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Recognition of U1 and U2 Small Nuclear RNAs Can Be Altered by a 5-Amino-Acid Segment in the U2 Small Nuclear Ribonucleoprotein Particle (snRNP) B″ Protein and through Interactions with U2 snRNP-A′ Protein

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We have investigated the sequence elements influencing RNA recognition in two closely related small nuclear ribonucleoprotein particle (snRNP) proteins, U1 snRNP-A and U2 snRNP-B″. A 5-amino-acid segment in the RNA-binding domain of the U2 snRNP-B″ protein was found to confer U2 RNA recognition when substituted into the corresponding position in the U1 snRNP-A protein. In addition, B″, but not A, was found to require the U2 snRNP-A′ protein as an accessory factor for high-affinity binding to U2 RNA. The pentamer segment in B″ that conferred U2 RNA recognition was not sufficient to allow the A′ enhancement of U2 RNA binding by B″, thus implicating other sequences in this protein-protein interaction. Sequence elements involved in these interactions have been localized to variable loops of the RNA-binding domain as determined by nuclear magnetic resonance spectroscopy (D. Hoffman, C. C. Query, B. Golden, S. W. White, and J. D. Keene, Proc. Natl. Acad. Sci. USA, in press). These findings suggest a role for accessory proteins in the formation of RNP complexes and pinpoint amino acid sequences that affect the specificity of RNA recognition in two members of a large family of proteins involved in RNA processing.

The specific recognition of RNA by proteins involves a variety of amino acid sequences that differ widely among the known RNA-binding proteins (reviewed in references 25, 33, 38, and 47). One family of proteins involved in RNA processing shares a primary sequence motif of approximately 80 amino acids, which we have termed an RNA recognition motif (RRM) (for reviews, see references 2, 16, and 21). This motif contains the strongly conserved ribonucleoprotein particle (RNP) octamer consensus sequence (1) and is present as single or multiple copies in a given protein. Specific RNA-binding domains have been defined for two members of this family, the U1 small nuclear RNP (snRNP) 70K and A proteins, and the domain corresponds closely to the RRM in each protein (18, 30, 36). The role of specific sequence elements within the RRM in determining the RNA recognition properties of this family of proteins has recently been investigated by site-directed mutagenesis of the RNP consensus octamer (19, 31a) and of sequences adjacent to the octamer (35).

The U1 and U2 snRNPs are components of the spliceosome, which removes introns from pre-mRNAs (reviewed in references 39 and 42). The U1 snRNP recognizes pre-mRNAs in part through base pairing of U1 RNA with the 5′ splice site (49), while the U2 snRNP recognizes the intron branch point in part through base pairing with the conserved branch point sequence (27, 48, 50). In addition to the Sm proteins common to most U snRNPs, the U1 snRNP contains three unique proteins (70K, A, and C), while the U2 snRNP contains only two unique proteins (B″ and A′). The U1 snRNP-A (A) and U2 snRNP-A′ (A′) proteins are unrelated in sequence but are so named because they migrate at similar positions in sodium dodecyl sulfate-polyacrylamide gels (3, 28); for clarity we will use the designation A^{prime} for the U2 snRNP-A′ protein.

The A (40) and U2 snRNP-B″ (13) proteins each contain two RRMs. The sequences within the corresponding motifs of these proteins are highly conserved (40); their amino-terminal RRMs are 75% identical, and their carboxy-terminal RRMs are 86% identical. Despite the high degree of primary amino acid sequence similarity, these two proteins associate with different RNAs in vivo (reviewed in reference 51). Thus, A and B″ are useful models for the study of the determinants of RNA recognition within the RRM (12, 40).

We have constructed a series of recombinant cDNA molecules encoding permutations between the RRMs of A and B″ and found that substituting a 5-amino-acid sequence from B″ into A confers the ability to recognize U2 RNA. These results are similar to those reported by Scherly et al. (35), who found that an 8-amino-acid sequence in the same region determined the specificity of recognition of U1 and U2 snRNAs. Our findings show that this region is not sufficient to interconvert the A and B″ proteins but represents only one such determinant. In addition, we found that B″ requires A^{prime} for specific high-affinity binding of B″ to U2 RNA in vitro but that the 5-amino-acid change uncouples the recognition of U2 RNA from the ability to respond to A^{prime}. The molecular interactions that control U2 RNA recognition by B″ demonstrate that the function of an RRM as an RNA-binding domain can be regulated by specific sequences within the RRM as well as by intermolecular interactions with other proteins. These results are interpreted in light of our recent determination of the tertiary structure of the U1 RNA-binding domain of the A protein (15) and the possibility that the pentamer segment interacts directly with RNA.

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MATERIALS AND METHODS

Enzymes, host strains, and vectors. Enzymes were obtained from United States Biochemical Corp. and New England BioLabs. *Escherichia coli* NM522 and the pGEM-3zf(+) plasmid vector were purchased from Promega. *E. coli* TG-1 was purchased from Amersham. *E. coli* BL21 and BL21(DE3)pLysS and the expression vectors pET-3c and pET-8c were gifts from William Studier (Brookhaven National Laboratory). The vector pGEX-2T was obtained from Amrad Corp. Ltd.

Cloning procedures and cDNAs. cDNA clones of the U1 snRNP-A (18), U2 snRNP-A^{prime} (8a, 9), U1 snRNP-70K (30), and La (4) proteins were obtained by screening expression libraries with antisera from patients with autoimmune diseases. cDNAs for the U2 snRNP-B″ protein were obtained from human fibroblast and endothelial-cell λgt11 libraries (Clonetech, Palo Alto, Calif.) by screening with oligonucleotides corresponding to the previously published cDNA sequence (13) according to standard techniques (34). The nucleotide sequence in the open reading frames of the B″ cDNAs was identical to the previously published sequence.

In vitro transcription and translation. DNA constructions were placed next to the promoter for T7 or SP6 RNA polymerase, transcribed in vitro, and translated in rabbit reticulocyte lysates as suggested by the supplier (Promega). Translation products were analyzed by electrophoresis on 15% sodium dodecyl sulfate-polyacrylamide gels and then subjected to fluorography or trichloroacetic acid precipitation. Clones of human genomic U1, U2, U5, and mouse U6 RNAs were gifts from Nouria Hernandez (Cold Spring Harbor Laboratory), Alan Weiner (Yale University), Jeff Patton (University of South Carolina), and Ram Reddy (Baylor College of Medicine), respectively. Inserts or portions of inserts from each of these clones were subcloned into pGEM-3Zf(+). In vitro transcripts containing full-length RNAs were synthesized from templates linearized with *Rsa*I (U1), *Hpa*II (U2), *Acc*I (U5), or *Dde*I (U6). A U2 RNA lacking an intact stem-loop IV (U2ΔSL4, nucleotides 1 to 152) was transcribed from *Taq*I-truncated template. Both the full-length and truncated U2 transcripts contained 29 additional 5′ genomic nucleotides, and the full-length transcript contained 5 additional 3′ nucleotides. Human β-globin template was truncated at the *Bam*HI site to produce a 493-nucleotide RNA.

Oligonucleotide-directed mutagenesis. Mutagenesis was performed by using pGEM-3zf(+) vectors according to the instructions of the supplier (Amersham). All mutants were completely sequenced on at least one strand throughout their coding regions. Specific mutations created are indicated in Results. U2 stem-loop IV (U2 SL4) template was synthesized by polymerase chain reaction and represents nucleotides 147 to 188 of human U2. After being cloned into the *Eco*RI site of pGEM-3Zf(+), the in vitro transcript contained nine and five additional vector nucleotides at the 5′ and 3′ ends, respectively. U1 RNA mutations have been described previously (18, 31). U1 SL2 transcript contained U1 nucleotides 48 to 92 plus nine additional 5′ nucleotides from the vector.

HeLa cell RNA bindings and immunoprecipitations. Immunoprecipitations were performed as previously described (30) except that the RNA-binding buffer contained NT-2 (50 mM Tris [pH 7.40], 150 mM NaCl, 0.05% Nonidet P-40) 1 mM dithiothreitol, 10 mM $MgCl_2$, 2% polyvinyl alcohol, 0.1 mg of bovine serum albumin per ml, 0.5 mg of *E. coli* tRNA per ml, 0.125 mg of poly(A) RNA per ml, and 50 U of RNasin (Promega) per ml. Precipitations were washed in NT-2. For some of the experiments shown, protein was immunoprecipitated from *E. coli* extracts on protein A-Sepharose (Sigma) prior to RNA binding. ^{32}P-labeled HeLa cell RNA was produced as previously described (17). RNA was bound to protein for 5 to 20 min at 37°C and then immunoprecipitated on ice with 1 μl of antiserum and 4 mg of protein A-Sepharose (Sigma) for each 50 μl of binding reaction mixture. For the competition experiments, the indicated in vitro-transcribed unlabeled RNAs were added at a concentration of 2.25 μM. HeLa cell S-100 extracts were prepared by standard techniques (6); heat inactivation was at 90°C for 5 min.

Overexpression of protein in *E. coli*. Proteins were produced in *E. coli* by using an inducible T7 RNA polymerase expression system (32) or the Glutagene system (41) (Amrad Corp.). Fusion proteins are denoted as containing the T7 12-amino-acid segment (g10) or glutathione reductase (gst) at the amino terminus. An *Nco*I site was created at the translation-initiating methionine of U2 snRNP-B″ and U2 snRNP-A^{prime}. *Nco*I-*Eco*RI inserts were isolated from these constructs as well as from the U1 snRNP-A protein and subcloned in frame into the *Bam*HI site of either pET-3c or pGEX-2T. The constructs were then transfected into BL21(DE3)pLysS (for pET-3c) or BL21 (for pGEX-2T). Following induction, bacteria were lysed by freeze-thaw and sonication, and the insoluble material was removed by centrifugation at 10,000 × *g* for 15 min. The crude extracts were used directly for immunoprecipitation and RNA bindings. Protein induction was evaluated after sodium dodecyl sulfate-polyacrylamide gel electrophoresis by Western blot (immunoblot) analysis and by Coomassie blue staining.

Mobility shift assays. ^{35}S-labeled translation products were incubated with in vitro-transcribed RNA in the binding buffer described above with the addition of 0.1 M EGTA and then electrophoresed in a nondenaturing 5% polyacrylamide–90 mM Tris-borate (pH 8.3) gel. Quantitative titrations by the mobility shift assay were performed as previously described (19). The highest RNA concentration (7.5 μM) was used with serial twofold dilutions. The K_d was estimated as the RNA concentration at which 50% of the protein was present in the slower-migrating complex.

RESULTS

B″ protein can bind directly to both U1 and U2 RNAs in vitro. B″ cDNAs were obtained by screening libraries with oligonucleotides from the known cDNA sequence (13), and the recombinant B″ protein was produced in reticulocyte lysates by in vitro transcription and translation or in *E. coli* by using the pET T7 expression system (32, 45). The pET constructs produced proteins fused to the first 12 amino acids of the phage T7 gene *10* protein (g10). Production of a rabbit antiserum against a synthetic g10 peptide (16a, 19) allowed us to utilize the fused g10 peptide as an epitope tag for immunoprecipitations. This method of RNA binding avoided the use of human autoimmune sera, which may contain cross-reactive or unrelated antibodies.

When total HeLa cell ^{32}P-labeled RNA was incubated with the full-length recombinant g10-B″ fusion protein synthesized in *E. coli* and immunoprecipitated with g10 peptide antiserum, weak binding to both U1 and U2 RNAs was detected (Fig. 1A, lane 4; for similar results using in vitro-translated B″, see Fig. 2, lane 4). In contrast, the g10 antiserum alone or an extract from *E. coli* containing only the pET vector showed no detectable binding (Fig. 1A, lanes 2 and 3). The binding of B″ to U1 as well as U2 RNAs was

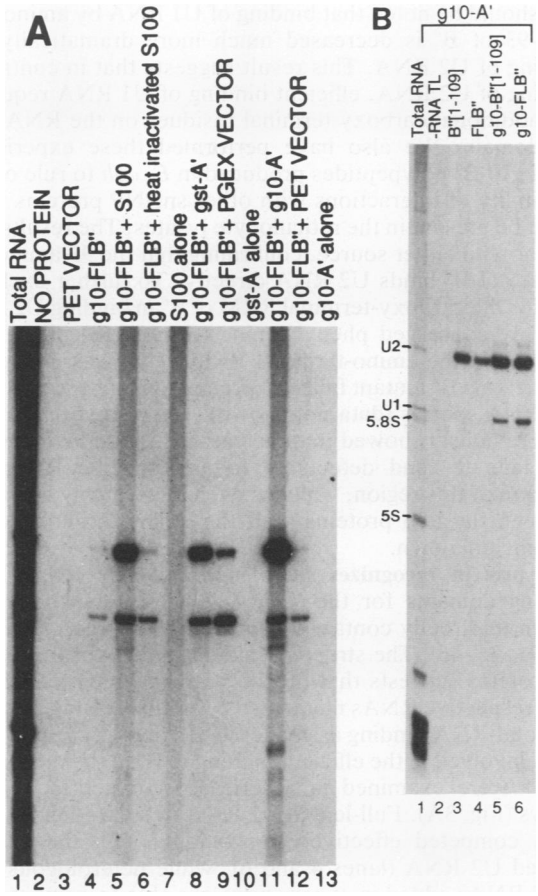


FIG. 1. Binding of recombinant U2 snRNP-B″ protein to HeLa cell U1 and U2 RNAs and enhancement of U2 binding by U2 snRNP-A^{prime} protein. (A) Full-length U2 snRNP-B″ and U2 snRNP-A^{prime} (A′) proteins produced in *E. coli* as T7 g10 fusion proteins or as gst fusion proteins were incubated with total ^{32}P-labeled HeLa cell RNA. After binding as described in the text, the proteins were immunoprecipitated with the g10 antiserum and protein A-Sepharose, and the coprecipitated RNAs were examined by 5% polyacrylamide–8.3 M urea gel electrophoresis. Lane 1, Total HeLa cell RNA; lane 2, no extract added (antiserum and protein-A sepharose only). In lanes 3 to 12, bindings using *E. coli* extracts expressed the following: lane 3, pET vector alone; lane 4, full-length g10-B″(g10-FLB″) alone; lane 5, full-length g10-B″ plus HeLa cell S-100 extract; lane 6, full-length g10-B″ plus heat-inactivated S-100 extract; lane 7, HeLa cell S-100 extract alone; lane 8, full-length g10-B″ plus gst-A^{prime} (A′); lane 9, full-length g10-B″ plus pGEX-2T vector; lane 10, gst-A^{prime} only; lane 11, full-length g10-B″ plus g10-A^{prime}; lane 12, full-length g10-B″ plus pET vector; lane 13, g10-A^{prime} alone. (B) In vitro-translated g10-A^{prime} (A′) was incubated with ^{32}P-labeled HeLa cell RNA and the in vitro-translated B″ polypeptides indicated above each lane and then immunoprecipitated as for panel A. Lane 1, Total HeLa cell RNA; lanes 2 to 6, g10-A^{prime} plus an unprogrammed translation (lane 2), B″ amino acids 1 to 109 (lane 3), full-length B″ (lane 4), g10-B″ amino acids 1 to 109 (lane 5), or full-length g10-B″ (lane 6).

unexpected, because B″ has been reported to be part of the U2 snRNP complex and is not known to associate with U1 snRNPs (3, 11, 28). On the other hand, given the sequence similarities between the RRMs of the U1 snRNP-A and the U2 snRNP-B″ proteins (depicted in Fig. 3B), it was anticipated that some degree of cross-recognition of U1 and U2 RNAs might be possible.

U2 snRNP-A^{prime} protein provides accessory binding function for B″. B″ is not known to associate with U1 snRNPs, and so we investigated whether interaction with some other factor might affect the binding of B″ to U1 and U2 RNAs in vitro. Addition of a HeLa cell S-100 extract to the B″-binding reaction resulted in a dramatic enhancement of binding to U2 RNA (Fig. 1A, lane 5). Increased binding to U1 RNA was also occasionally noted. However, it was always at least 10-fold less than that seen with U2 RNA. As expected, S-100 extract alone did not precipitate any specific RNA, since none of the many RNA-binding proteins in the extract possess the g10 epitope tag (lane 7). The effect of the S-100 extract on g10-B″ RNA binding was dosage dependent (not shown) and heat sensitive (lane 6), suggesting the involvement of a protein factor. The only other U2 snRNP-specific protein, A^{prime}, is an obvious candidate for such a factor. Therefore, we produced recombinant A^{prime} protein by using the pGEX expression system, which produces gst fusion proteins (41).

The addition of recombinant gst-A^{prime} fusion protein produced in *E. coli* caused an increase in the apparent affinity of B″ for U2 RNA (Fig. 1A, lane 8) similar to that produced by the addition of S-100 cell extracts. Addition of a control extract from *E. coli* expressing only the gst fusion vector (pGEX) showed a slight effect on the binding of B″ to U1 and U2 RNAs but much less than the effect of gst-A^{prime} (lane 9). We do not understand why this *E. coli* extract affected the coprecipitation of U1 and U2 RNAs, but such coprecipitation was seen in several experiments. This effect was dependent on the presence of the pGEX vector, since the addition of extracts of *E. coli* carrying other expression vectors had no effect on the coprecipitation of RNA (compare lanes 12 and 4).

As a separate demonstration of the specificity of the U2 RNA-binding effect of A^{prime}, the protein was produced in *E. coli* with a different vector. A^{prime} expressed in pET expression vectors also stimulated B″ binding (Fig. 1, lane 11), demonstrating that this effect was dependent on A^{prime}. gst-A^{prime} alone failed to coprecipitate specific RNAs (lane 10). On the basis of these and other experiments not shown, we estimate that A^{prime} caused approximately a 100-fold stimulation of B″ binding to U2 RNA.

To exclude direct RNA-binding activity of the A^{prime} fusion proteins, an *E. coli* extract containing overexpressed g10-A^{prime} fusion protein was assayed for binding of ^{32}P-labeled HeLa cell total RNA. g10-A^{prime} did not bind to any RNA in this assay (Fig. 1, lane 13). Furthermore, RNA-binding activity of A^{prime} was not detected with any of a variety of other assays (8b).

In addition to the large increase in U2 RNA binding upon addition of S-100, gst-A^{prime}, or g10-A^{prime} extract, there was also a small stimulation of U1 RNA binding (Fig. 1A, lanes 5, 8, and 11; compare with lane 4). This effect was variable but reproducible. Therefore, a g10-A^{prime} fusion protein was used to determine whether A^{prime} was associated with U2 and U1 RNAs in the presence of B″. In vitro-translated g10-A^{prime} and B″ or g10-B″ polypeptides were used in combination to bind to HeLa cell total RNA (Fig. 1B). When the g10 epitope tag was present on A^{prime} only but not on B″ (lanes 3 and 4), only U2 RNA was coprecipitated by g10 antiserum. On the other hand, when the epitope tag was on both A^{prime} and B″, binding to both U1 and U2 RNAs was evident (lanes 5 and 6). Both full-length B″ (amino acids 1 to 225, lane 6) and a fragment lacking the carboxy-terminal RRM (amino acids 1 to 109, lane 5) were sufficient to bind U1 and U2 RNAs, and both

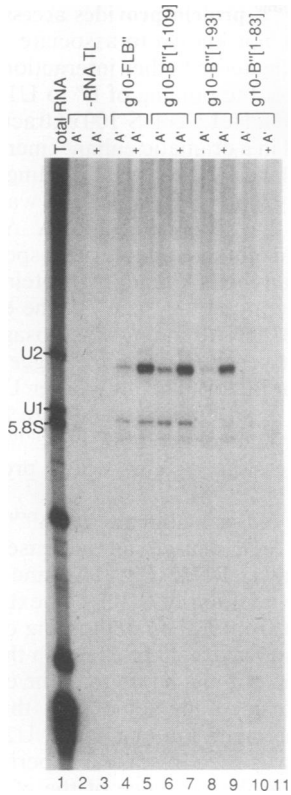


FIG. 2. Definition of the RNA-binding domain of U2 snRNP-B" protein. HeLa cell RNA binding assays were performed as for Fig. 1 in either the absence (−) or presence (+) of *E. coli* extract containing gst-A^{prime} (A'). The in vitro-translated g10-B" polypeptides were added in equimolar amounts. Lane 1, HeLa cell total RNA; lanes 2 and 3, unprogrammed translation; lanes 4 and 5, full-length g10-B"; lanes 6 and 7, g10-B" amino acids 1 to 109; lanes 8 and 9, g10-B" amino acids 1 to 93; lanes 10 and 11, g10-B" amino acids 1 to 83.

could interact with A^{prime} to allow specific coprecipitation of U2 RNA (lanes 3 and 4). B" is therefore similar to A in that the carboxy-terminal RRM is not needed as part of the U RNA-binding domain (19, 36). These results show that A^{prime} associates specifically with U2 snRNA but only in the presence of B", while B" itself is capable under these conditions of binding directly to U1 or U2 snRNAs through its amino-terminal RNA-binding domain.

Amino-terminal RRM of B" is sufficient for binding U2 RNA and for accessory response to A^{prime}. To determine whether regions flanking the RRM of B" are critical for A^{prime} enhancement, carboxy-terminal deletions of B" were prepared. Equimolar amounts of in vitro-translated g10 fusion proteins were used to bind total HeLa cell ^{32}P-labeled RNA. As shown in Fig. 2, amino acids 1 to 93 of B" were capable of binding U2 RNA (lane 8) but at a lower efficiency than amino acids 1 to 109 or full-length constructs (compare lanes 4, 6, and 8). In addition, U2 RNA binding by B" amino acids 1 to 93 was enhanced by the A^{prime} protein (lane 9). In contrast, amino acids 1 to 83 were not sufficient for either property (lanes 10 and 11). Separate immunoprecipitation experiments showed that the g10 antiserum precipitated all of the deletion constructs with comparable efficiency, thereby excluding inaccessibility of the epitope tag as a cause for the loss of apparent RNA binding (data not shown).

It should be noted that binding of U1 RNA by amino acids 1 to 93 of B" is decreased much more dramatically than binding of U2 RNA. This result suggests that in contrast to binding of U2 RNA, efficient binding of U1 RNA requires a few additional carboxy-terminal residues on the RNA-binding domain. We also have performed these experiments using g10-B" polypeptides produced in *E. coli* to rule out the possibility of interactions with other snRNP proteins which might be present in the reticulocyte lysates. The results were similar with either source, confirming that the 93-amino-acid domain of B" binds U2 RNA directly. To further exclude a role for the carboxy-terminal RRM of B" in binding U2 RNA, a highly conserved phenylalanine (residue 53) in the RNP octamer of the amino-terminal RRM of B" was changed to valine. This B" mutant failed to precipitate any specific HeLa cell RNA species (data not shown). Lutz-Freyermuth et al. (19) previously showed that the carboxy-terminal RRM of A also fails to bind detectably to any specific RNA. The function of this region, which is even more highly conserved between the two proteins than the amino-terminal region, remains unknown.

B" protein recognizes stem-loop IV of U2 RNA. RNA-binding domains for the A and 70K proteins have been shown to directly contact independent stem-loop structures (18, 31, 36, 46). The structural similarity between the A and B" proteins suggests that the sites which they recognize on their respective RNAs might also be similar. Using the HeLa cell total-RNA binding assay described above, regions of U2 RNA involved in the efficient binding to B" in the presence of A^{prime} were examined in a series of competition binding assays (Fig. 3A). Full-length U2 RNA or stem-loop IV of U2 RNA competed effectively for binding with the in vivo-labeled U2 RNA (lanes 4 and 6), while heterologous unlabeled RNAs added in excess were not able to compete with U1 or U2 RNAs (β-globin, U6, and U5 RNAs, lanes 1 to 3) and a mutant in U2 RNA lacking stem-loop IV (U2ΔSL4, lane 5) also did not compete. The efficiency of competition by stem-loop IV was less than complete, which may indicate that other regions of U2 RNA also participate in binding; this would be consistent with the conclusions of Hamm et al. (14) that regions other than stem-loop IV contribute to the efficiency of B" incorporation into U2 snRNPs. From these and other binding data (8b; data not shown) we conclude that stem-loop IV of U2 RNA forms the major binding site of B" protein in the presence of A^{prime}. The sequence in stem-loop IV of U2 RNA differs from that in stem-loop III of U2 RNA, which was noted previously to be similar to stem-loop II of U1 RNA (18).

The sequence present in the loop of U2 stem-loop IV is similar to that in the loop of U1 stem-loop II, which is the binding site for the A protein (18, 36) (Fig. 3B). This suggests that these two very similar proteins may recognize the similar loop sequences in each RNA. This model predicts that B" recognizes U1 RNA through cross-reactivity with stem-loop II of U1 RNA. We show below (see Fig. 5) that this stem-loop transcript bound B" in vitro with approximately the same affinity as U2 RNA. Further evidence of the similarity between stem-loop II of U1 RNA and stem-loop IV of U2 RNA is the observation that stem-loop IV of U2 RNA competed for binding of U1 RNA to B" (Fig. 3A, lane 4) while other RNAs did not (Fig. 3A, lanes 1, 2, 3, and 5).

A 5-amino-acid segment from the B" protein confers recognition of U2 RNA when placed in the A protein. The close primary sequence relationship between the A and the B" proteins (Fig. 3B) led us to test whether the element(s) controlling the specificity of RNA recognition might be

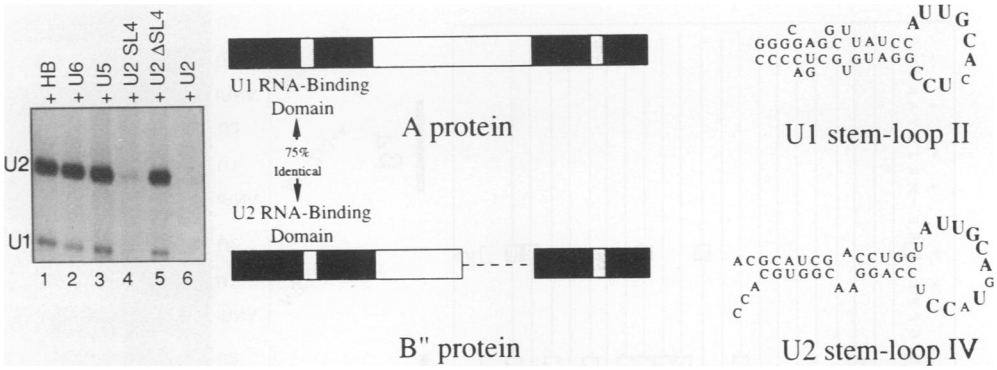


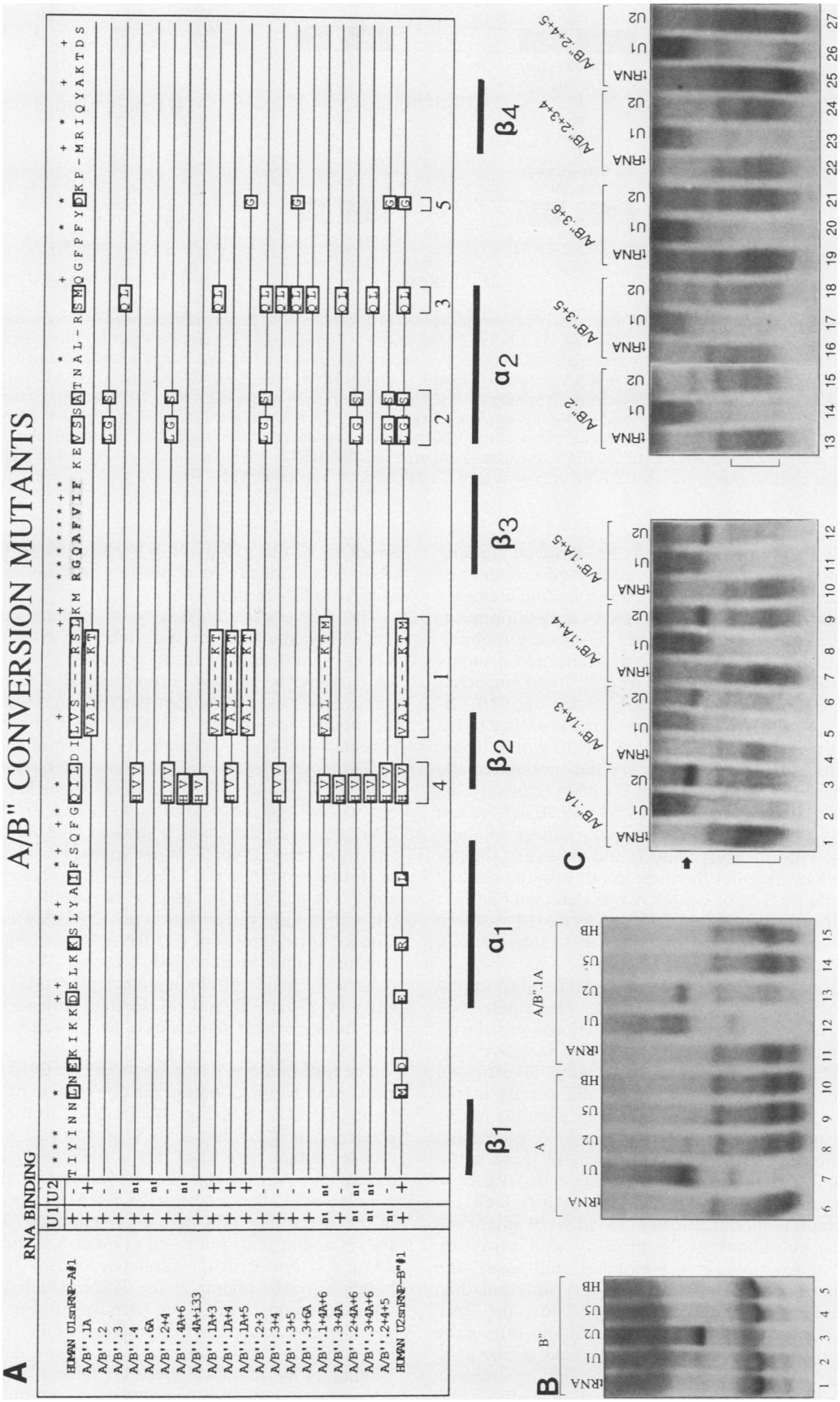
FIG. 3. U2 snRNP-B″ protein interacts primarily with stem-loop IV of U2 RNA. (A) In vitro-transcribed RNAs were tested for their abilities to compete against in vivo-labeled U1 and U2 RNAs. *E. coli* extracts containing overexpressed g10-B″ amino acids 1 to 109 were bound to ^{32}P-labeled HeLa cell total RNA and analyzed as for Fig. 1 except that each binding reaction mixture contained, in addition, 2.25 μM unlabeled in vitro-transcribed competitor RNA as follows: lane 1, β-globin RNA; lane 2, U6 RNA; lane 3, U5 RNA; lane 4, stem-loop IV of U2 RNA (U2 SL4); lane 5, U2 RNA lacking stem-loop IV (U2ΔSL4); lane 6, full-length U2 RNA. (B) Schematic diagram showing the similarity between the RRMs of A and B″ and between stem-loop II of human U1 RNA and stem-loop IV of human U2 RNA. Black boxes represent the RRMs, and grey boxes show the locations of the RNP octamer sequences. Nucleotides that are identical in the two loops of U1 and U2 RNA are shown in boldface. In U1 RNA, the loop sequence AUUGCACU is phylogenetically conserved in a number of species ranging from yeast cells to mammals. In U2 RNA, the sequence UUGCANU is conserved (10).

interchangeable. As shown in Fig. 4A, site-directed mutants were constructed that progressively converted elements of the A amino-terminal RRM into the corresponding elements of the B″ amino-terminal RRM. The RNA recognition properties of these mutants were analyzed by using a mobility shift assay in which ^{35}S-labeled in vitro-translated proteins were bound to RNA in the absence of A^{prime} and subjected to native gel electrophoresis. As shown in Fig. 4B, B″ formed a complex with slower mobility in the presence of U2 RNA (lane 3) than in the presence of tRNA, β-globin RNA, or U5 RNA (lanes 1, 4, and 5). A smaller amount of the protein was shifted in the presence of U1 RNA (lane 2). This finding confirmed that B″ binds only to U1 and U2 RNAs and not to other RNAs. In contrast, A formed a slower complex very efficiently with U1 RNA (lane 7) and only very slightly with U2 RNA (lane 8) under the same conditions; no binding to tRNA, β-globin RNA, or U5 RNA was detected (lanes 6, 10, and 9, respectively). When A^{prime} was added to these mobility shift assays, the labeled B″ failed to migrate as a discrete complex (data not shown).

The mobility shift analysis of B″ binding differed from that obtained by coimmunoprecipitation of total HeLa cell RNA in that the relative efficiency of binding of U2 compared with that of U1 appeared much higher when the mobility shift assay was used. Although we have not directly investigated this difference, we have noted similar differences in the results of these two techniques when other proteins in this family were used (unpublished observations). We envision several possible reasons for the differences in these methods. It is possible that the use of synthetic in vitro-transcribed small nuclear RNAs (snRNAs) (which lack the numerous nucleotide modifications made in vivo) and/or the use of different conditions in the mobility shift assay are responsible. For example, in the mobility shift assay, the ionic conditions in the native gels are very different than those used in the immunoprecipitations. Thus, the RNP complexes must be stable under these conditions during the 2-h migration time in the electrophoretic field of the gels. However, apart from this difference in the data, the results of these experiments are completely compatible. It is clear that this difference in RNP complex formation is not due to an effect of the g10 peptide because these fusion proteins behaved identically to nonfusion proteins in mobility shift assays (data not shown).

The mobility shift assay was used to screen the A/B″ conversion mutants for the ability to bind to U2 and U1 RNAs. The results of these assays are summarized in Fig. 4A, and representative examples are shown in Fig. 4C. Lanes 1 to 12 show the four conversion mutants that gained the ability to bind to U2 RNA in comparison with the parental wild-type A protein. For each of these mutants, a new complex of slower mobility was seen with both U1 RNA (lanes 2, 5, 8, and 11) and U2 RNA (lanes 3, 6, 9, and 12) but not with tRNA only (lanes 1, 4, 7, and 10). Representative examples of mutants which showed no U2 RNA binding are shown in lanes 13 to 27. What appears to be a small amount of shifted protein in the U2 RNA lanes (lanes 15, 18, 21, 24, and 27) is similar to the amount of parental A protein shifted when the same concentration of U2 RNA was used (Fig. 4B, lane 8); small effects on U2 RNA binding by some of these mutants cannot be excluded, however. All of these mutants remained capable of binding efficiently to U1 RNA (Fig. 4C, lanes 14, 17, 20, 23, and 26). Results with additional mutants that failed to bind significantly to U2 RNA are included in Fig 4A.

The four mutants which bound to U2 RNA in the mobility shift assay had in common the conversion of amino acids 44 to 48 of A (LVSRS; Fig. 4A, block 1) to the corresponding sequence of B″ (amino acids 41 to 45; VALKT). In the case of one mutant (A/B″.1A) this was the only mutation introduced into A, thus directly demonstrating that this simple 5-amino-acid change alters the RNA recognition specificity of the A protein. This pentamer sequence (which is part of the six amino acids indicated as block 1 at the bottom of Fig. 4A and is boxed in the sequence of A/B″.1A) is part of a highly variable region in the RRM which we have called variable region-1 (VR-1; see Discussion). Figure 4B, lanes 11 to 15, shows a mobility shift analysis of the pentamer mutant performed in parallel to wild-type B″ and A (lanes 1 to 10). Binding of the mutant to both U1 (lane 12) and U2 (lane 13) RNAs was relatively efficient and was not due to nonspecific binding, since unrelated β-globin or U5 RNAs failed to form


A
A/B'' CONVERSION MUTANTS
RNA BINDING
U1
U2
HUMAN U1snRNP-A#1
A/B''.1A
A/B''.2
A/B''.3
A/B''.4
A/B''.6A
A/B''.2+4
A/B''.4A+6
A/B''.4A+13
A/B''.1A+3
A/B''.1A+4
A/B''.1A+5
A/B''.2+3
A/B''.3+4
A/B''.3+5
A/B''.3+6A
A/B''.1+4A+6
A/B''.3+4A
A/B''.2+4A+6
A/B''.3+4A+6
A/B''.2+4+5
HUMAN U2snRNP-B''#1
β1
α1
β2
β3
α2
β4
B
B''
A
A/B''.1A
tRNA
U1
U2
U5
HB
C
A/B''.1A
A/B''.1A+3
A/B''.1A+4
A/B''.1A+5
A/B''.2
A/B''.3+5
A/B''.3+6
A/B''.2+3+4
A/B''.2+4+5

complexes (lanes 14 and 15). In addition, when the pentamer mutant was expressed as a g10 fusion protein in *E. coli* and used to bind HeLa cell total RNA, U1 and U2 RNAs were the only species specifically precipitated (see Fig. 6B, lane 4). We conclude that conversion of the 5-amino-acid sequence in A to that of B″ results in a gain of specific recognition of U2 RNA rather than a general relaxation of the specificity of RNA recognition.

Relative affinities of A, B″, and pentamer mutant for binding U1 and U2 RNAs. To quantitate the relative apparent affinities of wild-type A, wild-type B″, and pentamer mutant A/B″.1A for U1 and U2 RNAs, serial dilutions of U1 RNA stem-loop II or of U2 stem-loop IV were incubated with a constant amount of ^{35}S-labeled in vitro-translated protein. The amount of the labeled protein bound was then assayed by mobility shift analysis, and the results were quantitated by densitometry to obtain an estimate of the relative affinities for each RNA (19). An example of such dose response mobility shifts is shown in Fig. 5A and B. The pentamer mutant (A/B″.1A) retained the high affinity for U1 RNA ($K_d \approx 70$ nM) characteristic of its parent A protein ($K_d \approx 30$ nM); the affinity of B″ for U1 was much lower ($K_d \approx 800$ nM) (Fig. 5A). Conversely, the pentamer mutant (A/B″.1A) showed an affinity for U2 RNA ($K_d \approx 900$ nM) similar to that of wild-type B″ ($K_d \approx 400$ nM), while A showed only very low-affinity binding of U2 RNA ($K_d > 10{,}000$ nM) (Fig. 5B). These values are in accord with an earlier approximated K_d of 80 nM for binding of A to U1 stem-loop II of U1 (19). Figure 5C shows examples of mobility shift experiments that were scanned to generate the quantitation curves shown in Fig. 5A and B.

In summary, the pentamer conversion mutant retained a U1 RNA-binding affinity similar to that of its parental protein while acquiring the ability to bind U2 RNA with approximately the same relative affinity as wild-type B″. Therefore, other B″-specific sequences are not necessary for the binding of U2 RNA by B″.

Site of protein-protein interaction of A^{prime} within the RRM of B″ is distinct from the pentamer segment. To determine whether the pentamer sequence in the conversion mutants is also sufficient for the enhancement of U2 RNA binding by A^{prime}, the A/B″ conversion mutants were assayed for their abilities to interact with A^{prime}. In vitro-translated A, B″, or A/B″ conversion mutant polypeptides were added to *E. coli*-produced g10-A^{prime} fusion protein, incubated with labeled HeLa cell total RNA, and immunoprecipitated with the g10 antiserum (Fig. 6A). It is evident that the wild-type B″ protein enabled the g10 peptide-tagged A^{prime} protein to complex with and coprecipitate U2 RNA (lane 11), while none of the conversion mutants were able to do so (lanes 3 to 10). As expected, the A protein could not enter into an RNP complex with the A^{prime} protein (lane 12). These data demonstrate that the site on B″ that allows it to interact properly with A^{prime} is distinct from the pentamer sequence that controls U2 RNA recognition.

Specificity of reverse construct: pentamer segment in A confers increased U1 RNA binding when substituted into B″. To further confirm the importance of this 5-amino-acid sequence in U1 and U2 RNA recognition, we exchanged the sequence from A (LVSRS) into B″ (replacing VALKT). This construct (B″/A.1A) represents the reverse of the A/B″.1A exchange construct. All of the constructs were expressed as g10 fusion proteins in *E. coli* and used to bind HeLa cell ^{32}P-labeled total RNA as in Fig. 1. As expected from the quantitative estimates of binding efficiency, the A/B″.1A mutant bound U1 RNA at high efficiency, similar to that of wild-type A, but in addition acquired the capacity to bind U2 RNA (Fig. 6B, compare lanes 3 and 4). On the other hand, the B″/A.1A mutant bound U2 RNA with a low efficiency, similar to that of wild-type B″, but bound U1 with increased efficiency (Fig. 6B, compare lanes 5 and 6), thus confirming the importance of this region in determining the affinity of these two closely related proteins for their respective RNAs. This construct showed a tendency to bind nonspecifically to some large RNAs, as shown by an increased background in lanes 5 and 10. The ability of B″/A.1A to continue binding U2 RNA suggests that other B″-specific sequences may participate in U2 RNA recognition.

Comparison of the A/B″.1A and the B″/A.1A exchange constructs demonstrated that only wild-type B″ was significantly affected by the addition of A^{prime} (Fig. 6B, compare lanes 3 to 6 with lanes 8 to 11). These data indicate that the sequence VALKT in B″ is necessary for A^{prime} enhancement (since its replacement in B″/A.1A abolished the effect) but not sufficient (since it failed to confer the effect in A/B″.1A) and that additional sequence differences outside this segment must be required for the A^{prime} accessory function.

FIG. 4. A change of five amino acids in the U1 snRNP-A protein allows it to bind to U2 RNA. (A) Summary of site-directed mutants that progressively convert the U1 snRNP-A protein into the U2 snRNP-B″ protein. The first and last sequences in the table show the amino-terminal RRMs of the U1 snRNP-A and U2 snRNP-B″ proteins as taken from Query et al. (30). Between A and B″ are shown the amino-terminal RRMs of each of the A/B″ conversion mutants. Each mutant contains the wild-type A sequence (solid line) except at the boxed positions, where the sequence has been converted to that of B″. The numbered blocks at the bottom of the table indicate groups of amino acids converted as a block to the B″ sequence. Block 1 corresponds to sequences in VR-1 including residues LV in β2 of the domain. Block 6 is not shown; it changed amino acids 1 to 6 (MAVPET) of A to amino acids 1 to 3 (MDI) of B″. The shaded region in A highlights the RNP 1 octamer sequence. The columns at the left summarize the results of RNA-binding assays. The abilities of mutants to bind (+) or not to bind (−) to full-length U1 or U2 RNAs are indicated. nt, Mutants not tested directly for U1 or U2 RNA binding but only for their ability to coprecipitate U2 RNA in the presence of A^{prime} (Fig. 6A). Greek symbols at the bottom indicate the secondary structure of A as determined by nuclear magnetic resonance spectroscopy (see reference 15 and Fig. 7). (B) Mobility shift assays of B″, A, and pentamer mutant A/B″.1A. ^{35}S-labeled in vitro-translated polypeptides representing B″ amino acids 1 to 109 (lanes 1 to 5), A amino acids 1 to 119 (lanes 6 to 10), or A/B″.1A amino acids 1 to 119 (lanes 11 to 15) were incubated with 1.5 mg of the indicated in vitro-transcribed RNAs and assayed for specific complex formation on a nondenaturing polyacrylamide gel. Lanes 1, 6, and 11, No additional RNA (tRNA only); lanes 2, 7, and 12, full-length U1 RNA; lanes 3, 8, and 13, full-length U2 RNA; lanes 4, 9, and 14, U5 RNA; lanes 5, 10, and 15, β-globin RNA. (C) Mobility shift assays of selected A/B″ conversion mutants were performed as for panel B. The ^{35}S-labeled in vitro-translated polypeptides represented amino acids 1 to 142 of the constructs indicated above each panel. The RNAs added to each lane are indicated above each lane and are identical to those in panel B. Lanes 1 to 12 show all mutants which formed a complex with U2 RNA; lanes 13 to 27 show representative examples of those which did not detectably bind U2 under these assay conditions. The brackets show the location of the labeled protein in the presence of tRNA only (which migrates as a smear); the arrow shows the slower-migrating band formed upon addition of U2 RNA to mutants containing the exchanged pentamer segment. The results of all the mutants tested are summarized in panel A.

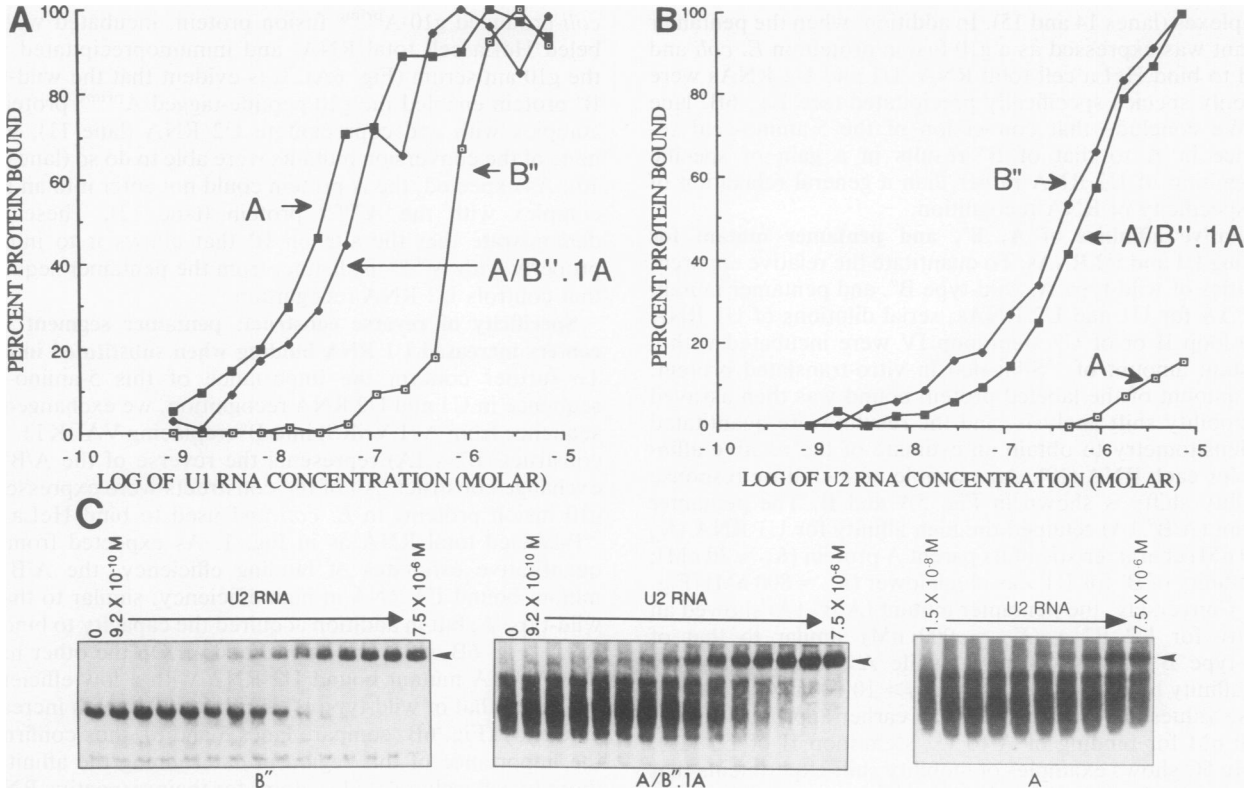


FIG. 5. Quantitative demonstration that the pentamer mutant A/B".1A acquires affinity for U2 RNA similar to that of the U2 snRNP-B" protein. Relative binding affinities were measured by using the mobility shift method as described elsewhere (19). In brief, a constant amount of in vitro-translated protein was incubated with serial twofold dilutions of an in vitro-transcribed RNA. The graphs show the percent protein present in the specific RNP complexes formed by stem-loop II of U1 RNA (A) and by full-length U2 RNA (B). The B" polypeptide used represents amino acids 1 to 109; the A and A/B".1A polypeptides represent amino acids 1 to 142. (C) Examples of autoradiograms for quantitation of U2 RNA binding; the labeled polypeptide for each gel is indicated below the gel, and the concentrations of U2 RNA are indicated above. The large arrowhead indicates the position of the slower-migrating complex.

DISCUSSION

Determinants of specific RNA recognition within the RNA-binding domain. Although the RRM has been shown to represent all or most of an RNA-binding domain for the 70K, A, and B" proteins (see reference 16 for a review), the determinants within this motif important for recognition of specific RNAs are not understood. We have exploited the similarity between the RRMs in two RNA-binding proteins to determine which of the sequence differences are responsible for their differing RNA-binding properties. We have shown that the U1 snRNP-A protein can be altered to recognize U2 RNA by modification of a 5-amino-acid segment to the corresponding sequence in a related protein, B" (Fig. 4A). This region includes the most divergent positions within the RRM family, both in amino acid composition and in length, which varies from 1 to 14 amino acids. We call this region VR-1; it encompasses the sequence indicated in Fig. 4A as block 1. The presence of this poorly conserved element of sequence within the larger motif suggests either that the element is extraneous and unnecessary or that the element is involved in functions unique to individual proteins. The demonstration here that the poorly conserved VR-1 region is responsible for at least some of the differing RNA recognition properties of A and B" suggests that it might play a similar role in other RRM-containing proteins.

It is likely that, in such a diverse family of proteins, multiple sequence elements have the potential to influence RNA-binding specificity. Cross-species comparisons among members of the RRM family in which phylogenetically diverse sequences are available show conservation of VR-1 in some but not all family members. The La protein, for example, is almost identical in this region in the human (4), bovine (5), and frog (24) sequences, whereas other regions in the La RRM are more divergent, suggesting that VR-1 may play an important role in RNA recognition by La. In contrast, the U1 snRNP-70K protein VR-1 sequence is not phylogenetically conserved in either length or sequence between human (see reference 30 and references therein), frog (7), and fly (20), even though the remainder of the RRM is almost identical. Thus, VR-1 may not be a critical determinant of RNA specificity in all RRM proteins.

Our recent determination of the structure of the U1 RNA-binding domain of the A protein using nuclear magnetic resonance spectroscopy has revealed that VR-1 is partially in a loop that connects two strands of a four-stranded antiparallel β sheet (15). In the domain, the RNP 1 and RNP 2 sequences are directly adjacent to one another in the β sheet (Fig. 7). Specific aromatic amino acids within RNP 1 (the RNP consensus octamer) and RNP 2 are necessary for RNA binding (19, 31b), and by using the hnRNP-A1 protein, a conserved phenylalanine within each can be cross-linked to oligodeoxythymidine (22), suggesting that they might directly contact the bound RNA. As shown in Fig. 7, our structural analyses indicate that two conserved

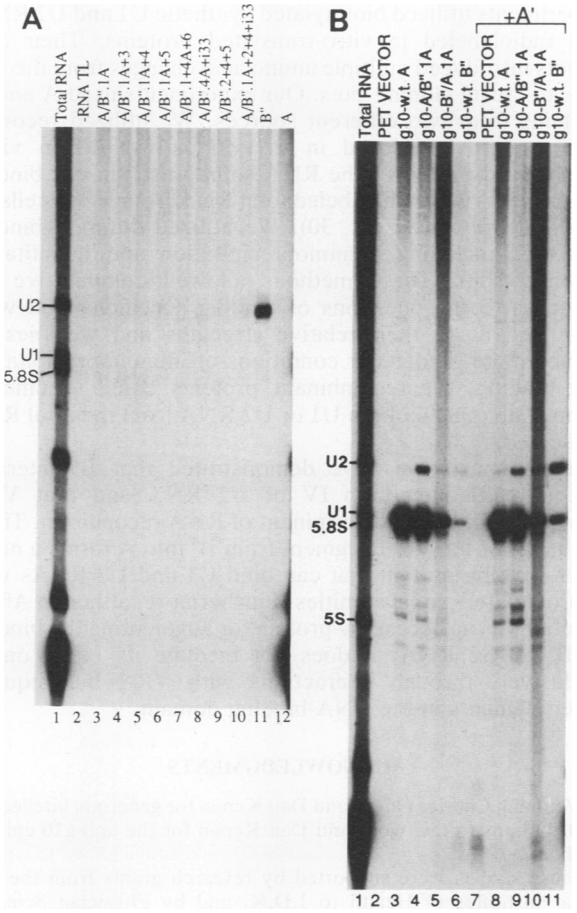


FIG. 6. Regions of U2 snRNP-B″ outside the pentamer sequence responsible for U2 RNA recognition are necessary for interaction with A^{prime} and enhancement of U2 RNA binding. (A) *E. coli* extract containing g10-A^{prime} was bound to ^{32}P-labeled HeLa cell total RNA in the presence of the following in vitro-translated polypeptides and analyzed as for Fig. 1B: lane 1, HeLa cell total RNA; lane 2, unprogrammed translation; lane 3, A/B″.1A; lane 4, A/B″.1A+3; lane 5, A/B″.1A+4; lane 6, A/B″.1A+5; lane 7, A/B″.1+4A+6; lane 8, A/B″.4A+i33; lane 9, A/B″.2+4+5; lane 10, A/B″.1A+2+4+i33; lane 11, B″ amino acids 1 to 109; lane 11, wild-type A. Except as noted, all the translated polypeptides represented full-length proteins. (B) *E. coli* extracts containing the indicated overexpressed g10 fusion proteins were used to bind ^{32}P-labeled HeLa cell total RNA in the absence (lanes 2 to 6) or presence (lanes 7 to 11) of gst-A^{prime} (A′) and the coprecipitated RNAs were analyzed as for Fig. 1. Lane 1, Total HeLa cell RNA; lanes 2 and 7, pET vector alone; lanes 3 and 8, g10–wild-type A; lanes 4 and 9, g10-A/B″.1A; lanes 5 and 10, g10-B″/A.1A; lanes 6 and 11, g10–wild-type B″.

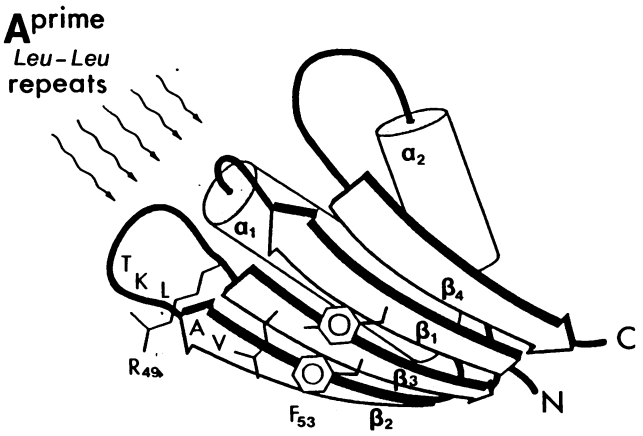


FIG. 7. Model of the tertiary structure of the RNA-binding domain of the U2-B″ protein as determined for the A protein by nuclear magnetic resonance spectroscopy (15), showing the amino acid residues in regions that affect the recognition of U1 and U2 RNAs by A and B″ proteins. VR-1, representing residues 44 to 48 of A and 41 to 45 of B″, lies between β-strand 2 and β-strand 3, affects the specificity of RNA recognition directly, and is involved also in interaction with A^{prime}. Residues 17 to 28 in A, connecting β-strand 1 and α-helix 1, correspond to residues 14 to 25 in B″ and appear to be sites of protein-protein interaction between A^{prime} and B″ (37) that affect binding to U2 RNA. Amino acids are shown in single-letter code, and A^{prime} is shown as the 5-leucine repeats found to be required for reconstitution into U2 snRNPs (8).

aromatic residues in RNP 1 and RNP 2 project outward to the surface of the RNA-binding domain. VR-1 may contact the RNA directly or may influence the orientation of the domain for recognition of the RNA by residues in the RNP 1 and RNP 2 regions. The observation that B″ continued to bind U2 RNA even when VR-1 was substituted from A (construct B″/A.1A; Fig. 6B) showed that additional B″-specific sequences are involved in binding to U2 RNA and that VR-1 is not the only determinant of recognition. An important caution is that in the case studied here, A and B″ proteins and the U1 and U2 RNAs possess very similar sequences (Fig. 3B) and, therefore, probably have the same higher-order structure. Thus, segments of one sequence when substituted into the other can still function properly. The degree to which this is a general feature of the family remains to be determined.

Results presented here are compatible with models in which VR-1 plays either a positive or a negative role in RNA binding. As an example of the negative mode, VR-1 in A might act by inhibiting the binding of U2 RNA to other elements in the domain. When exchanged with VR-1 from B″ (as in mutant A/B″.1A), binding of U2 RNA might become "unmasked." Therefore, in this case, the VR-1 sequence element need not play a direct role in RNA binding. This possibility appears less likely in light of the observation that the reverse exchange of VR-1 from A into B″ does not inhibit U2 binding. However, such an inhibitory effect could be dependent on A-specific sequences. In either case, VR-1 plays an important role in the recognition and binding of U1 and U2 RNAs by A and B″ proteins. In addition, we have ruled out the possibility that exchange of VR-1 might distort the folding of the RNA-binding domain by nuclear magnetic resonance spectroscopic analysis of the pentamer mutant domain A/B″.1A. In this experiment, when a highly ordered two-dimensional spectrum was compared with that of the wild-type RNA-binding domain of the A protein, only five distinct differences were evident (unpublished data).

Accessory factors influencing formation and activity of an RNA-binding domain. B″ requires the action of an accessory protein, A^{prime}, to function as a high-affinity U2 RNA-specific binding protein. Accessory factors that modulate the nucleic acid-binding activity of proteins in other systems are known, including transcription factors that function as heterodimers (reviewed in references 26 and 29). Protein-protein interactions among RNA-binding proteins have also been reported. For example, two proteins in the signal recognition particle, SRP9 and SRP14, form a heterodimer which binds specifically to 7SL RNA (44). In this case, SRP9

can bind weakly to 7SL RNA in the absence of SRP14, but the presence of SRP14 enhances the binding of SRP9. In addition, at least two 30S ribosomal proteins (S6 and S18) have a mutual requirement for binding to 16S rRNA (23, 43). B″ is unique in that it represents an RRM-containing protein with intrinsic affinity for its cognate RNA but which nevertheless requires an accessory factor (A^{prime}) to optimize its affinity for that RNA.

We have demonstrated that the minimal RNA-binding domain in B″ (amino acids 1 to 93) remains capable of enhancement by A^{prime}. In addition, we have shown that the VR-1 sequence VALKT in B″ is necessary but not sufficient for this interaction. The experiments reported here do not address the mechanism by which B″ interacts with A^{prime}. However, A^{prime} contains a repeating motif of leucine and asparagine residues (8) common to several proteins known to be involved in protein-protein interactions. Fresco et al. (8) demonstrated recently that the leucine repeats in A^{prime} are required for its reconstitution into U2 snRNPs in cell extracts as well as for its accessory function in the binding of B″ protein to U2 RNA. Furthermore, A^{prime} had no detectable intrinsic RNA-binding activity in several assays (Fig. 1A, lanes 11 and 12; 8b). Therefore, we propose that A^{prime} acts primarily through protein-protein interactions with B″, as depicted in Fig. 7. The finding of an accessory factor for B″ has implications for studies of RNA binding by other members of the RRM family. For example, it may be necessary to include accessory factors or cell extracts in binding reactions involving other RRM-containing proteins of unknown RNA specificity. Given the diverse nature of the RNA-binding proteins of this family, it will be important to understand the role of both conserved and nonconserved elements in the RRM that control the recognition of specific RNA sequences.

While this paper was being prepared, Scherly et al. (35) reported that an exchange of eight amino acids between B″ and A reversed binding specificity. There are similarities between the results of their study and the data presented here, but important differences must be noted. For example, one of the mutants reported here (A/B″.1A+4) contains seven of these same amino acids but showed significantly different binding properties. Mutant 5 depicted in Fig. 5 of reference 35 was found to have complete reversal of binding specificity in that it behaved identically to B″. In contrast, our mutant retained high-affinity binding to U1 RNA while acquiring the ability to bind U2 RNA. Thus, the determinants of specificity appear to involve portions of the domain other than just this single protein segment. In addition, the results of Scherly et al. (35) showed transfer of A^{prime} enhancement of RNA binding in mutant 5 by the 8-amino-acid segment, while we found uncoupling of the A^{prime} response with our mutants. Subsequent work from their laboratory (37) has confirmed our finding and shown that the site of interaction of A^{prime} with B″ includes residues 14 to 25 in addition to residues 37 to 46 (Fig. 7).

Another difference in the data reported here and that reported previously (35) is that Scherly et al. did not detect RNA binding by the reverse construct (mutant 6 in Fig. 5 of reference 35). In contrast, our reverse construct (B″/A.1A) showed an increase in binding to U1 RNA (Fig. 6). Thus, the continued ability of this mutant to bind U2 RNA after substitution of the segment in VR-1 confirms that other regions of the RNA-binding domain are important in the recognition and specific binding of these proteins to RNA.

Discrepancies between our results and those of Scherly et al. (35) probably result from our different methods. Their experiments utilized biotinylated synthetic U1 and U2 RNAs and radiolabeled in vitro-translated proteins. Their constructs introduced multiple amino acid changes from the wild type to create cloning sites. Our experiments used A and B″ proteins from two different sources. We utilized recombinant proteins produced in *E. coli* as well as in vitro-synthesized proteins. The RNA source used in our binding experiments was radiolabeled total RNA from HeLa cells, as reported previously (19, 30). We utilized different binding methods, including immunoprecipitation and quantitative mobility shift. These methods allowed comparative approaches to the questions of binding specificity and were evaluated as to their relative strengths and weaknesses. Furthermore, under our conditions of immunoprecipitation and binding, the recombinant proteins could specifically remove up to 80% of the U1 or U2 RNA from the total RNA presented.

In summary, we have demonstrated that B″ interacts directly with stem-loop IV of U2 RNA and that VR-1 constitutes only one determinant of RNA recognition. Thus, exchange of the VR-1 segment from B″ into A forms a novel RNA-binding protein that can bind U1 and U2 RNAs with approximately equal affinities. Furthermore, although A^{prime} functions as an accessory protein for augmenting the binding of B″ to U2 RNA, it does not mediate its effect on B″ exclusively through interactions with VR-1 but requires other elements in the RNA-binding domain.

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