A weak interaction between the U2A' protein and U2 snRNA helps to stabilize their complex with the U2B'' protein

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ABSTRACT

The U2 snRNP complex contains two specific proteins, U2B'' and U2A'. We have analysed the interaction of U2A' with U2B'' and with U2 RNA. U2A' can form a weak but detectable RNA-protein complex with U2 RNA and a stable protein complex with U2B''. This protein-protein complex binds efficiently and specifically to U2 RNA. Binding experiments with mutant forms of U2A' shows that the region of U2A' essential for binding to U2B'' is extensive, being located between amino acid position 1 – 164. The behaviour of the wild type U2A' protein, and in particular of a mutant version of the protein in which amino acids 3,4 and 5 are mutated, suggests that U2A' forms a weak interaction with U2 RNA which helps to stabilize the U2A'-U2B''-U2 RNA complex. Mutants of U2 RNA were used to localize the region of U2 RNA important for interaction with U2A'. The results show that U2A' interacts with the stem of hairpin IV.

INTRODUCTION

RNAs do not exist in the cell as free RNA but function as ribonucleoprotein (RNP) particles (for review see 1). Some RNA binding proteins are able to bind a whole group of RNAs, e.g. the proteins that bind heterogeneous nuclear RNA (hnRNA) (2). Others are highly specific and bind to one type of RNA only, e.g. the U1-70K and U1A proteins which bind specifically to U1 RNA (3, 4). Some RNA binding proteins can bind to RNA on their own (e.g. U1-70K and U1A) but in other cases there is a requirement for more than one protein. Little is known about the details of protein-protein and protein-RNA interactions occurring in RNP complexes. A good model system to study this type of interaction is the U2 snRNP complex in which U2 RNA is associated with the U2-specific proteins U2A' and U2B'' (5–7). Recently the molecular basis of the binding of U2B'' protein to U2 RNA and to U2A' protein, respectively, has been reported (7,8). The results showed that U2B'' is able to bind non-specifically to several kind of RNAs, but in the presence of U2A' it binds specifically to U2 RNA. In the same studies the minimal

segments of U2B'' protein and of U2 RNA required for RNP complex formation were identified. The minimal segment of U2B'' lies between amino acids 1 and 88 and contains one copy of the conserved RNP-80 motif (7), which has also been referred to as the RNA binding domain (2) or the RNA recognition motif (3). Hairpin IV of U2 RNA is the site of U2B'' binding. The loop sequence of this hairpin is responsible for the specific binding of U2B'' protein in the presence of U2A' since placing this loop sequence in the context of another U RNA generates a strong binding site for the U2B''-U2A' complex (7). Recently two elements of U2B'' with different functions were found to be involved in the interaction with U2A' protein. One, between amino acid positions 37 and 46, permits specific binding to U2 RNA while the other, situated between amino acid positions 14 and 25, reduces non-specific binding to e.g. U1-RNA (8).

In this study we have analysed the binding of the U2A' protein to U2B'' and to U2 RNA. U2A' contains a leucine-rich region in the amino-terminal half of the polypeptide and an acidic region at the carboxy-terminal end. In contrast to U2B'', the U2A' protein does not contain a copy of the RNP-80 motif (11).

Our results indicate that U2A' is able to bind in a specific manner to U2 RNA, albeit very weakly. Using mutant forms of U2 RNA and of the U2A' protein, the site of this interaction is localized to the stem of hairpin IV of U2 RNA. This interaction helps to stabilize the U2B''-U2A'-U2 RNA complex.

U2A' binds very strongly to U2B'' alone and to U2 RNA in the presence of U2B''. We show that an extensive region of the protein (amino acids 1 – 163) is required for this interaction, but even this large segment is insufficient to produce the wild-type level of binding, suggesting that the structure of much of the protein must be intact in order to allow the interaction with U2B''.

MATERIALS AND METHODS

Plasmids

The human U2A' cDNA (11) was subcloned as an EcoRI restriction fragment into the EcoRI site of pGEM 3Zf+ (Promega) vector from which the BamHI restriction site was removed by cutting the vector with BamHI, filling the recessed

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3' termini and re-ligation. Single stranded DNA was produced with the helper phage M13KO7 and point mutations were produced using the oligo-directed mutagenesis system kit from Amersham (U.K.). Since each mutation introduced a unique BamHI site, the mutation was checked by a BamHI-EcoRI digestion of the recombinants and subsequent sequencing of the mutated area.

The human U2B'' cDNA (10) was subcloned as an EcoRI restriction fragment into the EcoRI site of pGEM 3Zf+. The U1 and U2 derivatives used in this work have been described earlier (7).

In vitro transcription

To produce T7 messenger RNA for U2B'', U2A' wild type and derivatives, 2 µg of linearized (HindIII or BamHI) template was incubated at 37°C for 60 min in a total volume of 50 µl containing 40 mM Tris-HCl pH 7.9 /6 mM MgCl₂/10 mM spermidine-HCl/10 mM NaCl/2 mM dithiothreitol (DTT)/0.1 mg/ml BSA/60 µM GpppG/1 mM ATP, UTP, CTP, GTP/60 U RNase inhibitor (RNasin, Promega)/30 U T7 RNA polymerase (Promega). After incubation the non-incorporated nucleotides were removed with a G50 spin column, the RNA was phenol extracted and precipitated with 0.1 volume 3 M Na-acetate pH 5.2 and 2.5 volume ethanol. The RNA pellet was resuspended in 10 µl sterile water. Approximately 2 µg mRNA was synthesized with this method.

Biotinylated U snRNAs were prepared as above except that 100 µM biotin-11-UTP (Enzo) was added to the transcription reaction mix.

In vitro translation

³⁵S-labelled or ³H-labelled U2A' wild type and derivatives were made in wheat germ extract (WGE, Amersham U.K.) by translation of their corresponding T7 mRNAs. 200 ng T7 mRNA were incubated in a total volume of 30 µl containing 15 µl WGE/120 mM K-acetate/67 µM of 19 amino acids mixture minus methionine or minus leucine together with either 30 µC L-[³⁵S]methionine (1200 Ci/mmol, Amersham) or 30 µC L-[4,5-³H]leucine (120–190 Ci/mmol, Amersham). When cold U2B'' was made, 30 µC [³⁵S]methionine was replaced by 67 µM methionine.

Binding assays

Protein-protein binding

Usually, 8 µl KHN buffer (150 mM KCl/20 mM Hepes-KOH pH 7.9/0.05% NP-40) was mixed with 1 µl of radiolabelled U2A' derivative and 1 µl of unlabelled U2B'' and incubated for 60 min at 25°C. After dilution with 500 µl KHN buffer, the solution was transferred to a tube containing 15 µl packed protein A-agarose beads (Biozym) coupled to anti-B' monoclonal antibody 4G3 (10) via rabbit-anti-mouse IgG (Dakopatts). The solution was mixed for 90 min by rotating the tubes end over end at room temperature. The beads were pelleted by centrifugation, the supernatant aspirated and the pellet washed three times with 1 ml KHN buffer by rotating for 1 min. The beads were resuspended in 20 µl SDS-sample buffer and boiled for 4 min. After a brief centrifugation, the supernatant was loaded onto an SDS-polyacrylamide gel. After electrophoresis the gel was incubated with dimethylsulfoxide (DMSO, 2 × 30 min), 16% (w/v) 2,5-diphenyloxazol (PPO) in DMSO (1 × 60 min), water (3 × 30 min), dried by vacuum drying for 2 h at 60°C and exposed to Kodak X-omat film at -80°C.

Protein-RNA binding

(A) Precipitation via U2B''. The same protocol as for protein-protein binding was used except that 1 µl of a solution with 20 ng/µl U1 or U2 derivative and 2 µg/µl E.coli tRNA was added to the incubation mixture.

(B) Precipitation via biotinylated U1 or U2 derivatives. 8 µl KHN buffer was mixed with 1 µl of in vitro made radiolabelled U2A' derivatives, 1 µl unlabelled U2B'' and 1 µl 20 ng/µl biotinylated U1 or U2 derivative (in a solution with 2 µg/µl E.coli tRNA). After incubation for 60 min at 25°C the mixture was diluted with 500 µl KHN buffer and transferred to a tube containing 15 µl packed streptavidin-agarose beads (BRL) previously washed with KHN buffer. The solution was mixed for 90 min by rotating the tubes end over end at room temperature and further handled as described above in the procedure for protein-protein binding.

RESULTS

In vitro made U2A' binds weakly, but specifically, to U2 snRNA

Recently it has been shown that U2B'' protein requires U2A' protein for specific in vitro binding to U2 snRNA. In the absence of U2A', U2B'' binds non-specifically to RNA (7). We wished to establish assays to measure the interaction of U2A' with U2B'' and U2 snRNA, in order to examine the behaviour of U2A' mutants (see below). To demonstrate that in vitro made U2A' protein associates efficiently with U2 RNA in the presence of U2B'', radio-labelled U2A' protein was synthesized in wheat germ extract and incubated with biotinylated U2 snRNA

Fig. 1. Protein U2A' binds U2 snRNA but requires binding to U2B'' for efficient U2 RNA interaction. (A) Precipitation via biotinylated U-RNA. Unlabelled U2B'' and radiolabelled U2A' were synthesized separately in wheat germ extract. U2A' was incubated in KHN buffer with U1, U1.4 and U2 snRNA (as indicated above each lane) either alone or mixed with U2B'' in a ratio of 1:1. After incubation, the protein-RNA complexes were precipitated with streptavidin-agarose beads. Proteins were released by boiling in SDS-PAGE sample buffer and analysed by SDS-PAGE. In lane 7, 10% of the input protein (I) was loaded as a marker. The U1.4 mutant is U1-snRNA in which the loop sequence of the second hairpin was changed into the loop sequence of the fourth hairpin of U2-snRNA (7). (B) Precipitation via U2B''. Labelled U2A' was incubated in KHN buffer alone, with U2B'' or with U2B'' plus U2 RNA. After incubation the protein complexes were immunoprecipitated with anti-B' monoclonal antibody 4G3 (10) coupled via rabbit anti-mouse IgG to protein-A agarose beads. Proteins were analysed as described above.

transcripts in the absence and presence of unlabelled, *in vitro* made, U2B". The labelled RNA-bound U2A' protein was detected after precipitation of the biotinylated RNA with streptavidin linked to agarose beads (4).

In the presence of U2B", U2A' association with U2 RNA or with U1.4 RNA is readily detectable above the background level of non-specific binding to U1 RNA (fig. 1A lanes 4 to 6). In the U1.4 mutant of U1 RNA the loop sequence of the second hairpin was changed into the loop sequence of the fourth hairpin of U2 RNA (7). In the presence of U2B", U2A' can be precipitated equally well by U2 RNA or U1.4 RNA (fig. 1A lanes 5 and 6, see also ref. 7). This result is a confirmation of the fact that the main U2A'-U2B" binding site of U2 RNA is located in the loop sequence of hairpin IV.

We have previously reported (7) that U2A' alone does not bind to U2 RNA. However, in the buffer conditions used here a consistent, but very weak binding of U2A' to U2 RNA in the absence of added U2B" was observed (fig. 1A, lanes 1 till 3). Under these conditions, binding to U1.4 was identical to the non-specific U1 background (fig. 1A, lanes 1 and 2). Since the binding was not to the loop sequence of hairpin IV (see lane 2), and thus had a different specificity to that seen in the presence of U2B", it seemed possible that it might be due to direct U2A'-U2 RNA interaction.

The increased non-specific precipitation of U2A' protein with U1 RNA after addition of U2B" (fig. 1A, lanes 1 and 4) is probably mediated by U2B" which has a tendency to bind non-specifically both to the agarose beads and to U1 RNA (data not shown).

U2A' and U2B" can form a complex in the absence of U2 RNA. This was shown by immunoprecipitation of radio-labelled U2B" bound to U2A' with a U2A'-specific antibody (8). U2A' can also be precipitated with the anti-B' monoclonal antibody 4G3 (10), after incubation in the presence of unlabelled U2B" (fig. 1B). The presence of U2 RNA during the incubation did not enhance the U2A' signal (fig. 1B, lane 3).

Thus, assays have been established by which the interaction of mutant U2A' proteins with U2B" protein and of the resulting complex with U2 RNA can be measured.

Interaction of U2A' point mutants with U2B" in the presence or absence of U2 RNA

Thirteen mutant forms of the U2A' cDNA were constructed by generating unique BamHI restriction sites. As a consequence two amino acids were substituted by glycine and serine in the mutant U2A' proteins (the positions of the substituted amino acids are indicated by numbers). Only one mutant had an additional point mutation, namely mutant U2A'3/4/5, in which the amino acid sequence from position 3 to 5 was changed from LysLeuThr into MetGlySer. The interaction of the U2A' point mutants with U2B" was tested by immunoprecipitation of radio-labelled U2A' point mutants after incubation with (+) or without (–) unlabelled U2B" using anti-B" monoclonal antibodies (fig. 2B). The binding of the point mutants to U2 RNA was tested by precipitation of biotinylated U2 RNA with streptavidin linked agarose beads, after its incubation with a labelled U2A' mutant and non-labelled U2B" (fig. 2C). As internal control, the deletion mutant U2A'(Δ225–244), which is able to bind to U2B" and to U2

Fig. 2. Interaction between radio-labelled U2A' point mutants and U2B" in the presence and absence of U2 RNA. (A) The amount of radiolabelled U2A' protein used per binding assay (input). The numbers of each mutant indicate the position of the two amino acids which were changed into GlySer. Note that mutant A'204/205 showed an aberrant and yet unexplained electrophoretic behaviour. (B) Protein-protein interaction. U2A' and U2B" interaction was tested in the absence of U2-snRNA. Radiolabelled U2A' BamHI point mutants were incubated in KHN buffer either alone (– lanes) or mixed with U2B" in a ratio of 1:1 (+ lanes). After incubation, the protein complexes were immunoprecipitated with anti-B" monoclonal antibody 4G3 coupled via rabbit anti-mouse IgG to protein-A agarose beads. Proteins were analysed as described in the legend to fig. 1. As a positive internal control deletion mutant U2A'(Δ225–244) was used (arrowhead). (C) Interaction of U2A' with U2B" in the presence of U2 RNA. Each U2A' derivative was incubated in the presence of U2B" with biotinylated U1 RNA (U1 lanes) or biotinylated U2 RNA (U2 lanes). Internal positive control is U2A'(Δ225–244) (arrowhead). After incubation the protein-RNA complexes were precipitated with streptavidin-agarose beads and the proteins were analysed as described above.

Fig. 3. Binding of truncated and internally deleted U2A' derivatives to U2B'' and to U2 RNA in the presence of U2B''. (A) The amount of C-terminally truncated U2A' protein used for the assay (input). (B) The C-terminally truncated U2A' derivatives were tested for binding to U2B'' alone and to U2B'' in the presence of U2 RNA by immunoprecipitation of the complex with the anti-B' monoclonal antibody 4G3 as described in the legend to fig 1B. As a positive internal control wt U2A' was used (indicated by arrowhead). (C) The binding of the internally deleted U2A' derivatives to biotinylated U1-RNA (U1 lanes) and biotinylated U2-RNA (U2 lanes) in the presence of U2B'' was analysed as described in figure 1. As a positive internal control wt U2A' was used (arrowhead).

RNA, was added (figs 2B and 2C arrowhead). Binding to U1 RNA was used as a control of specificity (fig. 2C). The relative amounts of input protein used for the assay are shown in the input panel (fig. 2A).

Mutants A'64/65, A'84/85, A'104/105 and A'164/165 to A'244/245 were able to bind both to U2B'' (fig. 2B) and to U2 RNA in the presence of U2B'' (fig. 2C). The mutants A'124/125 and A'144/145 were not able to bind to U2B'' nor to U2 RNA in the presence of U2B'' (fig. 2B and 2C). Mutant A'3/4/5 showed an unexpected behaviour. It did not bind U2B'' in the absence of U2 RNA but could be precipitated via U2 RNA in the presence of U2B'' (fig 2B and 2C). Mutants U2A'24/25 and U2A'44/45 were very difficult to analyse because their T7 mRNAs translated very poorly giving rise to very low levels of protein (fig. 2A and unpublished data). However, on very long exposure of the gels, A'24/25 appeared to behave similarly to A'3/4/5 while A'44/45 did not bind either U2B'' or U2 RNA (data not shown).

A large portion of U2A', including the leucine-rich region, is required for RNA binding

To define which region of U2A' was required for binding to U2 RNA, truncated U2A' mutants were generated using the 13

BamHI mutant forms of U2A' cDNA (fig.2). In the first type of experiment the binding of C-terminal truncated U2A' derivatives to U2B'' and to U2 RNA in the presence of U2B'' protein, was tested by co-precipitation with U2B'' (fig. 3B). The negative control is the incubation without U2B'' and the internal control is the full length U2A' protein (fig. 3B, indicated by an arrowhead).

U2A' fragment containing the first 203 amino acids complexed efficiently with U2B'' and U2B'' plus U2 RNA. Deletion of the 20 amino acids between 203 and 183 reduced this interaction considerably. A fragment of U2A' corresponding to the N-terminal 163 amino acids, A'(1-163), was the shortest truncation mutant tested still able to bind to U2B'' (fig. 3B). Further deletion beyond position 163 was not done because the A' mutant 144/145, needed for this truncation, had already been shown not to be able to bind to U2B'' or U2 RNA (fig. 2B and 2C). The addition of U2 RNA had a slight enhancing effect on the binding efficiency of the two shortest truncation mutants, suggesting that it could help to stabilize the weak U2B''-U2A' interaction formed with these mutants.

An N-terminal deletion between amino acid positions 4 and 25 destroyed the specific binding of U2A' to U2 RNA and U2B'' (fig. 3C), the wild-type U2A' internal control is indicated with

Fig. 4. The influence of the stem of hairpin IV of U2 RNA. (A) Determination which U-RNA mutant stabilizes the binding of A'3/4/5 to U2B''. A'3/4/5 was incubated in the presence of U2B'' with several types of U RNAs (as indicated). After incubation the protein complexes were immunoprecipitated with the anti-B' monoclonal antibody 4G3. The proteins were analysed as described above. As a positive internal control deletion mutant U2A'(Δ 225–244) was used (arrowhead). (B) Location of the region of U2 RNA which stabilizes U2A'3/4/5-U2B'' complex. Mutant A'3/4/5 was incubated with U2B'' and several deletion mutants of U2 RNA. The U2 mutants are indicated according to which stem-loops they retained (III + IV, IV) or which had been deleted (Δ III, Δ IV). After incubation the RNA-protein complex was precipitated with anti-B' monoclonal antibody 4G3 and analysed as described above.

an arrowhead. This result was not unexpected because the mutations in mutant A'3/4/5 and A'24/25, which were used to construct this deletion, had already a strong effect on the binding capacity of the mutants. Our conclusion is that the minimal segment of U2A' protein capable of binding specifically to U2 RNA in the presence of U2B'' lies between amino acids 1–163. This region of U2A' protein contains the leucine-rich region, but not the C-terminal cluster of acidic residues (fig. 5).

We also tested U2A' mutants with deletions of 20 amino acids inside the leucine-rich region which are situated between positions whose mutation had not affected binding (figs 2B and 2C). None of these mutants were able to form a complex with U2B'' and U2 RNA (fig. 3C). This indicates that the leucine-rich region is important for the function of U2A'. Deletions of 20 amino acids in the U2A' region shown not to be essential for U2 RNA interaction (amino acids 163–255) did not detectably affect U2 RNA binding (the internal control in fig. 2 and data not shown).

Mutant A'3/4/5 requires the stem of U2 hairpin IV for complex formation

Mutant A'3/4/5 did not bind to U2B'' in the absence of U2 RNA, but when U2 RNA was present an RNP complex was formed (fig. 2B and 2C, first two lanes). This effect might be due to either i) a conformational change of U2B'' caused by binding of U2 RNA to U2B'', resulting in a stronger U2A'-U2B'' interaction, or ii) a direct interaction of U2A' with the RNA that stabilized the binding between U2A' and the U2-RNA-bound U2B''.

The wt U2A'-U2B'' complex only requires the loop sequence of hairpin IV for binding (7, Fig. 1) whereas U2A' interacts weakly with U2 RNA outside the loop sequence of hairpin IV (fig. 1A). To distinguish between the possibilities raised above we tested whether the binding of mutant A'3/4/5 to U2B'' was

Fig. 5. Diagrammatic representation of the effect of mutations in U2A' on binding to U2B'' alone or in the presence of U2 RNA. The region essential for U2B'' binding (amino acid 1–163) is cross-hatched while the Leu-rich region (amino acid 57–121) is black. Also the C-terminal acidic amino acid cluster is indicated.

also dependent on U2 RNA regions outside the loop sequence of hairpin IV, using mutant U1.4 (fig. 4A). The negative RNA controls were U1 and U6 RNA, the positive control U2 RNA and the internal protein control was the deletion mutant A'(Δ 224–245). The results showed that mutant A'3/4/5 could not form a stable complex with U1.4 RNA and as noted above it could form a complex with wt U2 RNA (fig 4A). A similar result was obtained with mutant U2A'24/25 (data not shown). This indicates that the stabilizing effect on the interaction of U2A'3/4/5 with U2B'' is due to a structure within the U2 RNA which is located outside the loop sequence of hairpin IV, i.e. that direct interaction between U2A' and U2 RNA stabilizes the U2A'-U2B'' interaction.

To localize the U2 RNA region which is involved in this interaction more precisely, we used the U2A'3/4/5 mutant and four deletion mutants of U2 RNA (7). The U2 fragments were named according to which stem-loop they retained (III + IV and IV) or which had been deleted (Δ III, Δ IV). The protein-RNA complex was precipitated with the anti-B' monoclonal antibody 4G3. Deletion of hairpin III did not abolish U2A'3/4/5 binding (fig. 4B) whereas RNA transcripts containing only hairpin IV showed normal complex formation with U2A'3/4/5. Thus the U2A'3/4/5 mutant needs U2 hairpin IV to form a stable complex with U2B''. These results, together with those in figs. 2C and 4A, indicate that the region of U2 RNA which stabilizes the U2A'3/4/5-U2B'' complex is located in the stem of hairpin IV.

DISCUSSION

Interaction of U2A' with U2B''

U2A' can form a stable complex with U2B'' in the absence of U2 RNA (8 and this paper). The minimal region of U2A' essential for binding to U2B'' has been defined, and lies between amino acid positions 1 and 163. The binding efficiency of this U2A' fragment to U2B'' is slightly increased when U2 RNA is present, suggesting that the conformation of U2A'(1–163) is comparable, but perhaps not identical, to the N-terminal part of wt U2A'. The strength of interaction between U2A' and U2B'' is increased, and made independent of the presence of RNA, by additional amino acids which lie between positions 183 and 203. U2A'(1–163) contains a leucine-rich region located between amino acid position 57–121. Part of this region has a sequence with a remarkable periodicity, namely (LXX)₈. Double point mutations inside the leucine-rich region did not markedly effect the binding of U2A' to U2B'' and U2 RNA (fig. 5), but deletions of 20 amino acids within the leucine-rich region abolished the

binding to U2B'' and U2 RNA. It thus seems that the native structure of this region is important for the binding capacity of the U2A' protein.

Amino acid substitutions at position 44/45, 124/125 or 144/145 abolished the binding of U2A' to U2B'' and U2 RNA. The effects of these mutations may be due either to non-specific structural effects or to the fact that these amino acids in U2A' make direct contact with U2B''.

Mutant U2A'3/4/5 showed an impaired U2A'-U2B'' interaction such that it was only able to bind to U2B'' in the presence of U2 RNA (fig. 5). This suggests that the N-terminal region of U2A' is involved in the interaction with U2B''. Recently, two charged amino acids in the U2B'' protein, E21 and R25, were identified as having a great influence on the association of U2B'' with U2A' (8). Mutation of these amino acids, even the very conservative changes of Glu to Asp and Arg to Lys, significantly reduced the interaction. Similarly, substitution of these two amino acids in the U1A protein (which is closely related to U2B'' but does not bind to U2A') allowed the U1A protein to form a strong interaction with U2A' (8). Chou and Fasman secondary structure prediction (14) and comparison with the X-ray structure of the N-terminal region of the U1A protein (15) suggests that E21 and R25 together with K24 are situated on the same face of a generally hydrophilic α -helix. According to Chou and Fasman secondary structure prediction, the N-terminal sequence 1–14 of the U2A' protein may also form a α -helix structure with three charged amino acids, K3, E7 and E10 situated in the α -helix (K3 is one of the three amino acids changed in mutant A'3/4/5). Observations made by us using the computer-graphics program Insight II/Discover (from Biosym Technologies Inc.), indicate that the charged amino acids K3, E7 and E10 could potentially form an electrostatic interaction with respectively E21, K24 and R25 of U2B'' (data not shown). So it seems possible that interactions between the two helices may be involved in U2B''/U2A' complex formation. However, more and detailed structure information will be required to support this hypothesis.

Interaction of U2A' with U2 RNA

Our earlier experiments did not reveal a direct interaction of U2A' with U2 RNA (7). In this study however, we found that U2A' indeed can interact directly with U2 RNA, albeit very weakly, in buffers containing Hepes and Potassium salts rather than Tris and Sodium. The stem structure of hairpin IV is probably important in this interaction with U2A'. This was revealed by making use of the mutant A'3/4/5 which could only interact with U2B'' in the presence of U2 RNA (fig. 4), indicating that the U2B''-U2A' interaction was disturbed but could be stabilized sufficiently to allow detection by the binding of U2 RNA. It was possible to determine that the U2 sequence required for this stabilization lies outside the loop sequence necessary and sufficient for binding the wt U2B''-U2A' complex (7) but within the region required for the (weak) binding of the wt U2A' protein on its own (fig. 1A).

The stem of hairpin IV is not as highly conserved as the loop sequence of hairpin IV during evolution (12). For example, the only base-pairs which are conserved between *Schizosaccharomyces pombe* and human are G156-C173 and U155-G174 (13). This could indicate that the U2A'-U2 RNA interaction has not been conserved.

The direct interaction of U2A' with the stem structure of hairpin IV probably has a minor role in the formation of the U2-RNP complex, since the U2B''-U2A' complex binds efficiently and specifically to the U1.4 mutant, which contains

only the loop sequence of U2 hairpin IV. The proposed U2A' interaction with U2 RNA however, reveals the role of a weak interaction which stabilizes the protein-RNA complex. This is one of the many protein-RNA and protein-protein interactions whose sum results in the stability of the U2 snRNP particle.

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