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The U2B″ RNP motif as a site of protein-protein interaction

Daniel Scherly, Nina A.Dathan, Wilbert Boelens[1], Walther J.van Venrooij[1] and Iain W.Mattaj

European Molecular Biology Laboratory, Meyerhofstrasse 1, 6900 Heidelberg, FRG and [1]Department of Biochemistry, University of Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands

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The U2 snRNP contains two specific proteins, U2B″ and U2A′. Neither of these proteins, on its own, is capable of specific interactions with U2 RNA. Here, a complex between U2B″ and U2A′ that forms in the absence of RNA is identified. Analysis of mutant forms of U2B″ shows that the smallest fragment able to bind specifically U2 RNA (amino acids 1-88) is also the minimal region required for complex formation with U2A′, and implies that this region must be largely structurally intact for U2A′ interaction. Although this truncated U2B″ fragment is capable of making specific protein-RNA and protein-protein interactions its structure, as measured by the ability to bind to U2A′, appears to depend on the rest of the protein. Hybrids between U2B″ and the closely related U1A protein are used to localize U2B″ specific amino acids involved in protein-protein interaction. These can be divided into two functional groups. U2A′ interaction with U2B″ amino acids 37-46 permits binding to U2 RNA whereas interaction with U2B″ specific amino acids between positions 14 and 25 reduces non-specific binding to U1 RNA. These two proteins may serve as a general example of how RNA binding may be modulated by protein-protein interaction in the assembly of RNPs, particularly since the region of U2B″ involved in interaction with U2A′ consists mainly of a conserved RNP motif.

Key words: protein-protein interaction/protein-RNA interaction/RNA processing/U2 snRNP

Introduction

RNA-protein complexes, also called ribonucleoprotein (RNP) particles, are abundant and play important roles in DNA, RNA and protein metabolism (see e.g. Dreyfuss *et al.*, 1988, for a recent review). They can be formed of multiple proteins and RNAs (ribosome, spliceosome), multiple proteins with one RNA (SRP, snRNP) or one protein with one RNA (tRNA-aminoacyl-tRNA synthetase, RNase P). The architecture of the first two classes of RNPs is maintained by both protein-protein and RNA-protein interactions. The molecular nature of those interactions is poorly understood but, with the number of known RNPs increasing rapidly, some common motifs which may be involved have been discovered.

The best characterized protein motif (for reviews see Bandziulis *et al.*, 1989; Mattaj, 1989) consists of ~80 amino acids. First called the RNA binding domain (Dreyfuss *et al.*, 1988) it was later also named the RNA recognition motif (RRM) (Query *et al.*, 1989) or the RNP-80 motif (Scherly *et al.*, 1989). Within this motif, the most conserved sequence is a segment of eight amino acids called the RNP consensus sequence (RNP-CS, Adam *et al.*, 1986) or RNP1 (Dreyfuss *et al.*, 1988). For three snRNP proteins that are members of this family, U1 70K (Query *et al.*, 1989), U1A (Scherly *et al.*, 1989) and U2B″ (Scherly *et al.*, 1990), it has been shown that the RNP-80 motif is part of the minimal protein element able to bind specifically to RNA. A major determinant of both U1A and U2B″ RNA binding specificity has been located immediately N-terminal to the RNP-CS (Scherly *et al.*, 1990), which is one of the most variable regions when RNP-80 motifs are compared (Bandziulis *et al.*, 1989; Query *et al.*, 1989). In contrast to 70K and U1A, U2B″ requires a second protein, U2A′, to bind specifically to U2 RNA. In the absence of U2A′, U2B″ binds RNA non-specifically (Scherly *et al.*, 1990).

Since there is no example in which the molecular basis of protein-protein interaction between RNP proteins and the resultant effects on RNP assembly is well understood, a detailed examination of the U2B″-U2A′ interaction has been undertaken. Using rabbit antibodies raised against recombinant U2A′, we show that U2B″ and U2A′ form a complex in the absence of RNA. Amino acids 1-88 of U2B″, corresponding to a copy of the RNP-80 motif together with a few additional amino acids, are defined as the minimal protein element which can bind to U2A′. U2A′ is shown to modulate the binding of U2B″ to RNA in two ways. First, the interaction is essential for specific binding to U2 RNA. Second, U2A′ decreases the binding of U2B″ to U1 RNA. These two effects of U2A′ are shown to be separable and to be due to interaction with different regions of the minimal RNA binding segment of U2B″.

Results

An intact RNP motif is required for interaction of U2B″ with U2A′

To analyse the interaction of the U2B″ and U1A snRNP proteins with their cognate RNAs, we previously constructed a series of mutant forms of both cDNAs. The primary mutations were made by generating unique *Bam*HI restriction sites, which subsequently allowed the construction of N- or C-terminal deletions, internal deletions or hybrids between the U1A and U2B″ cDNAs (Scherly *et al.*, 1989, 1990; W.B. and D.S., unpublished data). The U2B″ derivatives in which mutations had been introduced into the minimal RNA binding segment, amino acids 1-88, (Scherly *et al.*, 1990) were translated in wheat germ extract (WGE) in the presence of [^{35}S]methionine. Their association with *in vitro* made U2A′ protein was tested by coprecipitation with U2A′ using either of two specific anti-U2A′ rabbit

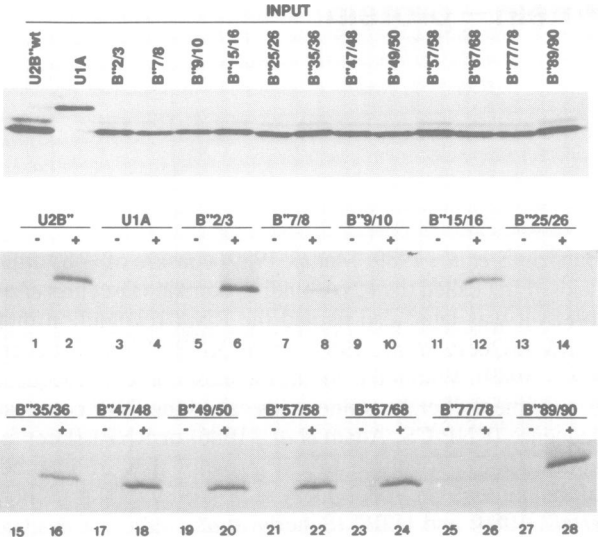

Fig. 1. Protein−protein interaction between U2A′ and radiolabelled U2B″ point mutants. The amount of protein used per binding assay is shown in the INPUT panel. Unlabelled U2A′ and radiolabelled U2B″ *Bam*HI point mutants (see text) were synthesized separately in wheat germ extract. Each U2B″ derivative was incubated either alone (− lanes) or mixed with U2A′ in a ratio of 1:1 (+ lanes). After incubation, the protein complexes were immunoprecipitated with a rabbit anti-U2A′ antibody coupled to protein−A sepharose beads. Proteins were released by boiling in SDS−PAGE sample buffer and analysed by SDS−PAGE. The protein derivatives analysed are indicated. U1Awt protein was used as a negative control (lanes 3 and 4) and U2B″wt as a positive control (lanes 1 and 2).

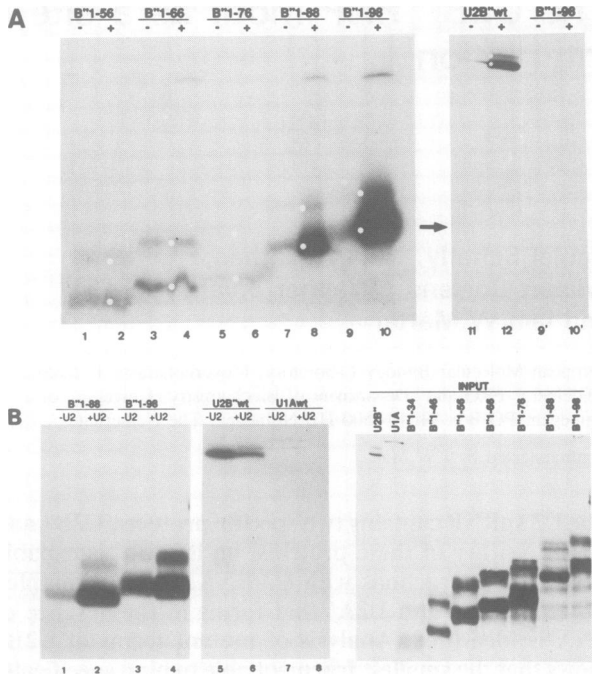

Fig. 2. Protein−protein interaction between U2A′ and truncated U2B″ derivatives. The amount of protein used is shown in the INPUT panel. U2B″wt and each truncated derivative appear as a doublet (white dots). The reason for this has not been studied in detail, but appears to be due to upstream initiation at a non-methionine codon *in vitro* (unpublished observations). (**A**) The truncated U2B″ proteins were tested in the presence and absence of added U2A′ protein as described in Figure 1. To illustrate the difference in binding efficiency between U2B″(1−98) and U2B″wt, lanes 11, 12, 9′ and 10′ show a shorter exposure of the relevant lanes of the same gel. (**B**) The effect of U2 RNA on interaction between U2B″wt and truncated U2B″ derivatives with U2A′. U2 RNA was (lanes +U2) or was not (lanes −U2) added to the indicated protein mixtures. These were then immunoprecipitated with anti-U2A′ antibodies as described.

antisera. The antibodies were raised against recombinant U2A′ protein produced in *Escherichia coli* (as described in Materials and methods). In the following figures the relative amounts of input protein are shown together with the protein immunoprecipitated either in the absence (−) or presence (+) of added, unlabelled U2A′ protein.

U2A′ and U2B″ are found associated within U2 snRNP particles *in vivo*. However, *in vitro*, U2B″ forms a specific complex with U2A′ in the absence of U2 snRNA, as shown by the fact that it is immunoprecipitated by anti-U2A′ antiserum in the presence of added U2A′ protein (Figure 1, lane 2) but not in its absence (Figure 1, lane 1). As a specificity control, the interaction of the closely related U1A protein with U2A′ was tested and found to be negative (Figure 1, lanes 3 and 4). In the U2B″ point mutants two amino acids, whose positions are indicated by numbers, were substituted by glycine and serine. Of the mutants only three, B″9/10, B″25/26 and B″77/78, did not associate with U2A′ and one, B″7/8, reproducibly bound more weakly (Figure 1, lane 8). These four mutants are incapable of binding to U2 RNA *in vitro* while the other eight mutants can either bind both to U2A′ and to U2 RNA or can still bind to U2A′ but not to U2 RNA (Figure 1 and W.B. and D.S., unpublished data).

A protein consisting of only the first 88 amino acids of U2B″ can still bind specifically to U2 RNA (Scherly *et al.*, 1990). The minimal RNA binding protein segment was also found to be capable of specific interaction with U2A′ (Figure 2a, compare lanes 7 and 8 with lanes 1−6). The signal coming from B″(1−76) and shorter derivatives is background binding to protein A−sepharose beads and is unaffected by the addition of U2A′. It was previously reported that U2B″ 1−98 bound U2 RNA more strongly than U2B″ 1−88 (Scherly *et al.*, 1990). As seen in lanes 7−10 the longer truncated derivative also interacts more strongly with U2A′, which may explain its higher affinity for U2 RNA.

Next, a series of deletions, each 10 amino acids long, were made throughout the first 90 amino acids of U2B″. None of the deletion mutants was capable of interaction with U2A′ (data not shown). This indicated that the intact structure of the minimal RNA binding segment was both required, and sufficient, for complex formation with U2A′. However, this interaction was rather weak. When compared with U2B″ wild-type (wt), the binding of the truncated derivatives of U2B″ was much reduced (Figure 2a, lanes 11, 12, 9′ and 10′). The position of the B″1−98 protein, which is not visible at this level of exposure, is marked by an arrow. This was somewhat surprising because we have previously shown that the truncated U2B″ proteins bound to U2 RNA indistinguishably from U2B″wt (Scherly *et al.*, 1990). We therefore tested the effect of adding U2 RNA to the protein−protein interaction assay. U2B″wt was immunoprecipitated either from a standard protein−protein interaction assay or one to which U2 RNA had been added (Figure 2b, lanes 5 and 6). In the presence of RNA U2B″wt was immunoprecipitated by anti-U2A′ antibodies to a slightly reduced extent. In contrast, the addition of U2 RNA

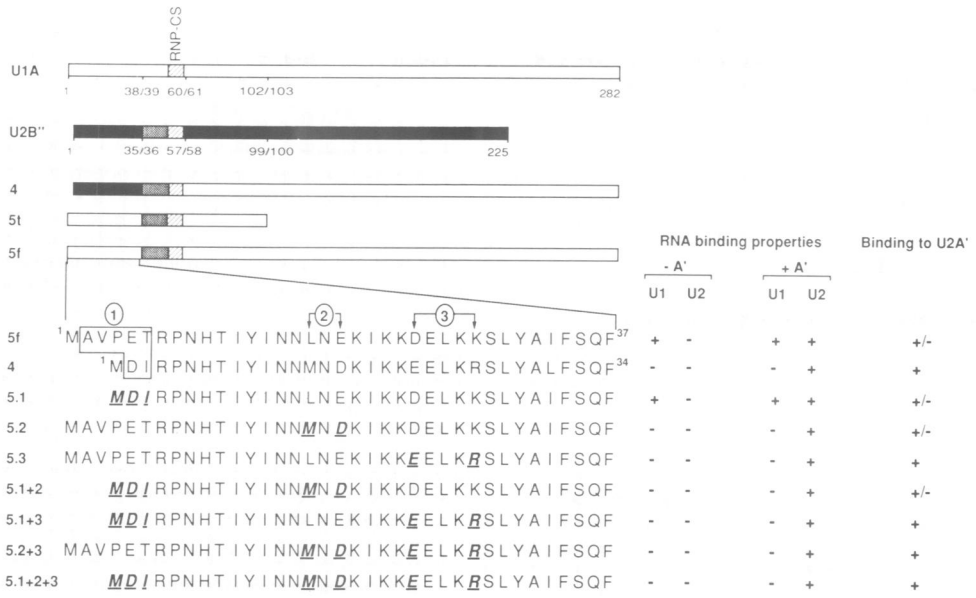

Fig. 3. Diagrammatic representation of U1A, U2B″ and hybrid proteins 4, 5t and 5f. U1A protein segments are represented by open boxes, U2B″ protein elements by black boxes. The conserved RNP consensus sequence (RNP-CS) is represented by a box with diagonal lines and the major determinant of U2B″ RNA binding specificity by a grey box. The N-terminal sequence of the indicated proteins is shown. In this region the main differences between U1A and U2B″ are grouped in three areas marked 1, 2 and 3. The RNA binding and U2A′ association properties of the indicated mutants are listed. In the case of RNA binding only strong interactions are listed. Weak binding is discussed in the text. The RNA and protein binding levels of constructs 5f and 4 are the references for the values assigned to the other constructs.

increased considerably the binding of B″(1−88) and B″(1−98) to U2A′ (Figure 2b, compare lanes 1 with 2 and 3 with 4. The left and right panels of Figure 2b are long and short autoradiographic exposures of the same gel). This effect was only observed when either U2 RNA or U1.4, a mutant of U1 RNA which binds to U2B″ (Scherly *et al.*, 1990) were added. Other RNAs like U1, U6 or tRNA had no effect on the interaction (data not shown). This result is discussed further below.

Two amino acid changes in U1A allow U2A′ interaction

Chimeras between the U1A and U2B″ proteins defined a small region of U2B″ (amino acids 37−46, grey boxes in Figure 3) as being a major determinant of the specificity of interaction with U2 RNA. When exchanged into U1A (Figure 3, 5t; construct 5 in Scherly *et al.*, 1990) it allowed binding to U2 RNA in the presence of U2A′. As expected, 5t (data not shown) and the full-length version of it, construct 5f (Figure 3), were able to interact with U2A′ (Figure 4, lane 2).

The binding of construct 5f to U2A′ was, however, reproducibly weaker than the binding of construct 4 (Figure 4, compare lanes 2 and 4) or of U2B″wt (Figure 4, lane 20). Construct 4 consists of the N-terminal 56 amino acids of U2B″ joined to the corresponding C-terminal portion of U1A (Figure 3). This suggested that the part of U2B″ N-terminal to amino acid 37 was also involved in U2A′ binding. The differences between constructs 5f and 4 can be grouped into three regions (denoted 1−3 in Figure 3). To test the involvement of the amino acids in these regions in interaction with U2A′, we systematically exchanged the U1A specific amino acids in construct 5f for those of U2B″, either singly or in combination, resulting in a progressive conversion of construct 5f into construct 4 (Figure 3). The exchange of region 1 (construct 5.1) or 2 (construct 5.2)

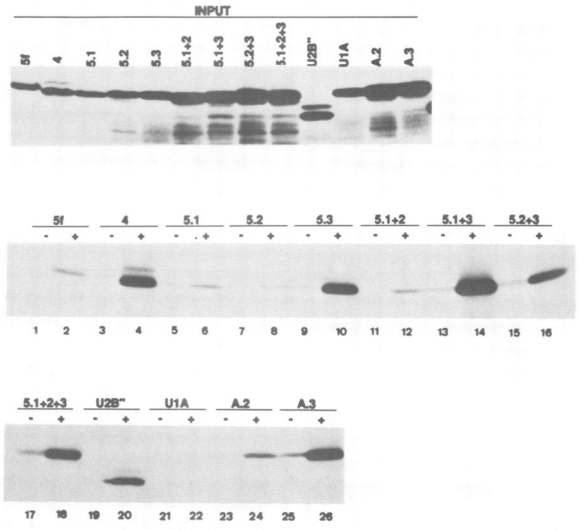

Fig. 4. Protein−protein interaction between U2A′ and N-terminal 5f mutants. The amount of radiolabelled proteins used is shown in the INPUT panel. The protein binding behaviour of the mutants listed in Figure 3 is shown. The presence or absence of U2A′ in the immunoprecipitation is indicated by the + or − sign above each lane.

had little or no effect on the binding to U2A′ in the 5f background (Figure 4, lanes 6, 8 and 12). However, the exchange of region 3 (construct 5.3) increased the binding capacity of 5f to the same level as construct 4 (Figure 4, compare lanes 10 and 4). To determine if the two amino acids of U2B″ (E21 and R25) introduced in construct 5.3 were sufficient to allow an interaction with U2A′, the same two amino acid substitutions were made in U1A wt (construct A.3). Mutant A.3, identical to U1A wt at all but these two amino acid positions, bound strongly to U2A′ (Figure 4, lane 26). The effect of altering the U1A amino acids of

region 2 to those of U2B″ (construct A.2, lane 24) also allowed U2A′ binding, although more weakly. Why the effect of changing M14 and D16 should be more easily detectable in A.2 than in 5.2 or 5.1+2 using this assay is not known. Thus, amino acids E21 and R25 of U2B″ play a major role in promoting the interaction between U2B″ and U2A′. However, constructs 5f and A.2 show that M14, D16 and U2B″ specific amino acids between positions 37 and 46 also influence the interaction.

Protein–protein interaction and RNA binding

Having shown that the A.2 and A.3 proteins could bind to U2A′, we wished to test if the presence of the U2B″ amino acids, or complex formation with U2A′, also enabled the mutants to bind U2 snRNA. In the streptavidin precipitation assay described previously (Scherly *et al.*, 1989) A.2 and A.3 bound specifically to U1 RNA in the absence of U2A′ (Figure 5a, lanes 2 and 9). The position of the two proteins is shown by a white spot in the input [I] lanes, the two smaller proteins A(1−101) and B″(1−134) are internal specificity controls (arrowheads). A(1−101) binds to U1 RNA both in the presence and absence of U2A′ while B″(1−134) binds to U2 RNA but only in the presence of U2A′. In the presence of U2A′, the binding of both A.2 and A.3 to U1 RNA is reduced (Figure 5a, lanes 5 and 12). However, neither protein bound to U2 RNA (Figure 5a, lanes 6 and 13). This indicated that U2A′ interaction with the U1A mutants could reduce U1 RNA binding, but that the protein−protein interaction had no effect on the specificity of RNA binding. We wished to examine the role of U2A′ in RNA binding in more detail, and for this purpose, made use of the mutant series shown in Figure 3.

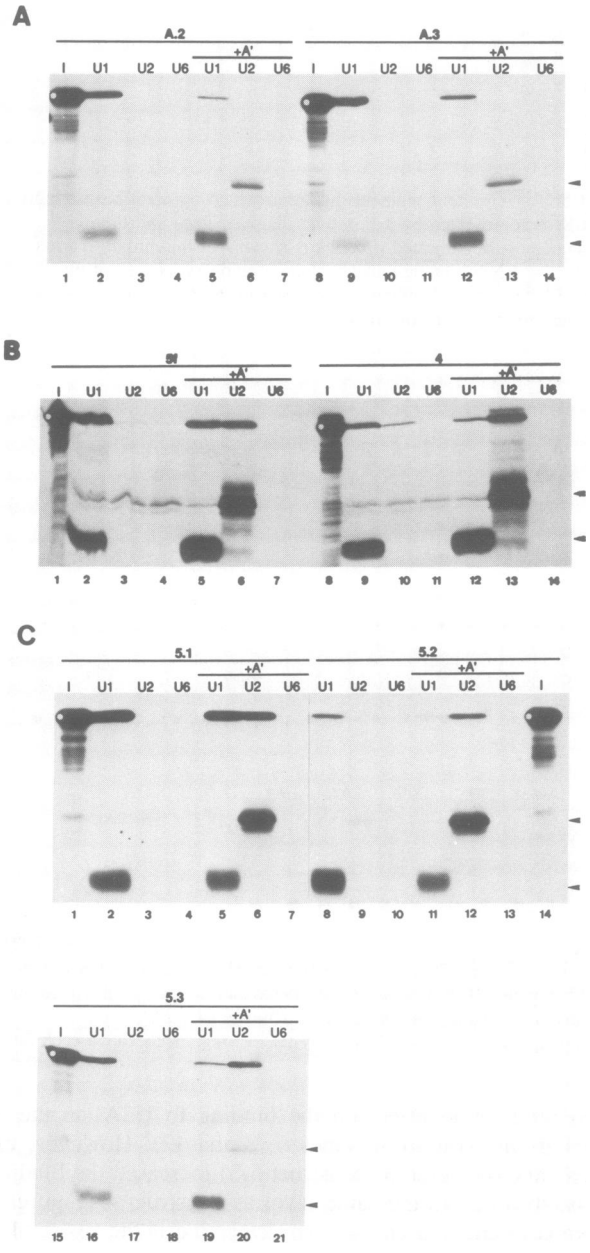

Fig. 5. RNA−protein binding assay. The RNA binding behaviour of the indicated proteins is shown. INPUT (I) lanes show the amount of the test protein used per binding assay. For each protein, the binding capacity to biotinylated U1, U2 or U6 RNA was tested in the absence or presence of U2A′ protein. In addition to the test protein (white dot), U1A(1−101) and U2B″(1−134) (arrowheads) were used as internal controls for RNA binding specificity.

At one extreme of the series is construct 4, which includes the first 56 amino acids of U2B″. As previously reported (Scherly *et al.*, 1990) this protein binds strongly to U2 in the presence of U2A′ (Figure 5b, lanes 10 and 13) and weakly to U1 on its own (Figure 5b, lane 9). This weak U1 interaction is further decreased in the presence of U2A′ (Figure 5b, lane 12) but is still higher than the binding to the negative control, U6 RNA (Figure 5b, lanes 11 and 14). Similarly, a very low level of binding to U2 RNA is seen in the absence of U2A′. This may be due to the presence of a small amount of U2A′ in the wheat germ extract. Alternatively, it is possible that the structure of construct 4 allows for a very weak interaction with U2 in the absence of U2A′.

At the other extreme of the series is 5f. This protein binds to U1 RNA in the absence of U2A′ (Figure 5b, lane 2) and equally to U1 and U2 RNA in the presence of U2A′ (Figure 5b, lanes 5 and 6). This protein behaves differently from the previously described 5t (Scherly *et al.*, 1990) which binds only to U2 RNA and requires U2A′. The reason for this difference is a region of U1A protein, present in the C-terminal portion of 5f, which has no detectable effect on the binding of U1A itself to U1 RNA (Scherly *et al.*, 1989) but which causes 5f, and a number of other U1A−U2B″ hybrid proteins including construct 4, to bind to U1 RNA (D.S. and W.B., unpublished data). These two proteins were used as reference standards against which the extra U2B″ amino acids present in mutants 5.1, 5.2 and 5.3 could be tested. Mutant 5.1 bound to RNA indistinguishably from 5f (Figure 5c, lanes 1−7) while mutants 5.2 and 5.3 bound with indistinguishable specificity, but with decreased affinity, compared with protein 4 (Figure 5c, lanes 8−21). Thus, the effects of two regions of interaction with U2A′ can be distinguished. The weak interaction involving amino acids 37−46 is essential for U2 RNA binding while the stronger interaction, involving in particular E21 and R25, but also M14 and D16, has the effect of reducing the (non-specific) association with U1 RNA.

Discussion

The U2B″ protein segment interacting with U2A′

Previous *in vivo* studies had shown that the two U2 snRNP specific proteins, U2B″ and U2A′, join the RNP separately from the pre-assembled U2 snRNP core proteins (for a

review see Mattaj, 1988). However, the order of assembly and the intracellular location in which the U2 specific proteins join the particle was still uncertain. Recently (Feeney and Zieve, 1990) suggested that U2B″ may assemble with the U2 core particle in the cytoplasm before it enters the nucleus and that U2A′ then enters the particle in the nucleus, in the final step of U2 snRNP assembly. In apparent contrast with this we have previously shown that, *in vitro*, the association with U2A′ is a prerequisite for specific binding of U2B″ to U2 RNA (Scherly *et al.*, 1990). In the same report we also showed that the N-terminal U2B″ RNP-80 motif (amino acids 9–81), plus a few amino acids on either side (amino acids 1–88), formed the minimal protein element to bind specifically to U2 snRNA.

Using rabbit anti-U2A′ antibodies we have now shown that U2B″ and U2A′ can form an immunoprecipitable complex in the absence of U2 RNA. The minimal segment of U2B″ required for this complex was the same (amino acids 1–88) as the minimal region able to bind to U2 RNA. Hybrid proteins between U2B″ and U1A containing U2B″ amino acids 59–225 did not detectably bind either to U2 RNA (Scherly *et al.*, 1990) or to U2A′ (data not shown) implying that no U2B″ specific amino acids downstream of position 59 are involved in the interaction. However, internal deletions of 10 amino acids each throughout the region from 1–90 all abolished U2B″ interaction with U2A′ (unpublished data) indicating that the structure of the whole region must be intact for U2A′ binding. Of 12 double amino acid mutations spread throughout the first 88 amino acids, three abolished U2A′ association: B″(9/10), B″(25/26), B″(78/79) and one, B″(7/8), weakened it. While we believe that the effects of most of these mutations are likely to be due to non-specific structural effects, one mutation, B″(25/26), altered an amino acid that was shown to be of direct importance for the specificity of U2A′ binding (see below).

Truncated forms of U2B″ (amino acids 1–88 or 1–98) bound less well to U2A′ than did full-length U2B″. The weak binding of the truncated proteins was improved greatly by the addition of U2 RNA, while this treatment had little effect on the U2A′ binding of full-length U2B″. Thus, although the N-terminal 88 amino acids of U2B″ contain all the information required for specific interaction with U2A′ protein and for U2 RNA binding, this protein segment seems to behave differently from the complete protein. Two explanations for this can be envisaged. The first is that U2A′ forms stabilizing contacts with U2 RNA once both are associated with U2B″, or at least with the truncated U2B″ derivative. Since U2A′ does not detectably bind to RNA *in vitro* (Scherly *et al.*, 1990), we consider this possibility unlikely. A second possibility, that the structure of the N-terminal region adopts a different structure in the absence of amino acids 89–225, seems more likely.

This implies that amino acids 1–88 cannot be considered as an independent domain (Rossmann and Argos, 1981) within the U2B″ protein, but rather their folding must depend upon amino acids 89–225. Since U2B″(1–88) was capable of both U2A′ and U2 RNA binding this is somewhat surprising and of interest for two reasons. First, it implies that detailed structural studies of isolated RNP motifs via NMR or X-ray crystallography may provide misleading information. Second, the 'cut and paste' approach to the analysis of functional 'domains' within proteins, e.g. transcription factors, is in widespread use. Our results suggest that even functioning units within chimeric proteins may be structurally disturbed, and that caution is required when interpreting data from such hybrids.

Two major elements; two different functions?

We have recently shown that amino acids 37–46 of U2B″ are able to confer specificity of interaction with U2 RNA when substituted into the U1A protein. In the same report, we noted that a construct having the first 34 amino acids of U2B″ fused to the corresponding carboxyl segment of U1A protein (construct 3 in Scherly *et al.*, 1990) had a reduced affinity for U1 RNA in the presence of U2A′. This was explained by proposing that the first 34 amino acids of U2B″ interacted with U2A′.

A fine dissection of this N-terminal region has confirmed this prediction and allowed the identification of U2B″ amino acids E21 and R25 as having the greatest influence on U2A′ association, although M14 and D16 also play a minor role. It is remarkable that the two most crucial discriminatory amino acids (E, R) are replaced in U1A by the very similar D24 and K28. Making two highly conservative changes (D → E, K → R) within the U1A protein allowed for strong interaction with U2A′. The resulting mutant (A.3), while able to bind strongly to U2A′, did not bind U2 RNA although it showed reduced affinity for U1 RNA in the presence of U2A′ (Figure 5a). This result provides strong support for the hypothesis that U2B″, rather than U2A′ is the protein which directly contacts U2 RNA (Scherly *et al.*, 1990).

In the absence of U2A′, U2B″ binds non-specifically to RNA. It displays similar affinity for U1, U2 or U6 RNA (Scherly *et al.*, 1990). Specific binding is conferred on U2B″ by U2A′ in two steps. The first step, caused by interaction with the U2B″ specific amino acids between positions 37 and 46, is sufficient to allow binding to U2 RNA in preference to e.g. U6 RNA. This is presumably the result of a conformational change in U2B″ induced by contact with U2A′. However, when only this interaction is possible, strong binding to U1 RNA is still observed (Figure 5b). This remaining non-specific RNA binding is likely to be due to the close resemblance between U2 hairpin IV, the binding site of U2B″, and the second hairpin of U1 RNA (Scherly *et al.*, 1990). The second step, which results in the reduction of non-specific U1 RNA binding, is due to interaction of U2A′ with the U2B″ specific amino acids between positions 14 and 25. This may be due either to a second conformational alteration in the U2B″ protein or to steric hindrance of the interaction with U1 RNA. The interactions, and their effects on RNA binding, are summarized in diagrammatic form in Figure 6. Since, as mentioned above, the binding site of U2B″ in U2 RNA is very similar to the second hairpin loop of U1 RNA, this additional discrimination is likely to play an important role in preventing non-productive binding of the U2B″–U2A′ complex to U1 RNA. It is therefore possible to consider U2A′ as being both an essential cofactor for U2B″ binding to U2 RNA and a negative modulator of its interaction with U1 RNA.

Secondary and tertiary structure models (Ghetti *et al.*, 1989, 1990) for the RNP-80 motif have been proposed. The model consists of a sheet of four antiparallel β-strands behind which two intercalated α-helices are found. The sequence of those elements is $\beta\alpha\beta\beta\alpha\beta$. Although the second $\beta\alpha\beta$ repeat does not seem to be present in U2B″ (unpublished observations), Chou and Fasman (1978) secondary structure prediction suggests the presence of the first $\beta\alpha\beta$ motif. Interestingly, this would place E21 and R25 next to each

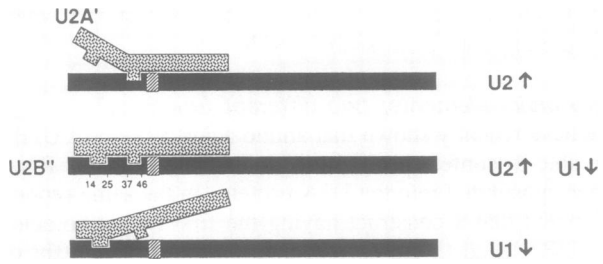

Fig. 6. Schematic of the U2A′–U2B″ interaction. U2B″ is represented by a black rectangle, U2A′ by a spotted rectangle. The proteins are not drawn to scale. The RNP-CS is indicated by the diagonally striped box. The two regions of interaction between U2A′ and U2B″ specific amino acids are shown, and the effect of the various possible interactions on RNA binding is given on the right of the figure.

other on the same face of a generally hydrophilic α-helix. Amino acids 37–46 form the most hydrophobic stretch of the U2B″ RNP motif and, if the model is correct, would be located at the end of the second β-strand.

The U2B″–U2A′ complex and other RNP systems

U2B″ and U2A′ now represent the best understood example of the involvement of protein–protein interaction in RNP assembly. However, they are by no means the only proteins which must form a complex before interacting with RNA. The signal recognition particle (SRP) is a cytoplasmic ribonucleoprotein which translocates nascent secretory proteins to the rough endoplasmic reticulum membrane. This RNP is made of six proteins and one RNA (7SL). The RNA binding of two SRP proteins, SRP9 and SRP14, shows similarity to U2B″ and U2A′ (Strub and Walter, 1990). Neither SRP9 nor SRP14 efficiently binds to RNA alone but, when mixed, they can form a complex which binds 7SL RNA efficiently and specifically. Like U2B″ and U2A′, SRP9 and SRP14 can also form a complex in the absence of the RNA. Neither of the proteins, however, contains an RNP-80 motif, and the structural basis of their interaction is not understood.

Another intensively studied RNP complex is the 30S subunit of the *E.coli* ribosome (for a review see Stern *et al.*, 1989). While two proteins, S18 and S6, seem to be interdependent for their binding to 16S RNA (Mizushima and Nomura, 1970) other examples of one protein being dependent on another have been shown not to be due to a requirement for protein–protein interaction *per se*, but rather to result from the fact that the binding of one protein induces a structural alteration of 16S RNA necessary to generate the binding site for the second protein.

The region of U2B″ required for interaction with U2A′ lies within the conserved RNP motif. Many RNPs are complex, allowing for the formation of multiple protein–protein interactions. Other members of the family of RNP motif-containing proteins may also use the same protein structural elements for protein–protein interactions. While the mutational analysis of U2B″, U1A and U1 70K has yielded a certain amount of useful information, it is now clear that a structural analysis of both RNA–protein and protein–protein complexes via NMR or X-ray crystallography is required in order to reveal the details of the interactions involving the RNP-80 motifs of the snRNP proteins.

Materials and methods

Antisera

The *Eco*RI restriction fragment of the U2A′ cDNA (Sillekens *et al.*, 1989) was subcloned into the *Eco*RI site of a T7 expression vector (Tabor and Richardson, 1985). The recombinant protein starts with a methionine and three additional amino acids coming from the vector, followed by 18 amino acids from the untranslated leader sequence of the cDNA and then by the entire U2A′ coding sequence. The *E.coli* strain BL 21 LysS (Studier *et al.*, 1990) was used. Three hours after induction with IPTG, the cells were harvested, pelleted, resuspended in SDS–PAGE sample buffer and loaded on a preparative SDS–polyacrylamide gel. The band containing U2A′ protein was excised from the gel, crushed and mixed with PBS buffer. This colloidal solution was mixed with Freund's adjuvant and injected into two different rabbits following standard techniques. To test the specificity of this antibody, we *in vitro* translated U2A′, U2B″ and U1A separately in the presence of [^{35}S]methionine and performed immunoprecipitations with the rabbit anti-U2A′ antibody. In the first rabbit anti-U2A′ activity appeared coincident with anti-U1A and anti-U2B″ activity. In the second rabbit, after two negative bleeds and three bleeds which only recognized U2A′, anti-U1A and anti-U2B″ activity also appeared. This was surprising since there is no obvious resemblance between U2A′ and the other two proteins (Sillekens *et al.*, 1987, 1989). For the experiments shown here we utilized either the U2A′ specific bleeds from the second rabbit or serum from the first rabbit from which the anti-U1A and anti-U2B″ antibodies had been removed by passage over an affinity column to which recombinant U1A protein, produced as described above, was coupled.

Plasmids

The human U2A′ cDNA (Sillekens, 1989) was subcloned as an *Eco*RI restriction fragment into the *Eco*RI site of pGEM1 (PROMEGA). The entire human U1A cDNA (Sillekens, 1987) was inserted as an *Eco*RI–*Eco*RI fragment into pGEM-3z(+) (PROMEGA) vector from which the *Bam*HI site was deleted by cutting the vector with *Hin*cII and *Sma*I and religation. The human U2B″ cDNA (Habets, 1987) was inserted as an *Eco*RI restriction fragment into the pBS(+) (PROMEGA) vector from which the *Bam*HI site was deleted as described for U1A subclone. The orientation of all three cDNAs was such that transcription was under the control of the T7 RNA polymerase promoter.

The U2B″ derivatives used in this work have been described (Scherly *et al.*, 1990). Construct 5f is the full-length version of construct 5 in Scherly *et al.* (1990). Constructs 5.1, 5.2, 5.3, 5.1+2, 5.1+3, 5.2+3, 5.1+2+3 were made by site directed mutagenesis of construct 5f. The U1A(102/103) clone (Scherly *et al.*, 1989) was used to create mutants A.2 and A.3.

In vitro transcription and translation

Messenger RNA for U2A′ and U2B″wt or derivatives was produced *in vitro* using T7 RNA polymerase as described (Scherly *et al.*, 1989). These synthetic mRNAs were used for *in vitro* translation in wheat germ extract (WGE) as described (Scherly *et al.*, 1989). U2A′ was translated in the absence of radiolabelled amino acids whereas U1A and U2B″ derivatives were synthesized in the presence of [^{35}S]methionine.

Protein–protein interaction assay

Each new radiolabelled translation product was tested by SDS–PAGE for incorporation efficiency before the protein–protein interaction assay. Usually, 1 μl of radiolabelled U2B″ derivative was mixed with 1 μl of unlabelled U2A′ and incubated for 30 min at room temperature. For testing the weak interactions of the truncated derivatives, a 10-fold volume excess of each truncated U2B″ derivative was mixed with U2A′. The binding solution was diluted with 500 μl of Ipp150 (150 mM NaCl/10 mM Tris–HCl pH 8.0/0.1% v/v NP-40/0.1% w/v sodium azide) and transferred to a tube containing ~20 μl of packed protein A–sepharose beads coupled to rabbit anti-U2A′ antibodies. The solution was mixed for 90 min by rotating the tubes end over end at room temperature. The beads were pelleted by 30 s centrifugation, the supernatant aspirated and the bead pellet washed three times with 1 ml Ipp150 by rotating for 10 min. The bead pellet was resuspended in 25 μl SDS–PAGE sample buffer, vortexed briefly and boiled for 3–5 min. After a brief centrifugation, the supernatant (~30 μl) was loaded onto an SDS–polyacrylamide gel. After electrophoresis the gel was treated with Entensify (NEN), dried by vacuum drying for 2 h at high temperature and exposed to Kodak XOMAT film at −80°C for 1–3 days.

RNA–protein binding assay

The streptavidin precipitation assay described previously was used (Scherly *et al.*, 1989). Briefly, labelled U2B″ derivatives with or without unlabelled U2A′ were mixed with either biotinylated U1, U2 or U6 RNA substrates.

After incubation, proteins bound to the RNA were precipitated with streptavidin coupled to agarose beads. After several washes, the bead pellet was resuspended in SDS–PAGE sample buffer and boiled to release the RNA-bound proteins. The supernatant was fractionated by SDS–PAGE, fluorographed, dried and autoradiographed. In each binding reaction the two following internal standards were used: U1A(1–101), a protein binding specifically to U1 RNA and U2B″(1–134), a protein binding specifically to U2 RNA in the presence of U2A′ (Scherly *et al.*, 1989, 1990).

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