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Loop I of U1 Small Nuclear RNA Is the Only Essential RNA Sequence for Binding of Specific U1 Small Nuclear Ribonucleoprotein Particle Proteins

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The binding of the U1 small nuclear ribonucleoprotein (snRNP)-specific proteins C, A, and 70K to U1 small nuclear RNA (snRNA) was analyzed. Assembly of U1 snRNAs from bean and soybean and a set of mutant *Xenopus* U1 snRNAs into U1 snRNPs in *Xenopus* egg extracts was studied. The ability to bind proteins was analyzed by immunoprecipitation with monospecific antibodies and by a protein-sequestering assay. The only sequence essential for binding of the U1-specific proteins was the conserved loop sequence in the 5′ hairpin of U1. Further analysis suggested that protein C binds directly to the loop and that the assembly of proteins A and 70K into the RNP requires mainly protein-protein interactions. Protein C apparently recognizes a specific RNA sequence rather than a secondary structural element in the RNA.

What is the basis of specific protein binding to RNA? To answer this question, two extreme types of model systems have been analyzed. Studies performed on ribosomes based on nuclease protection experiments and phylogenetic comparison have defined several classes of RNA structural motifs recognized by proteins (25). Ribonucleoproteins (RNPs) containing only a single protein (1, 7, 16, 21) have been easier to study, but their analysis cannot give information about the role of protein-protein interactions in the assembly of RNPs. The major U small nuclear RNPs (snRNPs), which are essential cofactors in the splicing of mRNA precursors (17), are of intermediate complexity. Various RNA-protein and protein-protein interactions occur in these RNPs, which consist of a small number of proteins and fewer than 200 nucleotides of RNA. The availability of cDNAs (5, 14, 15, 18, 20, 22) enables the synthesis of many of the U snRNP proteins in vitro. In vitro systems to assemble U1 snRNPs have also been developed and characterized (6, 12; J. Hamm and I. W. Mattaj, Methods Enzymol., in press). The in vitro assembly of a set of mutant U1 snRNAs designed to lack single secondary structural elements has defined two discrete protein-binding sites on the RNA (6). The proteins common to all major U snRNPs require only the conserved Sm-binding site (3, 11). The essential contact points of the U1 snRNP-specific proteins A and 70K were localized to the 5′-most hairpin structure of the U1 snRNA. Although binding of the U1 snRNP-specific proteins is stabilized by protein-protein interactions with the proteins bound to the Sm-binding site, both groups of proteins can bind independently. The location of the third U1 snRNP-specific protein, C, could not be determined because no monospecific antibody was available.

Almost identical secondary structures can be proposed for the U1 snRNAs of bean (19), soybean (V. L. van Santen, W. Swain, and R. A. Spritz, Nucleic Acids Res., in press), and *Xenopus laevis* (23), although the primary structure conservation is only 50% (Fig. 1a). The nonconserved nucleotides are mostly located within the three stems at the 5′ end of the RNA, but the potential base pairing is maintained. Most of the single-stranded regions are absolutely conserved; these regions include the 5′ end of the RNA required for interaction with the 5′ splice site (24), the Sm-binding site, and the loop sequences.

We have assembled bean and soybean U1 snRNAs and a set of *Xenopus* U1 snRNA mutants in vitro into RNPs. We analyzed binding of the *Xenopus* proteins to these RNAs by immunoprecipitation and by a protein-sequestering assay. The essential RNA element for binding of the U1 snRNP-specific proteins C, A, and 70K is the conserved loop sequence of the 5′-most hairpin structure. The protein recognizing this sequence is likely to be C, and this protein can bind to the RNA in the absence of A and 70K.

MATERIALS AND METHODS

In vitro synthesis of RNA and assembly. Templates to synthesize RNA in vitro were generated by site-directed mutagenesis (9). T7 promoter sequences were inserted at the 5′ ends and restriction sites were inserted at the 3′ ends of the coding sequences (6) (*X. laevis*, *Bam*HI; bean, *Sna*BI; soybean, *Dra*I). RNA fragments containing only the first 117 or 118 nucleotides of the RNAs were generated by linearizing the templates at restriction sites present in the coding sequences (*Xenopus*, *Taq*I; bean and soybean, *Mae*II). RNA was prepared as previously described (6). RNPs were assembled in *Xenopus* egg extracts for 30 min at room temperature and immunoprecipitated at high stringency (6).

Antibodies. Specificities of antisera were determined by immunodecoration of Western (immuno-) blots of nuclear extracts or purified U snRNPs. U1 snRNP-specific sera were tested for cross-reactivity with Sm antigens by immunoprecipitation of ^{32}P-labeled RNPs from oocytes that had been injected with U5 snRNA genes (6). Immunoprecipitations were performed with the following antibodies: anti-Sm serum Küng (4), anti-A serum Schleumuss (R. Lührmann, personal communication), anti-C serum B152 (W. van Venrooij, personal communication), and monoclonal antibody 2.73 (anti-70K) (2).

Protein-sequestering assay. RNA fragments containing only the first 117 or 118 nucleotides were analyzed by a protein-sequestering assay (6; Hamm and Mattaj, in press).

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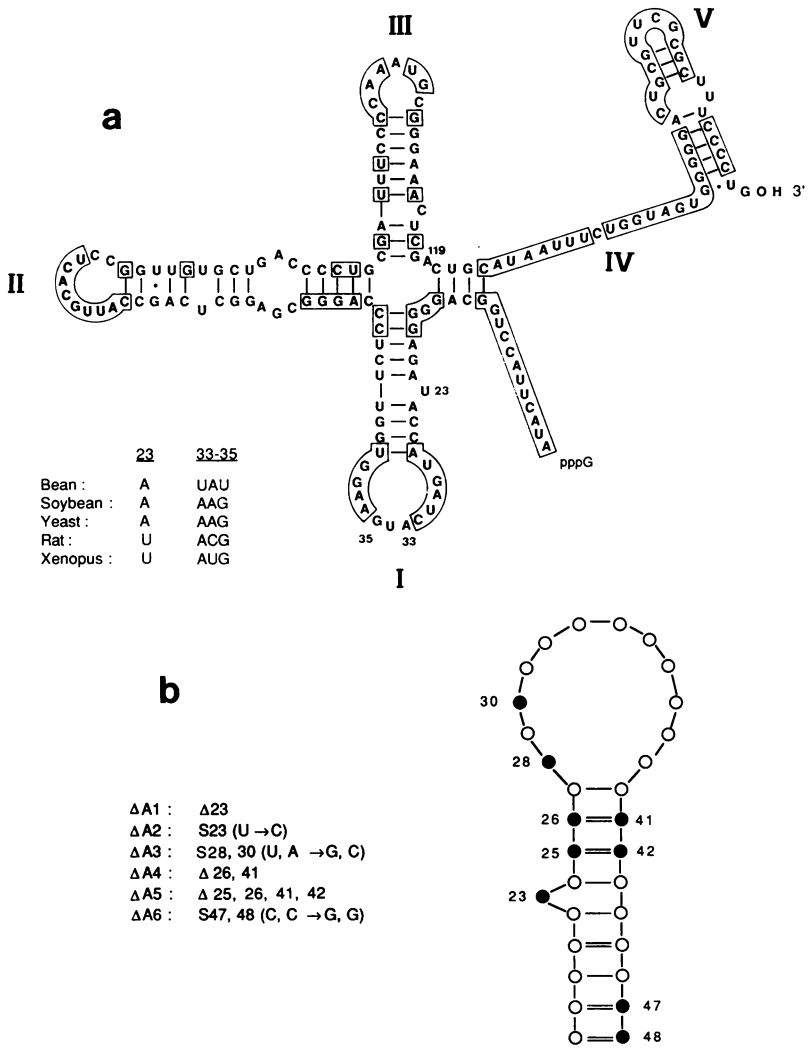

FIG. 1. (a) Conserved nucleotides of U1 snRNAs. The primary and the proposed secondary structure of the *Xenopus* U1 snRNA is shown. Structural elements are numbered I to V. Nucleotides that are identical in *X. laevis*, rat, human, bean, and soybean are boxed. Variable bases of loop I (nucleotides 33 to 35) and of the unpaired nucleotide 23 are specified for U1 snRNAs of various species at lower left. (b) Mutations within hairpin I of *Xenopus* U1 snRNA. Mutations were introduced into a U1 snRNA gene-T7 RNA polymerase promoter construct (6) by site-directed mutagenesis (9). Numbers denote nucleotide positions; mutated positions are indicated by black dots. Double lines represent G-C base pairs in the wild-type U1 snRNA. Substituted (S) and deleted (Δ) nucleotides of the mutant ΔA1 to 6 are given at left.

Briefly, unlabeled RNA fragments were preincubated under assembly conditions, and subsequently labeled wild-type U1 snRNA was added. The labeled RNA could then be immunoprecipitated only with antibodies against proteins that had not been sequestered by the preincubated unlabeled RNA. This assay is more sensitive in detecting weak RNA-protein and protein-protein interactions than is direct immunoprecipitation at high stringency.

RESULTS

Binding of *Xenopus* proteins to bean and soybean U1 snRNA. It has previously been demonstrated that U1 snRNAs assemble in vitro into U1 snRNPs when added to extracts of *Xenopus* eggs. Under optimal conditions, 90% of the RNA is immunoprecipitable with antibodies directed against either common or U1-specific snRNP proteins (6).

By analyzing the assembly of wild-type and mutant U1 snRNAs, it has been shown that hairpin I (Fig. 1a) is essential for the binding of the U1 snRNP-specific proteins A and 70K. Using a monospecific serum recognizing protein C, the third U1 snRNP-specific protein, we found that it also requires only hairpin I for binding (data not shown). The fact that the loop sequences but not the stem compositions were conserved among *Xenopus*, bean, and soybean U1 snRNAs offered a simple way to analyze whether the *Xenopus* proteins recognize specific stem or loop sequences. Bean and soybean U1 snRNAs were synthesized in vitro and assembled into RNPs in *Xenopus* egg extracts. The RNPs were then immunoprecipitated with anti-Sm, anti-C, anti-A, or anti-70K antibodies, and the RNA was extracted and analyzed (Fig. 2a). The surprising result was that the soybean U1 snRNA, like the *Xenopus* RNA, interacted with all proteins while the bean U1 snRNA was only immunoprecipitated with antibodies against the common U snRNP proteins (anti-Sm). To test whether binding of the U1 snRNP-specific proteins to the bean RNA was weakened rather than

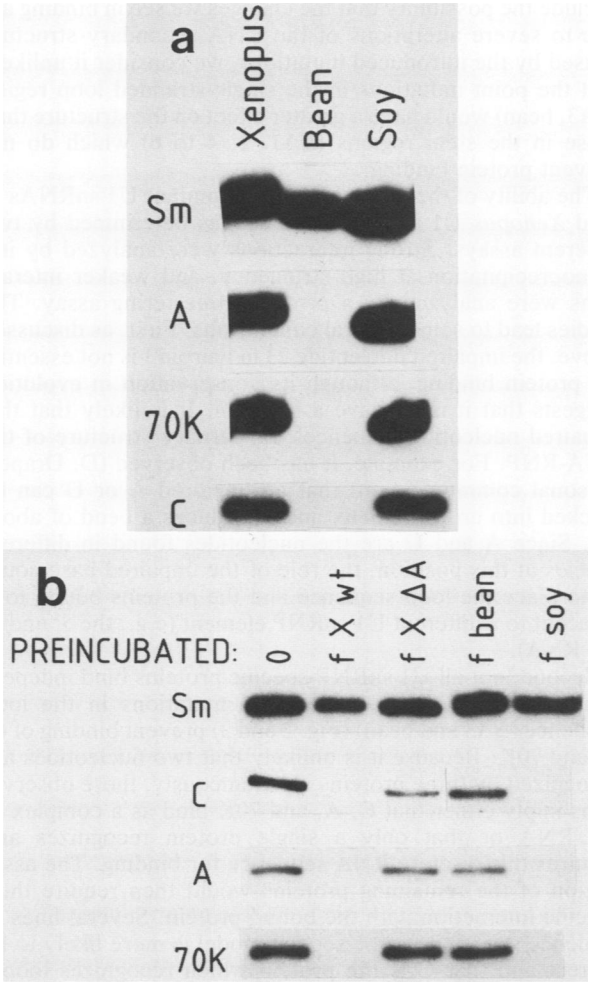

FIG. 2. (a) Immunoprecipitation of in vitro-assembled U1 snRNPs. *Xenopus*, bean, or soybean U1 snRNA was synthesized and assembled in vitro into RNPs (see Materials and Methods). RNPs were immunoprecipitated with antibodies against Sm, C, A, and 70K as indicated. The RNA was extracted and analyzed by denaturing polyacrylamide gel electrophoresis. (b) Protein-sequestering assay with U1 snRNA fragments of different organisms. Unlabeled U1 snRNA fragments of bean (lane f bean [nucleotides 1 to 117]), soybean (lane f soy [nucleotides 1 to 117]), *Xenopus* wild-type RNA (lane f Xwt [nucleotides 1 to 118]) or *Xenopus* ΔA RNA (lane fXΔA [nucleotides 1 to 118; ΔA is lacking nucleotides 18 to 48]) were analyzed with a protein-sequestering assay (see Materials and Methods). In the control experiment (lane 0), no RNA was preincubated. RNPs were immunoprecipitated with antibodies against Sm, C, A, or 70K as indicated at left, and the RNA was extracted and analyzed.

eliminated, a more sensitive assay was used. Unlabeled RNA fragments containing only the first 117 or 118 nucleotides were synthesized, and their ability to bind the U1 snRNP-specific proteins was analyzed in a protein-sequestering assay (Fig. 2b). In this assay, the RNA to be analyzed was unlabeled and preincubated with extract in molar excess over the protein of interest. Subsequently, radioactively labeled wild-type U1 snRNA was added. This labeled RNA will only be immunoprecipitated if the preincubated RNA is unable to bind the protein recognized by the antibody used. Even under these conditions the bean RNA fragment was unable to bind the U1 snRNP-specific proteins. This suggested that an element was changed in bean U1 and that this alteration had the same effect as deleting hairpin I of *Xenopus* U1 completely (mutant ΔA). Since hairpin I is identical in bean and soybean U1 snRNAs except for two positions in the loop sequence, and because these two nucleotides are identical in *X. laevis* and soybean (Fig. 1b), this identifies either one or both of the nucleotides 33 and 35 as being essential for binding U1 snRNP-specific proteins. These observations imply that proteins C, A, and 70K might not interact independently of each other with the RNA, because a double point mutation abolishes binding of all three proteins.

Structural requirements. In order to analyze structural requirements for binding of the proteins C, A, and 70K in more detail, mutations were introduced into hairpin I of the *Xenopus* U1 snRNA (Fig. 1b, ΔA1 to 6). The mutant U1 snRNAs were assembled in vitro into RNPs and immunoprecipitated with anti-Sm, anti-C, anti-A, or anti-70K antibodies. RNAs were then extracted and analyzed (Fig. 3a). Immunoprecipitation was performed at high stringency (6) and required strong RNA-protein interactions. A double point mutation at positions 28 and 30 had the same effect as deleting this stem loop completely (Fig. 3a; compare lanes ΔA3 and ΔA). None of the U1 snRNP-specific proteins bound detectably to mutant ΔA3. In contrast, deletion or substitution of the unpaired nucleotide 23 did not affect binding of U1-specific snRNP proteins (Fig. 2a, ΔA1 and 2). This nucleotide is therefore not essential for protein binding. The deletion of a single GC base pair from the upper part of the stem in ΔA4 did not interfere with protein binding. The deletion of two adjacent GC base pairs in ΔA5 prevented binding of 70K and reduced binding of A but did not affect the interaction with C. A mutation destroying two GC base pairs at the bottom of the stem (ΔA6) still allowed C binding but removed A and 70K.

Taken in combination with the previous results, these direct immunoprecipitations demonstrate that mutations in the conserved loop sequence prevent binding of all U1 snRNP-specific proteins. The results obtained with mutants ΔA5 and ΔA6 show that C does not require A or 70K for binding and that A may not need the 70K protein to stay in the RNP (Fig. 3a). Unlabeled RNA fragments of the mutant U1 snRNAs were analyzed by the protein-sequestering assay to detect weaker interactions. In this assay only ΔA3, the mutant with an altered loop sequence, was unable to sequester C, A, and 70K (Fig. 3b). The other mutants were able to sequester all U1 snRNP-specific proteins, although with different efficiencies (Fig. 3b and data not shown), supporting the conclusion that the loop sequence is essential for C, A, and 70K binding. The changes in mutants ΔA4 to 6 do not abolish A or 70K binding in this assay and show that neither the sequence of the stem nor its normal structure is an essential part of their interaction sites.

Next, assembly of *Xenopus* wild-type U1 snRNA was performed at high salt concentrations. When the sodium chloride concentration was adjusted to 750 mM before doing the assembly reaction, the RNA was immunoprecipitated only with anti-Sm or anti-C antibodies (Fig. 3c), demonstrating that C can bind to the wild-type RNA in the absence of A and 70K and indicating that binding of C is unlikely to be based solely on ionic interactions.

DISCUSSION

The secondary structure of U1 snRNA is highly conserved among different species. Moreover, the primary structure of regions that have functional roles like protein binding or

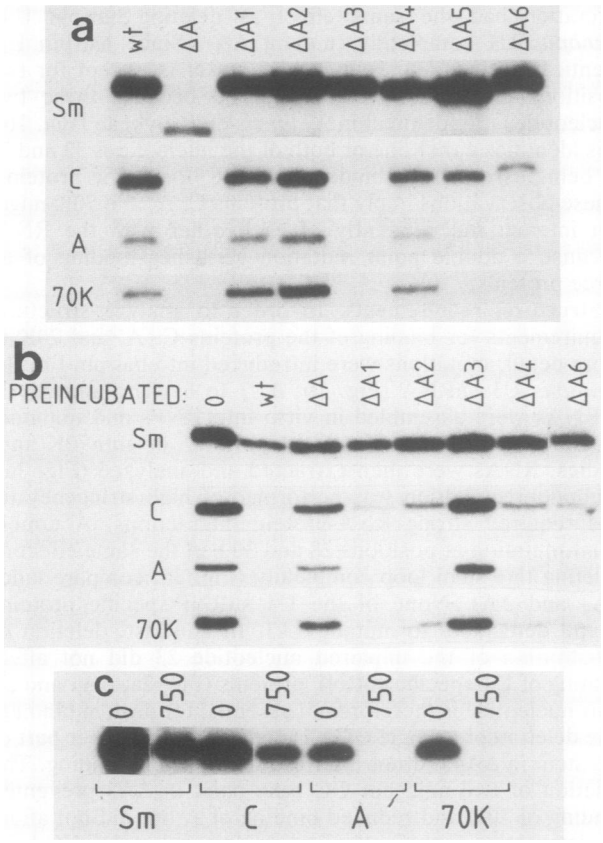

FIG. 3. (a) Immunoprecipitation of in vitro-assembled *Xenopus* U1 snRNPs. *Xenopus* wild-type or mutant U1 snRNAs were synthesized and assembled in vitro into RNPs. RNPs were immunoprecipitated with antibodies against Sm, C, A, or 70K, as indicated at left, and the RNA was extracted and analyzed. (b) Protein-sequestering assay with mutant U1 snRNA fragments. Unlabeled *Xenopus* U1 snRNA fragments were analyzed for their ability to sequester the U1 snRNP-specific proteins (see Materials and Methods). RNPs were immunoprecipitated with antibodies against Sm, C, A, or 70K as indicated to left, and the RNA was extracted and analyzed. (c) Salt sensitivity of assembly. *Xenopus* wild-type U1 snRNA was incubated either under standard assembly conditions (lanes 0 [no sodium chloride]) or at high ionic strength (lanes 750 [sodium chloride concentration, 750 mM]), and particles were immunoprecipitated with antibodies against Sm, C, A, or 70K as indicated at bottom.

interaction with 5′ splice sites of mRNA precursors are absolutely conserved (Fig. 1a). Binding of the U1 snRNP-specific proteins C, A, and 70K has been shown to require only hairpin I of U1 snRNA (6). Recent data on the increased protection of U1 snRNA against nuclease digestion after binding of anti-70K antibodies to U1 snRNPs (13) are consistent with the fact that 70K binds to hairpin I. Here we have taken advantage of the partial conservation of hairpin I among *X. laevis*, bean, and soybean and of mutations made in *Xenopus* hairpin I to identify essential RNA-protein contacts. The specific binding of C, A, and 70K has been shown to depend critically on the conserved loop sequence. The composition of the stem of hairpin I is clearly less important, since the stems of soybean and *X. laevis* have diverged considerably (Fig. 1) without affecting protein binding. In addition, mutants ΔA4 to A6, which have alterations in the individual structural elements of this stem, have only a weak effect on protein binding. Although we cannot exclude the possibility that the changes we see in binding are due to severe alterations of the RNA secondary structure caused by the introduced mutations, we consider it unlikely that the point mutations in the single-stranded loop region (ΔA3, bean) would have a greater effect on the structure than those in the stem regions (ΔA1, 2, 4 to 6) which do not prevent protein binding.

The ability of the heterologous and mutant U1 snRNAs to bind *Xenopus* U1 snRNP proteins was determined by two different assays. Strong interactions were analyzed by immunoprecipitation at high stringency, and weaker interactions were analyzed by a protein-sequestering assay. The studies lead to some general conclusions. First, as discussed above, the unpaired nucleotide 23 in hairpin I is not essential for protein binding, although its conservation in evolution suggests that it must have a function. It is likely that this unpaired nucleotide influences the tertiary structure of the RNA-RNP. For example, it has been observed (D. Draper, personal communication) that an unpaired A or U can be stacked into an RNA helix and introduces a bend of about 15°. Since A and U are the nucleotides found in different species at this position, the role of the unpaired base could be to place the loop sequence and the proteins bound to it adjacent to a different U1 snRNP element (e.g., the 5′ end of the RNA).

Second, not all U1 snRNP-specific proteins bind independently to the RNA. Double point mutations in the loop sequence (ΔA3 and bean) (Fig. 2 and 3) prevent binding of C, A, and 70K. Because it is unlikely that two nucleotides are recognized by three proteins simultaneously, these observations imply either that C, A, and 70K bind as a complex to the RNA or that only a single protein recognizes and requires this discrete RNA sequence for binding. The association of the remaining proteins would then require their specific interaction with the bound protein. Several lines of evidence suggest that the second model is more likely to be correct and that C is the protein which recognizes loop I specifically and independently. Most convincingly, C binds tightly to the stem mutant ΔA6 in the absence of proteins A and 70K (Fig. 3a), and studies of the assembly of wild-type U1 snRNP at different salt concentrations show that C and the common U snRNP proteins can bind to U1 at up to 750 mM sodium chloride (Fig. 3c), while A and 70K binding is sensitive to lower salt. These results show that C can bind to U1 snRNA in the absence of both A and 70K. Since binding of C is abolished by mutation of the loop sequence, C must recognize the loop of hairpin I. Mutation of other structural elements of U1 snRNA (6) or of the stem of hairpin I has failed to reveal any other RNA sequence which is essential for binding of U1 snRNP-specific proteins. We have not analyzed the conserved 3′ part of loop I. This region could therefore be involved in RNA-protein interactions.

Although we have no definitive data on the ordered assembly of A and 70K, several observations suggest that A may bind prior to 70K. First, A associates with U1 snRNPs at salt concentrations at which 70K does not (6). Second, mutant ΔA5 can be immunoprecipitated by anti-A or anti-C but not by anti-70K antibodies. In addition, it has previously been shown that A associates with wild-type U1 before 70K in vitro (6). The second U1-specific protein to associate with the RNP is therefore probably A. The ability of C to bind to the RNA at high ionic strength indicates that this association is not based primarily on ionic interactions with the phosphate backbone of the RNA. These results are all consistent with a model for U1 snRNP assembly in which C binds in a sequence-specific manner to the loop of hairpin I; this is

followed by the association of proteins A and 70K, whose binding is critically dependent on protein-protein interaction with C. However, binding of A and 70K might well be stabilized by nonessential RNA-protein interactions. This is also implied by the results obtained with mutants ΔA4 to 6, which bind A and 70K when analyzed by the protein-sequestering assay but not when analyzed by immunoprecipitation at high stringency. It is interesting to note that both binding of C to loop I and of the common U snRNP proteins to the Sm-binding site involves binding of proteins to single-stranded regions of the RNA in a sequence-specific manner.

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