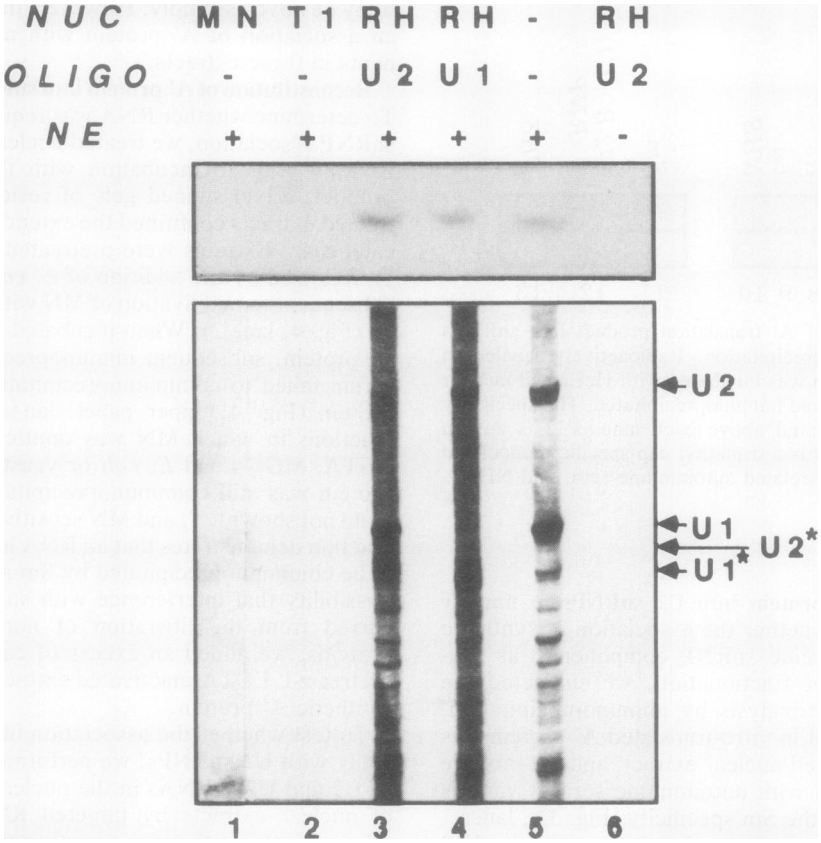
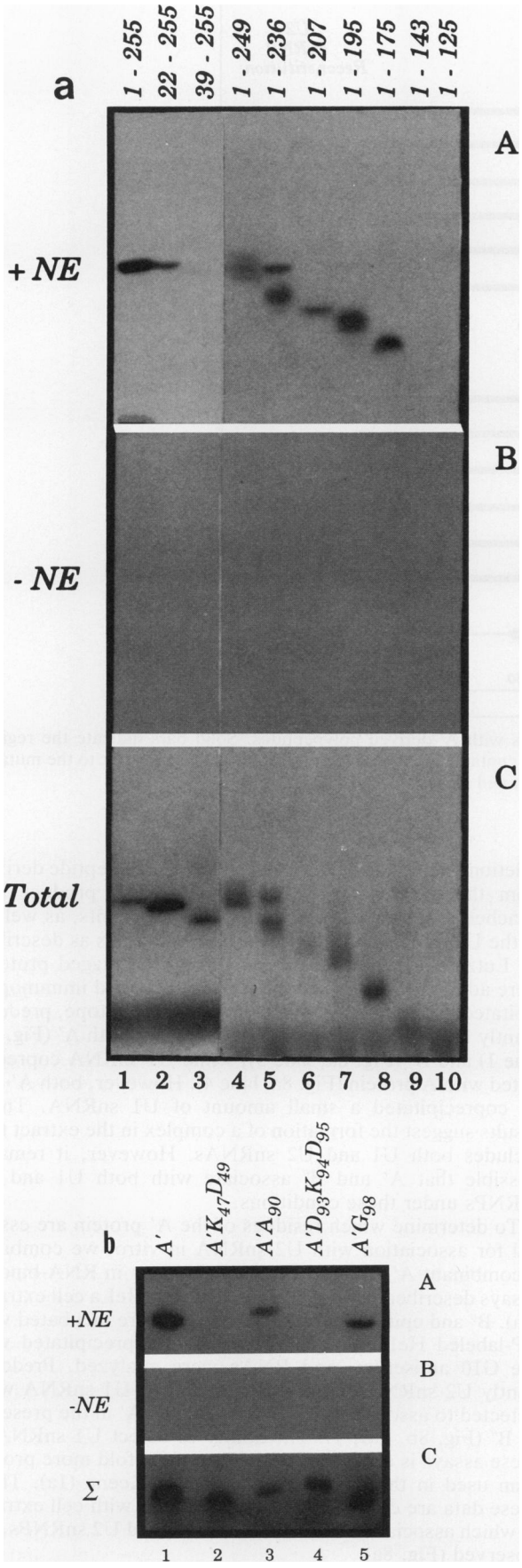


FIG. 4. Reconstitution of A′ into snRNPS is RNA dependent. Radioactively labeled in vitro-translated A′ was added to HeLa cell nuclear extract pretreated with MN (lane 1), T_1 nuclease (lane 2), U2-L15 complementary oligonucleotide and RNase H (lane 3), U1-16 complementary oligonucleotide and RNase H (lane 4), untreated nuclear extract (lane 5), or no nuclear extract (lane 6). Mixtures were immunoprecipitated with Sm antiserum and electrophoresed on an SDS-15% polyacrylamide gel (upper panel). To determine the extent of nuclease digestion, aliquots of the pretreated nuclear extracts were deproteinized, electrophoresed on a denaturing 10% polyacrylamide gel, and silver stained (lower panel). NE, Nuclear extract; NUC, nuclease; OLIGO, oligonucleotide.

antiserum (Fig. 5a, lanes 9 and 10). Note that in various repetitions of these experiments, products 1 to 143 and 1 to 125 were analyzed on higher-percentage acrylamide gels than those shown in the example of Fig. 5a, panel C, lanes 9 and 10, to achieve improved resolution. The N-terminal deletion mutant, representing amino acids 39 to 255 of A′ protein, was assayed and also failed to reconstitute (Fig. 5a, lane 3). However, a similar N-terminal deletion construct (22 to 255), in which residues 1 to 21 were deleted, was able to reconstitute but at a much reduced efficiency (Fig. 5a, lane 2).

Structural properties of the region of A′ protein required for reconstitution. The amino acid sequence of A′ protein reveals the occurrence of at least five uninterrrupted tandem arrays of leucine-rich sequence (repeats I to VI), which we refer to as the Leu-Leu motif, comprising the region of A′ protein required for its reconstitution (Fig. 6 and 7). Direct repeats of approximately 24 amino acid residues (repeat I appears to be truncated by deletion of its N-terminal half), defined by the conservative arrangement of hydrophobic residues, are localized between positions 22 and 152 and thus constitute over 50% of the molecule. Within these repeats, positions 4, 5, 8, 11, 14, 16, 21, and 24 are occupied mainly by leucine but, when occupied by a different residue, usually contain hydrophobic residues such as isoleucine, valine, phenylalanine, and, in a few instances, lysine, arginine, or glycine. Likewise, position 19 is occupied primarily by asparagine residues. An average of more than six charged residues are interspersed with conserved hydrophobic residues in each repeat, suggesting an amphipathic structure of the Leu-Leu repeat. In addition, there appears to be a subtle alteration of the repeats within A′ protein with respect to a general alteration of the basic and acidic character of each repeat, as well as to the occurrence of offset residues at a few of the variable positions. For example, in repeats II and IV of A′, the leucines at positions 4 and 5 are offset by one residue. This quality also occurs in some of the proteins described below that contain a Leu-Leu motif.

Similarity of A′ with other proteins. The periodicity of conserved residues in A′ protein resembles the consensus sequences of tandem leucine-rich intradomain homologies resident in a number of previously characterized proteins (Fig. 7): the leucine-rich α_2-glycoprotein of human serum of unknown function (61); the α (35, 64)- and β (34)-chains of the platelet integral plasma membrane heterodimer glycoprotein Ib, involved in platelet adhesion; *Drosophila* chaoptin, a cell surface glycoprotein with a role in photoreceptor cell membrane adhesion (54); the 90% homologous proteoglycan core proteins of human placenta fibroblasts (proteoglycan 40 or decorin) (23), fibromodulin (47), human bone (proteoglycan I or biglycan) (13), and bovine bone (proteoglycan II, or decorin) (7), that have collagen-binding and cell



attachment-inhibiting properties and are involved in platelet adhesion; *Drosophila* maternal effect gene product Toll that specifies embryonic dorsal-ventral fate (20); yeast adenylyl cyclase (22), a member of the G-protein-coupled receptor family, and rat lutropin-choriogonadotropin receptor (43); human carboxypeptidase N (62), which cleaves basic C-terminal amino acids from potent peptides; the myelomonocytic cell surface differentiation antigen CD14 (11, 59) (not shown in Fig. 7); and RNase/angiogenin inhibitor from porcine liver (21) and human placenta (28, 58). In addition, the putative *trans*-activating region of the human immunodeficiency virus type 1 *trans*-activator, rev (39), contains a similar sequence of leucine and asparagine residues (not shown in Fig. 7). Whether this region of Rev plays a role in U 2 snRNP assembly in human immunodeficiency virus type 1-infected cells is under investigation.

Alignment of the tandem repeats in A′ protein with the consensus sequences of Leu-Leu repeats found in those of other proteins showed that interspersed invariant positions of A′ and other proteins are brought into register (Fig. 7). Alternating A- and B-type repeats of RNase/angiogenin inhibitors (21, 28, 58) deviate slightly in length and composition from those in the aforementioned polypeptides. The introduction of a single gap in one sequence was required to place the conserved positions of all the consensus sequences into register. An overall consensus sequence of all these proteins was thus derived (Fig. 7). A′ protein displays greatest similarity to the human glycoprotein Ibα and *Drosophila* Toll consensus sequences and deviates from the overall consensus only in the most variable of the conserved positions. A′ protein differs from the others in that it lacks the highly conserved proline residue at position 1: however, it contains glycine residues at various positions surrounding position 1. Thus, structural features that tend to disfavor helix formation or bending of the backbone may characterize this region.

A computer-derived hydropathy profile (25) suggests that the repeated region of A′ protein is amphipathic. According to the algorithm of Garnier et al. (15), there is no predicted α-helical character in the repeated domains of any of these proteins. Rather, very short stretches of β-pleated sheet and turns prevail.

Reconstitution of A′ point mutants. To assess the involvement of various conserved residues of the Leu-Leu repeat motif in the assembly of snRNPs, we examined the reconstitution of four different A′-derived translation products containing point mutations. Substitution of a conserved leucine (Leu-90) by alanine (A′ Ala-90) (Fig. 5b, lane 3) and

FIG. 5. (a) Reconstitution of A′-derived truncation products into Sm-precipitable snRNPs. Radioactively labeled in vitro-synthesized wild-type A′ protein and A′-derived truncated polypeptide fragments (C) were incubated in a HeLa cell nuclear extract (+NE) (A) or in buffer D (B). Mixtures were immunoprecipitated with Sm antiserum, and products were electrophoresed on an SDS-15% polyacrylamide gel. The amino acid residues encompassed by each A′-derived translation product are indicated above each lane. (b) Reconstitution of A′ point mutants into Sm-precipitable snRNPs. Radioactively labeled in vitro-synthesized wild-type A′ protein and A′-derived point mutants (C) were incubated with a HeLa cell nuclear extract (+NE) (A) or in buffer D (B). Mixtures were immunoprecipitated with Sm antiserum and electrophoresed on an SDS-10% polyacrylamide-gel. The A′-derived translation products with mutated amino acids at the numbered positions are indicated above each lane.

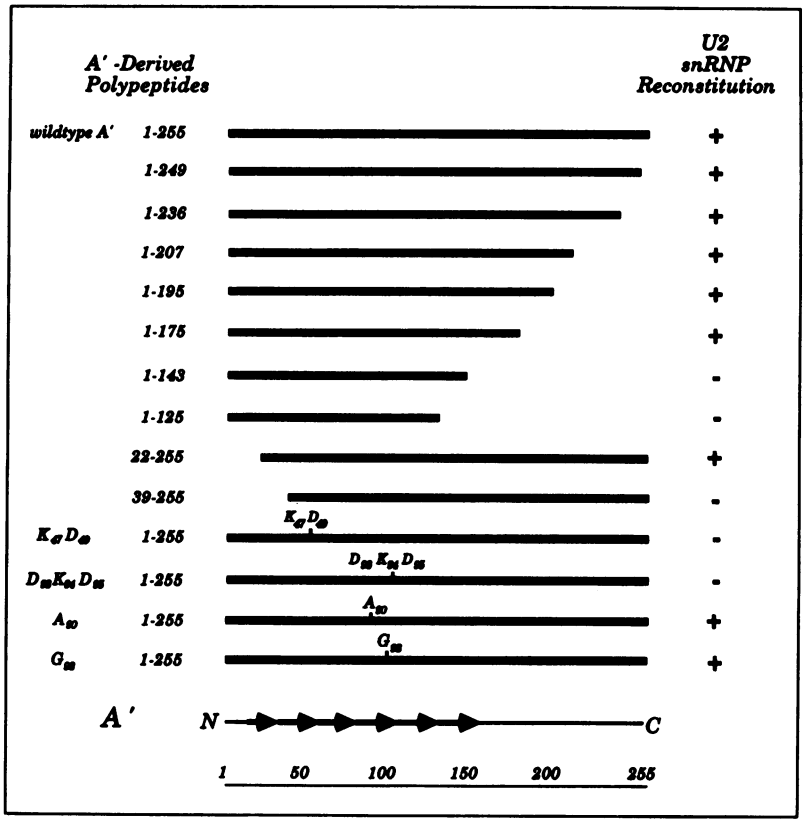
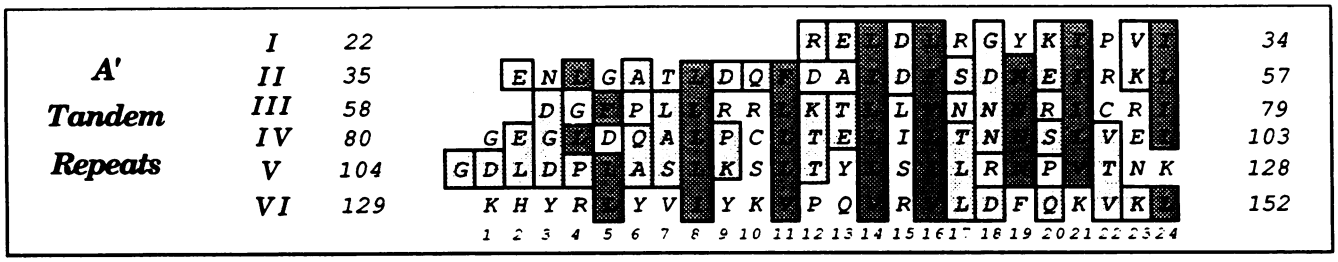


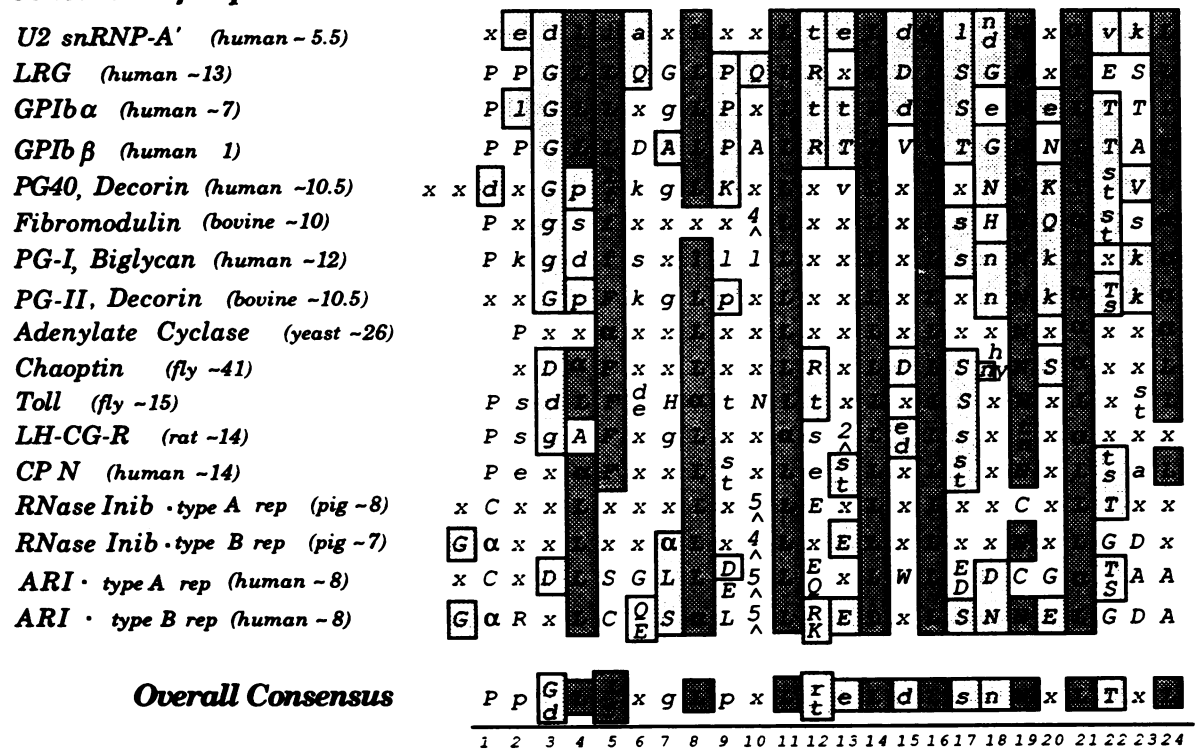
FIG. 6. Summary of Sm-precipitable snRNP reconstitution experiments with A'-derived polypeptides. Solid bars indicate the regions encompassed by the polypeptide. The numbers refer to amino acid residues contained in the polypeptide, and the letters refer to the mutated amino acid residues at the indicated position. The Leu-Leu repeats are denoted by horizontal arrows.

substitution of a conserved asparagine residue (Asn-98) by glycine (A' Gly-98) (Fig. 5b, lane 5) had little effect on the efficiency of reconstitution. Substitution of conserved hydrophobic residues, isoleucine and phenylalanine by lysine and aspartic acid, respectively (A' Lys-47–Asp-49) (Fig. 5b, lane 2) and of leucine-isoleucine-leucine by aspartic acid-lysine-aspartic acid, respectively (A' Asp-93–Lys-94–Asp-95) (Fig. 5b, lane 4) resulted in polypeptides that failed to be reconstituted. In summary, the smallest portion of A' protein that retained the ability to associate with U2 snRNP encompassed residues 22 to 175. Double and triple point mutants of A' protein, in which conserved hydrophobic amino acids were substituted by charged residues, were rendered inactive in the reconstitution assay. Although it remains possible that the overall structure of the A'-derived polypeptides has been perturbed by substitution of hydrophobic residues with charged residues, these data suggest that the Leu-Leu motif mediates reconstitution of A' into U2 snRNPs. As with all site-directed mutations, the structural integrity of the molecules could be compromised; however, analysis of the folded molecule will require conformational probes or X-ray crystallography. Thus, as summarized in Fig. 6, the 154-amino-acid region between residues 22 and 175 appeared to be essential for association of synthetic A' protein with Sm-precipitable snRNPs and with U2 snRNPs.

G10 epitope tag on A' demonstrates interaction with U2 snRNPs. As an additional confirmatory method to demonstrate interaction of the recombinant A' protein with U2 snRNPs in the cell extract, we attached an independent epitope tag to the protein and to a variety of terminal deletion constructs of A'. The 12-amino-acid peptide derived from the bacteriophage T7 gene 10 (G10) product was attached to the A' protein and protein fragments, as well as to the U2 snRNP B" and U1 snRNP A proteins as described by Lutz-Freyermuth et al. (38). When the tagged proteins were added to ^{32}P-labeled nuclear extracts and immunoprecipitated with a rabbit antibody to the G10 epitope, predominantly U2 snRNA was coprecipitated with both A' (Fig. 8a, lane 1) and B" (Fig. 8a, lane 3), while U1 snRNA coprecipitated with A protein (Fig. 8a, lane 4). However, both A' and B" coprecipitated a small amount of U1 snRNA. These results suggest the formation of a complex in the extract that includes both U1 and U2 snRNAs. However, it remains possible that A' and B" associate with both U1 and U2 snRNPs under these conditions.

To determine which residues of the A' protein are essential for association with U2 snRNA in vitro, we combined recombinant A', B", and total cell RNA as in RNA-binding assays described previously which lacked HeLa cell extracts (1a). B" and epitope-tagged A' proteins were incubated with ^{32}P-labeled HeLa cell RNA and immunoprecipitated with the G10 antiserum, and RNAs were analyzed. Predominantly U2 snRNA and a small amount of U1 snRNA were detected to associate with epitope-tagged A' in the presence of B" (Fig. 8b, lane 1). The ability to detect U1 snRNA in these assays is attributed to the use of fivefold more protein than used in the study of Bentley and Keene (1a). Thus, these data are consistent with the findings with cell extracts in which association of A' with both U1 and U2 snRNPs was observed (Fig. 8a).





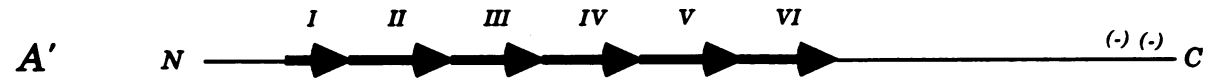


FIG. 7. Leucine periodicity in A' and in other proteins. Amino acid residues 22 to 152 of A' protein and the consensus sequences of the Leu-Leu repeats in known proteins have been arranged for optimal alignment to display the internal homology of A' and the approximately 24-residue periodicity of amino acids. The protein designations are described in the text, and the source of each protein and the approximate number of tandem repeats that occur in each protein are indicated in parentheses. The single-letter amino acid code is utilized. x indicates no consensus; α indicates an aliphatic residue; lower-case letters indicate amino acids that occupy at least one-third to less than one-half of the residues of all the repeats in a particular protein at that position; upper-case letters indicate amino acids that occupy at least one-half of the residues at that position. Residues within A' that are conserved at a particular position in the majority and in at least one-third of the proteins are darkly and lightly shaded, respectively. The A' protein is represented schematically at the bottom showing the six Leu-Leu repeats as horizontal arrows and the acidic carboxy terminus (−). LRG, Leucine-rich α_2-glycoprotein; GPIb, glycoprotein Ib; PG, proteoglycan; LH-CG-R, lutropin-choriogonadotropin receptor; CP-N, carboxypeptidase N; Inib, inhibitor; ARI, RNase/angiogenin inhibitor.

Analysis of deletion constructs of A' by the direct RNA binding assay was compared with those described above in Fig. 5a by reconstitution in cell extracts. Deletion constructs of A' that encompass residues 1 to 172 also were capable of reconstitution with U2 RNA (Fig. 8b, lane 2 to 7). Reconstitution of construct 1 to 162 with U2 RNA was evident also (lane 3), although the amount was decreased over the 1 to 172 construct (lane 2). Thus, in keeping with the results of reconstitution in cell extracts, we conclude that the C-terminal boundary of the domain required for reconstitution of A' resides between residues 162 and 172 at the C terminus of A' protein.

Analysis of requirements for the N-terminus by the direct RNA binding assay in the absence of cellular components (Fig. 8b, lanes 6 and 7) indicated that residues 1 to 22 were essential for association of A' with U2 snRNA. The Sm precipitation experiments of reconstituted extracts shown above (Fig. 5a, lanes 2 and 3) indicated also that the N

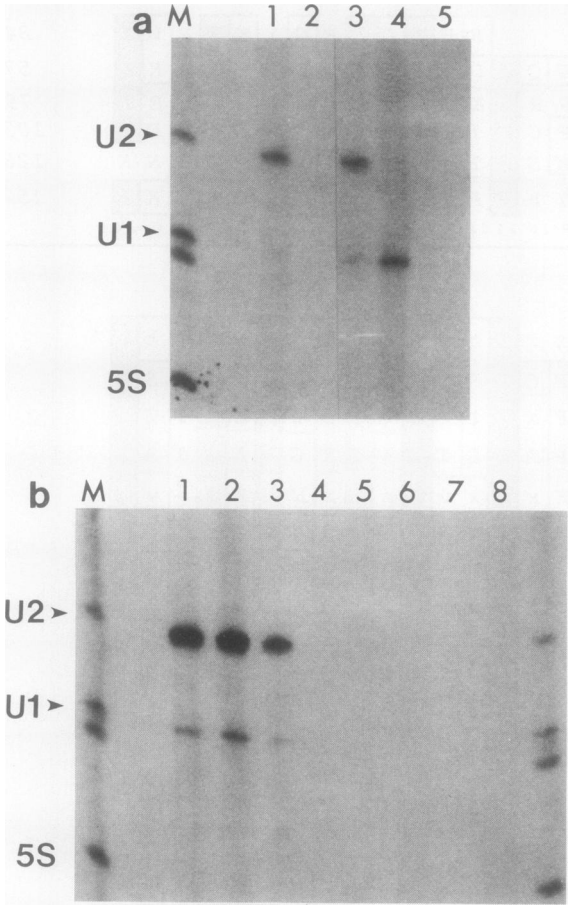


FIG. 8. Immunoprecipitation of epitope-tagged G10-A′ fusion proteins following reconstitution. HeLa cells were labeled with $^{32}P_i$ and extracts were prepared as described in the text. *E. coli*-produced unlabeled A′ protein attached to the T7 phage gene 10 (G10) peptide was combined with the extract or with phenol-extracted total cell RNA and immunoprecipitated with rabbit antiserum prepared against the 12-amino-acid G10 epitope as described previously (38). Washed pellets were extracted with phenol, precipitated with ethanol, and analyzed on 5% acrylamide-urea gels. (a) Reconstitution in cell extracts. Lanes: 1, tagged A′; 2, control, no added recombinant protein; 3, tagged B″; 4, tagged U1 snRNP A protein; 5, control, no added recombinant protein. (b) Reconstitution with total cell RNA. *E. coli*-produced B″ protein was added for RNA binding experiments. Lanes: M, marker RNA; 1, full-length A′ protein; 2, A′ residues 1 to 172; 3, A′ residues 1 to 162; 4, A′ residues 1 to 152; 5, A′ residues 1 to 142; 6, A′ residues 12 to 255; 7, A′ residues 22 to 255; 8, no A′ added.

terminus affected the efficiency of reconstitution, but it was not absolutely required. These results may reflect differences in the stringency of the RNA binding conditions or the presence of factors in the extract that compensate for defects in the N terminus of the A′ protein. Taken together, these findings demonstrate that the minimal region of the A′ protein required for optimal reconstitution is encompassed by amino acid residues 1 to 172. This sequence contains the Leu-Leu motif.

DISCUSSION

Reconstitution of A′ protein into an snRNP. We reconstituted U2 snRNPs with wild-type and mutant forms of A′ protein using both cell extracts and recombinant proteins in vitro. Several criteria were utilized to evaluate the reconstitution: (i) Following incubation in HeLa cell extracts, synthetic A′ protein was associated with an RNP complex containing trimethyl cap, Sm, and U2 snRNP B″ protein antigenic determinants; (ii) intact RNA was required for the reconstitution, and the reconstituted A′ translation product overlapped with U2 snRNPs in CsCl gradients; (iii) immunoprecipitation of recombinant A′ protein containing an epitope tag from reconstituted extracts demonstrated association with U2 snRNA. These experiments do not exclude the possibility that A′ protein associates with other snRNPs in these extracts, although the epitope tag experiments indicated that the main association was with U2 snRNA. At least five tandem amphipathic Leu-Leu repeats, encompassing a periodicity of conserved leucine and asparagine residues, compose the N-terminal half of A′ protein (Fig. 6 and 7) and are required for snRNP reconstitution (Fig. 5, 6, and 8). The inability of various truncated A′ polypeptides to reconstitute indicates that at least part of the region required for reconstitution is located in these deleted regions or, alternatively, that a required tertiary structure of a domain has been disrupted.

The Leu-Leu motif of A′ is implicated in protein-protein interactions. Several lines of evidence are compatible with the idea that the repeated Leu-Leu element promotes protein-protein interactions. For some proteins, such as chaoptin (54), the proteoglycan core proteins of proteoglycan 40 (23) and proteoglycan II (7), and RNase/angiogenin inhibitors (21, 28, 58), these tandem arrays compose the entire primary structure. Thus, the proposed functions of these molecules can be attributed to some aspect of the Leu-Leu motif. Experimental evidence for the involvement of regions containing this motif in intermolecular protein-protein interactions has been provided in a few cases. The thrombin and von Willebrand factor binding sites of the integral membrane glycoprotein Ib, the platelet receptor for von Willebrand factor and thrombin, have been mapped to the α-chain 43-kDa extracytoplasmic domain, half of which is composed of the seven tandem repeats (64). Dominant interfering mutations of yeast adenylyl cyclase that interfere with Ras activation of adenylyl cyclase map to the region containing 26 Leu-Leu repeats, which are suggested to interact directly with Ras protein (12). Similarily, the *trans*-activating region of the human immunodeficiency virus type 1 Rev protein has been mapped to a region (39, 45) that appears to conform to a single module of the Leu-Leu motif.

The findings that the smallest terminal truncation product of A′ able to associate with U2 snRNP encompasses the repeated motif and that deletion of a portion of repeats I and II or a portion of repeat IV and substitution of conserved hydrophobic residues preclude reconstitution are consistent with the hypothesis that the tandem repeats of the amphipathic Leu-Leu motif constitute a domain that mediates protein-protein interactions in U2 snRNP.

Modular structure of leucine-rich motif. The occurrence of the tandem arrays of the Leu-Leu motif in proteins of diverse origin and function implies a fundamental role as a functional domain that facilitates a common mode of protein-protein interaction. The Leu-Leu motif does not conform to the leucine zipper coiled-coil motif, a known site of intermolecular protein-protein interaction (26, 48), yet it too may represent an oligomerization or dimerization interface. The contribution of this motif alone can be expected to account for both the affinity and the specificity of the intermolecular interactions in which the host proteins participate as, for

some proteins, the entire molecule is composed of the tandem repeats. Evidence that this motif facilitates an extremely tight intermolecular association comes from the inhibition constants calculated for the inhibition of RNase A($K_i = 4 \times 10^{-14}$) and angiogenin ($K_i = 7 \times 10^{-16}$) by RNase/angiogenin inhibitor, which approximate the affinity of avidin for biotin (27, 29). The tolerance of substitutions of various repeats for one another with RNase/angiogenin inhibitor (30) and the 1:1 stoichiometry of RNase inhibitor to its substrates support the notion that the Leu-Leu repeats constitute structural modules that coordinately participate in the interaction, rather than individually mediating the interaction (3, 21, 28).

Implications for U2 snRNP assembly. It is not known whether A′ protein contacts U2 RNA directly; however, our attempts to detect binding of A′ protein to U2 RNA in the absence of B″ have proved unsuccessful (data not shown). No obvious homology to any known nucleic acid-binding motifs has emerged by computer or visual inspection. Most notable is the absence of an RNP consensus octamer sequence (1), referred to as RNP1, that resides within an 81-amino-acid RNA recognition motif that was shown to function as an RNA binding domain in the U1 snRNP 70-kDa (53), U1 snRNP A (38, 57), and U2 snRNP B″ proteins (1a, 57). However, addition of A′ protein results in a profound increase in the affinity and specificity of binding of B″ protein to U2 snRNA in vitro (1a). Data reported here using deletion constructs of A′ for snRNP reconstitution and U2 RNA binding indicate that the Leu-Leu motif of A′ protein mediates specific protein-protein interactions with the B″ protein. Thus, it appears that A′ is an accessory factor for RNA recognition that may interact with U2 RNA indirectly through protein-protein interactions or directly in the presence of B″.

Recent analysis of the structure of the RNA binding domain of these proteins by nuclear magnetic resonance spectroscopy (20a) indicates that the conserved RNP1 sequence and an aliphatic-aromatic-aliphatic sequence (53), referred to as RNP2, in the RNA recognition motif interact with one another in a four-stranded β-sheet. Amino acid residues adjacent to the RNP1 sequence were shown by Scherly et al. (57) and by Bentley and Keene (1a) to influence the specificity of RNA recognition. This segment resides between β2 and β3 strands of the domain and is termed loop 3. On the basis of our mutagenesis and RNA binding studies, we have proposed that A′ protein interacts with the B″ protein in regions additional to loop 3 (1a).

It is also possible that A′ protein interacts with cellular factors that mediate U2 snRNP interactions including those in the spliceosome. These include factors associated with pre-mRNA: the intron binding protein (16, 63), U2 auxiliary factor, which has been postulated to contact U2 snRNP presumably by protein-protein interactions (46, 56), as well as other factors.

The indications that A′ protein may exchange into snRNPs as presented here and previously (10) and that A′ may mediate protein-protein interactions within U2 snRNP suggests that U2 snRNPs are dynamic particles whose functions might be modulated by the presence of A′ protein. Reconstitution of A′-derived polypeptides into cellular extracts affords the opportunity to examine their roles within RNP complexes including the spliceosome by in vitro complementation.

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