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Thierry Forné, Ferdinand Rossi, Emmanuel Labourier, Etienne Antoine, Guy Cathala, et al.. Disruption of Base-Paired U4-Center-Dot-U6 Small Nuclear Rnas Induced by Mammalian Heterogeneous Nuclear Ribonucleoprotein-C Protein. *Journal of Biological Chemistry*, 1995, 270 (27), pp.16476–16481. 10.1074/jbc.270.27.16476 . hal-02193512

HAL Id: hal-02193512

<https://hal.science/hal-02193512v1>

Submitted on 8 Jun 2022

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Disruption of Base-paired U4·U6 Small Nuclear RNAs Induced by Mammalian Heterogeneous Nuclear Ribonucleoprotein C Protein*

(Received for publication, February 8, 1995, and in revised form, May 1, 1995)

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Due to 3' end modifications, mammalian U6 small nuclear RNA (snRNA) is heterogeneous in size. The major form terminates with five U residues and a 2',3'-cyclic phosphate, but multiple RNAs containing up to 12 U residues have a 3'-OH end. They are labeled in the presence of [α - 32 P]UTP by the terminal uridylyl transferase activity present in HeLa cell nuclear extracts. That these forms all enter the U6 snRNA-containing particles, U4·U6, U4·U5·U6, and the spliceosome, has been demonstrated previously. Here, we report an interaction between the heterogeneous nuclear ribonucleoprotein (hnRNP) C protein, an abundant nuclear pre-mRNA binding protein, and the U6 snRNAs that have the longest uridylyl stretches. This U6 snRNA subset is free of any one of the other snRNPs, since anti-Sm antibodies failed to immunoprecipitate hnRNP C protein. Furthermore, isolated U4·U6 snRNPs containing U6 snRNAs with long oligouridylyl stretches are disrupted upon binding of hnRNP C protein either purified from HeLa cells or produced as recombinant protein from *Escherichia coli*. In view of these data and our previous proposal that the U6 snRNA active in splicing has 3'-OH end, we discuss a model where the hnRNP C protein has a decisive function in the catalytic activation of the spliceosome by allowing the release of U4 snRNP.

Splicing of mRNA precursors occurs in a large ribonucleoprotein complex called the spliceosome. The reaction requires five U snRNAs¹ packaged into four individual snRNPs (U1, U2, U4·U6, and U5) and an as yet undetermined number of non-snRNP proteins. Spliceosome assembly involves the ordered interaction of U1 and U2 snRNPs through RNA base pairing with the 5'-splice site and the sequence around the branch point, respectively, and then the entry of the other snRNAs in the form of U4·U6·U5 tri-snRNP (for a recent review, see Ref. 1). It is known that U4 and U6 snRNAs are base-paired through two intermolecular helices (stem I and stem II) forming an evolutionary conserved secondary structure, the so-called Y structure (2). This structure is thought to occur in both U4·U6 and U4·U6·U5 complexes but to be disrupted once the U4·U6·U5 tri-snRNP has entered the spliceosome. Indeed, compelling results in both yeast and mammalian systems have led to the conclusion that U6 snRNA forms new base-pairing interactions with U2 snRNA and the pre-mRNA in the splice-

some (3–7) to yield a tripartite structure reminiscent of that formed in domains 5 and 6 of group II introns (1, 8). Extensive base pairing between U4 and U6 is an obstacle to the formation of this tripartite structure, and the U4·U6 snRNP is disrupted in the spliceosome before the splicing intermediates and products appear (9). The step(s) leading to the release of U4 snRNP is (are) not understood.

As a result of post-transcriptional 3' end modifications, metazoan U6 snRNA is heterogeneous (10, 11). In addition to a major form terminating with five Us and a 2',3'-cyclic phosphate (12), multiple minor RNAs exhibit, at their 3' end, an oligouridylyl stretch of variable length. All are part of the known U6 snRNA-containing particles, U4·U6, U4·U5·U6, and the spliceosome. The finding that U6 snRNA with a 2',3'-cyclic phosphate end is generated within the spliceosome as a consequence of pre-mRNA splicing (13) led us to propose that U6 snRNAs with elongatable ends are the active forms in splicing. As a first step toward the elucidation of the function of U6 snRNA elongation in splicing, we have obtained evidence that the hnRNP C protein, a potential partner of the splicing reaction (14), is bound exclusively to the U6 snRNAs having the longest oligouridylyl tails, and we have demonstrated that hnRNP C induces disruption of base-paired U4·U6 snRNAs in isolated U4·U6 snRNPs.

EXPERIMENTAL PROCEDURES

Materials—Micrococcal nuclease, RNase A and protein A-Sepharose 4B were from Pharmacia Biotech Inc. RNase H was from Life Technologies, Inc. The anti-C hnRNP (4F4), the anti-A1 hnRNP (4B10), and the anti-U hnRNP (3G6) antibodies and the recombinant hnRNP C1 protein and K94 mutant were generous gifts from Dr. G. Dreyfuss (Howard Hughes Medical Institute, Philadelphia, PA). The other antibodies used were the monoclonal anti-Sm Y12, and a patient serum of Ssb specificity (anti-La antibodies). All other chemicals were of analytical grade.

Extracts and Oligodeoxynucleotide-directed Cleavage of snRNAs—HeLa cell nuclear extracts were prepared by the method of Dignam *et al.* (15) except that triethanolamine buffer was used instead of HEPES. The DEAE-Sepharose-cleared extract was according to Hinterberger *et al.* (16). A U4·U6-enriched preparation was obtained from this extract by centrifugation in a glycerol gradient as described previously (13). The hnRNP C protein was prepared according to Pinol-Roma and Dreyfuss (17). Oligodeoxynucleotide cleavage of snRNAs was as described previously (18). Oligonucleotides were complementary to U4 (nucleotides 65–85), U5 (nucleotides 69–87) and U6 (nucleotides 77–95).

U6 snRNA Labeling and UV Cross-linking—Endogenous U6 snRNAs were 3' end-labeled by incubating 15 μ l of nuclear extract with 30 μ Ci of [α - 32 P]UTP in a final volume of 25 μ l containing 3.2 mM MgCl₂, 1 mM ATP, and 20 mM creatine phosphate for 30 min at 30 °C (13). For UV cross-linking experiments (18), the reactions were kept on ice for 10 min and then irradiated for 10 min with an UV transilluminator at 254 nm (7 milliwatts/cm² on the surface of the filter). The distance between the samples and the filter was 9 cm. The samples were finally digested with RNase A (0.6 μ g/ml) for 60 min at 37 °C before being precipitated by 5 volumes of acetone and electrophoresed in a 10% SDS-polyacrylamide gel followed by autoradiography on Kodak XAR films. RNase A digestion of immunoprecipitated samples was carried out directly on

* This work was supported by a grant from the Association pour la Recherche contre le Cancer given to C. Brunel (ARC 6952). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: sn, small nuclear; hn, heterogeneous nuclear; RNP, ribonucleoprotein; CS, consensus sequence.

protein A-Sepharose beads after they were washed with NET 2 buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Nonidet P-40, 0.5 mM dithiothreitol). Elution of the beads was in 50 μ l of Laemli buffer.

Glycerol Gradient Centrifugation and Detection of the snRNAs—200- μ l reactions containing 3' end-labeled U6 snRNAs were loaded onto 4 ml of 10–30% glycerol gradients made in 50 mM Tris-glycine buffer (pH 8.8). Centrifugation was in a SW 60 Beckman rotor at 41,000 rpm for 3 h at 4 °C. A total of 18 fractions of 220 μ l were recovered from the top. The RNAs present in each fraction were extracted with phenol after proteinase K (4 mg/ml) digestion and separated in a 10% polyacrylamide, 8 M urea gel. Labeled RNAs were first detected by autoradiography, and then the gel was electroblotted to Hybond membranes under the same conditions as described by Blencowe *et al.* (19). The membranes were UV-treated for 30 s, baked 1 h at 80 °C, and then prehybridized for 2 h before hybridization at 42 °C according to Church and Gilbert (20). DNA probes complementary to U4, U6, and U5 snRNAs were the same as those used in the oligodeoxynucleotide-directed cleavage experiments. Those complementary to U1 and U2 corresponded to nucleotides 1–15. All were 5' end-labeled with T4 polynucleotide kinase in the presence of [γ -³²P]ATP. Hybridizations were in the same buffer at 42 °C. One wash of 5 min in 2 \times SSC and then three washes of 15 min each in 1 \times SSC, 0.1% SDS were performed at room temperature before autoradiography.

Immunoprecipitations—Assays to detect immunoprecipitated protein adducts after UV cross-linking (see above) and to identify those of the 3' end-labeled U6 snRNAs that are immunoprecipitated were performed directly with 30 μ l of a 3' end-labeling reaction or from 100 μ l after fractionation of this same reaction in glycerol gradients. In all cases, antibodies were pre-bound to protein A-Sepharose in NET 2 buffer as described previously (21). The samples were added to 50 μ l of antibody bound to protein A-Sepharose, adjusted to 200 μ l, and incubated with gentle agitation for 1 h at 4 °C. After four washes with 1 ml of NET 2 buffer, bound material either was analyzed for protein adducts if UV cross-linked or digested with proteinase K for RNA detection. Released RNAs were extracted with phenol, separated by electrophoresis in 10% polyacrylamide-urea gels in Tris borate/EDTA (TBE) buffer, and finally detected by autoradiography.

RESULTS

hnRNP C Protein Interacts with U6 snRNAs with the Longest Oligouridylylate Stretches—Due to an endogenous terminal uridylyl transferase, incubation of HeLa cell nuclear extracts under splicing conditions in the presence of [α -³²P]UTP leads to preferential 3' end-labeling of U6 snRNA as well as other RNAs of smaller size (Ref. 13 and see "Experimental Procedures"). Such an assay reveals the presence of multiple U6 snRNAs, which differ by the length of their oligo(U) tail. All are assembled into U6, U4-U6, and U4-U6-U5 snRNPs (13). UV cross-linking experiments were done to determine whether spliceosomal proteins interact with the oligouridylylate stretch of the U6 snRNAs exhibiting a 3' (U)_n-OH end. Nuclear extracts were incubated in the presence of [α -³²P]UTP and irradiated at 254 nm. After digestion by RNase A, the presence of protein adducts was examined by electrophoresis in a SDS-polyacrylamide gel. Two proteins, one of about 50 kDa and a second one forming a doublet in the range of 42 kDa, were detected (Fig. 1, lane 2). On the basis of previously published data (22), we suspected that the 50-kDa protein adduct represented the La antigen. In agreement with this prediction, this protein adduct was immunoprecipitated with anti-La antibodies (lane 4). Two criteria led us to suggest that the second protein adduct might be the hnRNP C protein: its apparent size and the fact that the hnRNP C protein is known to bind avidly to poly(U) stretches (23, 24). This was verified by the finding that anti-C antibodies precipitate the protein adduct (lane 6). Since some other RNAs are labeled under the conditions used (13), it was necessary to identify the small RNAs that transferred their label to the La and C proteins. The extracts incubated with [α -³²P]UTP were immunoprecipitated as described above. Fig. 2A shows that anti-La, anti-Sm, and anti-C antibodies (lanes 4–6, respectively) immunoprecipitated labeled RNAs of a size compatible with U6 snRNA. Although we demonstrated previously by se-

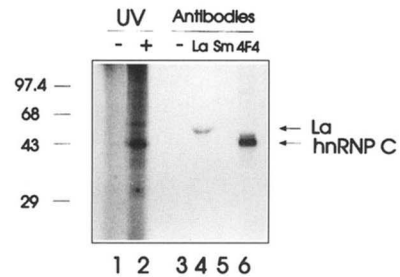


FIG. 1. Identification of U6 snRNA bound proteins by UV light-induced RNA-protein cross-linking. Reactions containing 15 μ l of nuclear extract were incubated for 30 min at 30 °C in the presence of 30 μ Ci of [α -³²P]UTP in a final volume of 25 μ l containing 3.2 mM MgCl₂, 1 mM ATP, and 20 mM creatine phosphate and then experimented, but not the control, to irradiation with UV light. Lane 1, control, no irradiation; lane 2, irradiation for 10 min with UV light and digested for 60 min at 37 °C with RNase A; lanes 4–6, UV light-irradiated and immunoprecipitated with anti-La, anti-Sm (Y12), and anti-C hnRNP (4F4) antibodies, respectively, before RNase A digestion; lane 3, the same assay using protein A-Sepharose beads without antibodies. The proteins were separated in a 10% SDS-polyacrylamide gel and revealed by autoradiography on XAR films. That the cross-linked product in lane 4 moved slightly faster than its equivalent in lane 2 is due to the presence of comigrating IgG from the serum used as source of antibody.

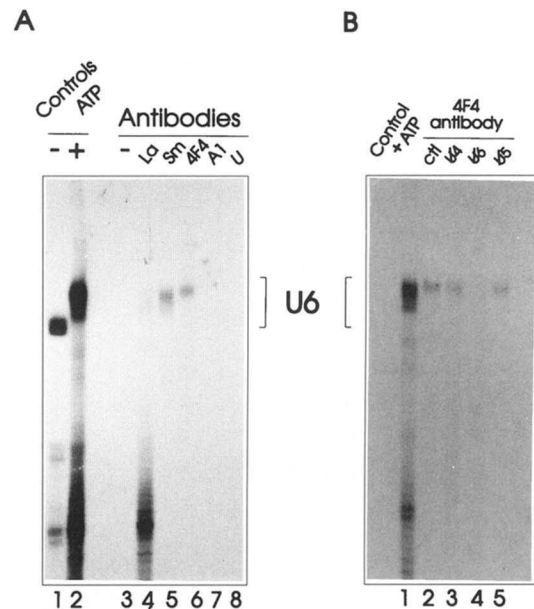


FIG. 2. Identification of a subset of U6 snRNAs immunoprecipitated by the anti-hnRNP C protein antibody. A, standard reactions containing 3' end-labeled U6 snRNA as described in Fig. 1 were subjected to immunoprecipitation with anti-La (lane 4), anti-Sm (Y12) (lane 5), anti-C (4F4) (lane 6), anti-A1 (4B10) (lane 7), and anti-U (3G6) (lane 8) antibodies. Lane 3, a control immunoprecipitation assay without antibodies. Total labeled RNAs are shown in lanes 1 and 2. Conditions of labeling were either in the presence (lane 2) or in the absence (lane 1) of ATP and creatine phosphate. The RNAs were extracted and separated in a 10% polyacrylamide urea gel. B, reactions as in A were immunoprecipitated by anti-C antibodies (4F4), and then the resulting samples were subjected to oligodeoxynucleotide-directed cleavage by RNase H. The oligonucleotides were complementary to U4 (lane 3), U6 (lane 4), and U5 (lane 5). Controls were direct analysis of nonimmunoprecipitated (lane 1), and immunoprecipitated but not cleaved (lane 2) samples.

quencing that the RNAs indicated U6 in lanes 1 and 2 (controls) are authentic (13), we carried out a complementary assay based on oligodeoxynucleotide directed cleavage by RNase H to be sure. As shown in Fig. 2B, among the three oligos used, only the antisense U6 oligo was efficient in directing cleavage of the RNAs precipitated by anti-C antibodies, thus confirming that they are U6 snRNAs. In Fig. 2A, one can see that anti-La, in

contrast to anti-Sm and anti-C antibodies, precipitated many other RNAs of smaller size than U6. They most likely represent La-bound RNA polymerase III transcripts (25), which no doubt contributed to labeling the La protein in the cross-linking assay (Fig. 1). A comparison of the U6 snRNAs precipitated by each class of antibodies (Fig. 2A, lanes 4–6) shows that they are not identical in length. It is known that they uniquely differ by the length of the added oligo(U) tail (13). Those recovered by anti-La antibodies have a short oligo(U) tail and are probably newly synthesized U6 snRNAs (26). Those recovered by anti-Sm antibodies are much more heterogeneous, comprising U6 snRNAs having oligo(U) tails containing up to 12 U residues. Finally, those recovered by anti-C antibodies appear to correspond to those with the longest oligo(U) tail among all the anti-Sm-precipitated U6 snRNAs, indicating that a minimum size (at least 8 residues) is required for the oligo(U) tail-hnRNP C protein interaction. Moreover, the possibility that the interaction between the hnRNP C protein and this subset of U6 snRNAs might be indirect, through some association with another hnRNP component, is unlikely because antibodies directed against hnRNP A1 and U proteins fail to precipitate U6 snRNAs from total nuclear extracts (lanes 7 and 8).

A striking result seen in Fig. 1 (lane 5) is that anti-Sm antibodies failed to precipitate any protein adduct, suggesting that the U6 snRNAs interacting with either the La or the hnRNP C protein are free of U4 and U5 snRNAs. Concerning the U6-La interaction, the result agrees with previously published data, *i.e.* those U6 snRNAs in U4·U6 complexes are not bound to the La protein despite having a La-binding site at their 3' ends (26). To verify that U6 snRNAs with long oligo(U) tails are free of U4 and U5 snRNAs when interacting with the hnRNP C protein, we subjected a nuclear extract containing 3' end-labeled U6 snRNAs to glycerol gradient centrifugation under conditions allowing for separation of free U6 from U4·U6 and U4·U6·U5 snRNPs (see "Experimental Procedures") and looked for immunoprecipitated snRNAs in the resulting fractions using anti-Sm and anti-hnRNP C protein antibodies. As expected, the result (Fig. 3, A and B) was that those labeled U6 snRNAs present in anti-C precipitates exclusively migrate as free U6 snRNPs, while those in anti-Sm precipitates are associated with U4 (U4·U6 snRNPs) and U4·U5 (U4·U6·U5 snRNPs). The Northern blot shown in Fig. 3C reveals how the major U2 to U6 snRNAs distribute through the gradient.

From these results, we conclude that the hnRNP C protein present in nuclear extracts interacts directly with those of the U6 snRNAs terminating with the longest oligouridylylate tail. Moreover, such an U6 snRNA-hnRNP C protein interaction does not exist when these U6 snRNAs are engaged with U4 and U5 snRNAs to form U4·U6 and U4·U6·U5 snRNPs.

hnRNP C Protein Induces Disruption *In Vitro* of Base-paired U4·U6 snRNAs—The above results raised the question of whether the association of the hnRNP C protein with free U6 snRNPs is a cause or a consequence of U4·U6 snRNP disruption. We performed a complementation assay using purified hnRNP C protein and U4·U6 snRNPs whose U6 snRNAs were elongated and labeled at their 3' end. To do this, a prerequisite was to prepare an extract sufficiently enriched with U4·U6 snRNPs compared with free U6 and U4·U5·U6 snRNPs. This was possible starting with a nuclear extract that has been cleared by DEAE-Sepharose chromatography as described previously (16). Indeed, most of its U4 and U6 snRNAs are in U4·U6 snRNPs (27). It has TUTase activity and contains the same elongatable forms of U6 snRNA as an extract active in splicing. It also contains U6 snRNA with 2',3'-cyclic phosphate end (13). Moreover, this extract turned out to be virtually free of hnRNP C protein on the basis of immunoblots assays (results

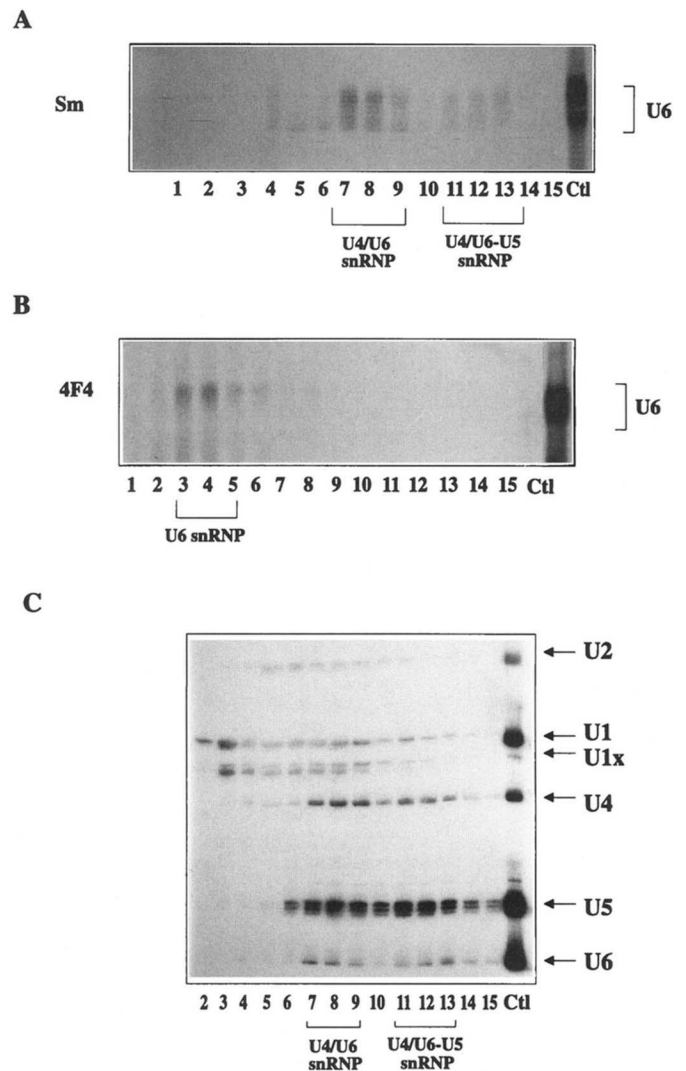


FIG. 3. Glycerol gradient fractionation of U snRNPs. Identical reactions to those described in Fig. 1 (150 μ l) were loaded onto a 10–30% glycerol gradient (see "Experimental Procedures"). 3' end-labeled U6 snRNAs present in each fraction were immunoprecipitated by anti-Sm (A) and 4F4 (B) antibodies. The Northern blot in C shows the anti-Sm precipitated U2 to U6 snRNAs through the gradient. The bands designed U1x are detected by the U1 probe and are, therefore, degraded forms of U1 snRNA.

not shown), thus allowing the effect of exogenously added hnRNP C protein to be monitored. This cleared nuclear extract was first incubated in the presence of [α - 32 P]UTP to label U6 snRNAs with 3'-OH end, and then the U4·U6 snRNPs were purified by centrifugation through a 10–30% glycerol gradient (see "Experimental Procedures"). These purified particles contained all of the forms of U4·U6 snRNPs, *i.e.* those with 3' end-labeled U6 snRNAs as well as those containing U6 snRNAs ending with 2',3' cyclic phosphate. The assay was carried out as follows. First, the amount of hnRNP C protein added was calculated to reconstitute approximately the U6 snRNA/hnRNP C ratio that exists in a splicing extract. Second, the reactions with or without hnRNP C protein added were subjected to immunoprecipitation by anti-C and anti-Sm antibodies. Third, immunoprecipitated materials were analyzed for U6 snRNA content by polyacrylamide-urea gels (Fig. 4, A and B). Addition of hnRNP C protein led to precipitation of labeled U6 snRNAs by anti-C antibodies (Fig. 4A, compare lanes 5 and 6), while the precipitation of these same U6 snRNAs by anti-Sm antibodies was largely decreased (Fig. 4A, compare lanes 1 and

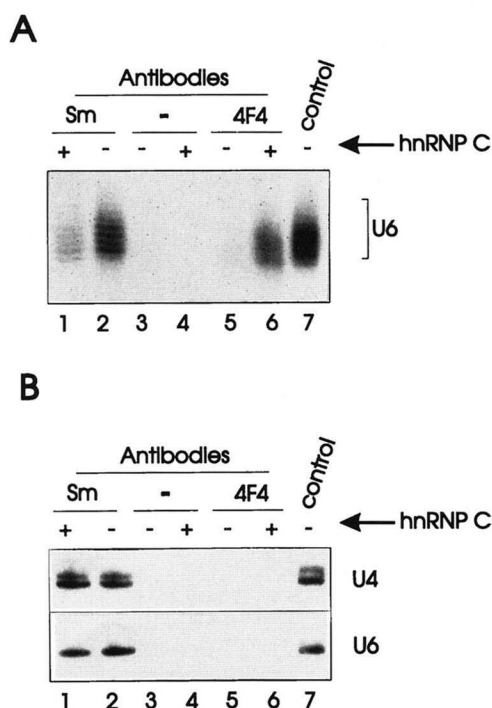


FIG. 4. hnRNP C protein induces *in vitro* disruption of U4·U6 snRNPs containing 3' end labeled U6 snRNA. A, U4·U6 snRNPs containing 3' end-labeled U6 snRNA were isolated by glycerol gradient centrifugation from a cleared nuclear extract (see "Experimental Procedures"). They were incubated for 30 min at 30 °C in the absence (lanes 2, 3, 5, and 7) or the presence (lanes 1, 4, and 6) of 10 nM of purified hnRNP C protein. The reactions (200 μ l) were subjected to immunoprecipitations by anti-Sm (lanes 1 and 2) and anti-C (lanes 5 and 6) antibodies and analyzed in a 10% polyacrylamide-urea gel. Lanes 3 and 4, controls treated on protein A-Sepharose beads without antibodies. Lane 7, a reaction without immunoprecipitation in order to indicate the input RNAs. B, the same gel as in A was transferred to hybond N⁺ membrane and then probed with U4 and U6 antisense oligodeoxynucleotide probes. The 3' end labeled U6 snRNAs cannot be seen here because they are much less radioactive than the probes. It is noteworthy that U4 snRNA appeared as a doublet in this gel, differently from the experiment shown in Fig. 4.

2). In contrast, as shown by Northern blots, hnRNP C protein has no effect on the major form of U4·U6 snRNPs containing U6 snRNAs terminating with a 2',3'-cyclic phosphate (Fig. 4B). It seems clear, therefore, that binding of hnRNP C protein only occurs in U4·U6 snRNPs whose U6 snRNAs have an elongated 3' end and that base pairing of U4 to U6 snRNAs is not an obstacle for hnRNP C binding. Finally, disruption of base-paired U4 and U6 snRNAs in these U4·U6 snRNPs provides the better explanation for the low immunoprecipitation of labeled U6 snRNAs by anti-Sm antibodies. To verify this, the following experiment was performed. The above reactions with or without hnRNP C protein were centrifuged through glycerol gradients to separate free U6 from U4·U6 snRNPs (Fig. 5). Probing for U4 and U6 snRNAs throughout the gradient shows that these snRNAs still comigrate if hnRNP C was added to U4·U6 snRNPs before centrifugation (Fig. 5A, compare *upper* and *lower* panels). This was expected since in the immunoprecipitation assay shown in Fig. 4B, all U6 snRNAs detected with the probe remained precipitable by anti-Sm antibodies. The 3' end-labeled U6 snRNAs, due to their low abundance, were not detected by the probe. They had a slower sedimentation when the U4·U6 snRNPs were incubated with the purified hnRNP C than without (Fig. 5B, compare the *two first panels*).

As the above experiment did not rule out the possibility that the dissociating activity could be contributed by some contaminant of the hnRNP C protein preparation, the same assay was

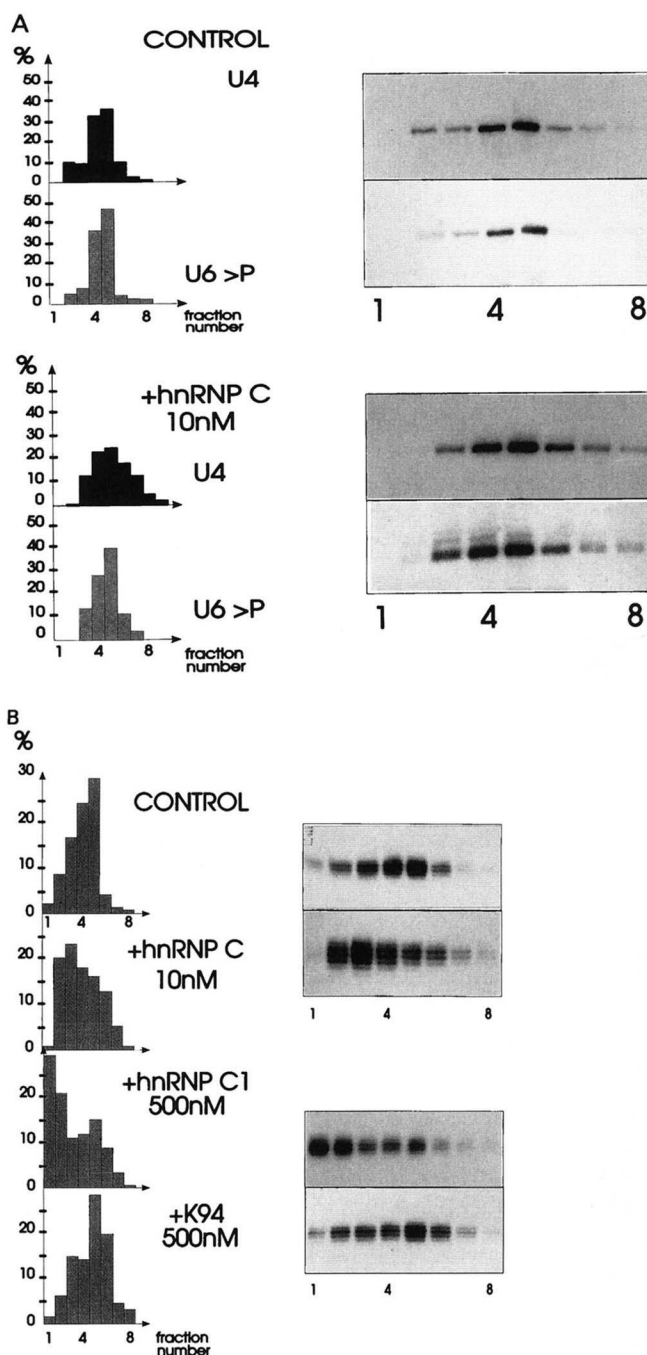


FIG. 5. Analysis of disrupted U4·U6 snRNPs by centrifugation in glycerol gradients. Identical reactions to those described in Fig. 3 (200 μ l) were loaded onto a 10–30% glycerol gradient (see "Experimental Procedures"). After fractionation from the top, the RNAs were extracted from each fraction and separated in a 10% polyacrylamide-urea gel. A shows the distribution of U4 and U6 snRNAs detected with the oligodeoxynucleotide probes. The autoradiographies were scanned by densitometry (*left panels*). For each snRNA, the values express a percentage of the sum of absorbancies throughout the gradient. B shows the distribution of 3' end-labeled U6 snRNAs from reactions with or without hnRNP C protein (*upper panel*). Lower panel shows the distribution of 3' end-labeled U6 snRNAs when the reactions were carried out in the presence of either recombinant C1 or K94 mutant in place of purified hnRNP C protein. The concentrations of protein used are indicated. Densitometry scanning of the autoradiographies was as above.

carried out using a recombinant hnRNP C1 protein. This protein again dissociated U4·U6 snRNPs containing 3' end-labeled and elongated U6 snRNAs despite being 50-fold less active than the purified hnRNP C protein (Fig. 5B, *third panel*). It is

possible that post-translational modifications (*i.e.* phosphorylations) and/or the presence of C2 isoform in the preparation from HeLa cells could account for this difference.

Specificity of the Dissociation Activity of hnRNP C Protein—It is true to say that U4·U6 snRNPs containing elongated U6 snRNAs are a weak part of the total U4·U6 particles. Besides, the hnRNP C protein is a very abundant nuclear protein and is one of the most avid pre-mRNA binding proteins in HeLa cell extracts. It is known to bind to oligo(U) stretches in natural RNAs (23, 24, 28, 29). It was therefore crucial to discover whether binding of hnRNP C protein to elongated U6 snRNAs followed by U4·U6 snRNP disruption is specific. We first determined the stoichiometry of the reaction of dissociation. To do this, different concentrations of purified hnRNP C protein were added to labeled U4·U6 snRNPs, and the dissociation was monitored by centrifugation in glycerol gradients (not shown). This led us to consider that dissociation is effective when four molecules of purified protein are added to one snRNA. It was assumed that 5% of the U6 snRNAs have a 3'-OH elongatable end. We have also tested the dissociating activity of the so-called K94 deletion mutant of hnRNP C protein. It has the same RNP binding domain as the hnRNP C protein and it binds to poly(U), but it lacks a large part of the hnRNP C1 protein sequence in the side of the carboxyl terminus (30). Used at the same concentration as the hnRNP C1 protein in our complementation assay, it failed to induce dissociation of U4·U6 snRNPs (Fig. 5B, *last panel*). However this mutant protein binds to U4·U6 snRNPs containing 3' end-labeled U6 snRNAs since, upon UV cross-linking of a reaction, it became labeled as did the hnRNP C protein (results not shown). It appears, therefore, that something in the sequence other than the canonical RBD of hnRNP C1 is required to induce dissociation. Other experiments using mutated proteins will be required to more precisely define the domain of the hnRNP C protein, which is implied in dissociation of U4·U6 snRNPs. Finally, other recombinant proteins with RNP-CS domains, namely U2AF, known to bind tenaciously to poly(U) (31) and SF2·ASF were tested in our complementation assay. As expected, they failed to disrupt U4·U6 snRNPs (results not shown).

DISCUSSION

We report here that the hnRNP C protein, belonging to the family of proteins exhibiting a RNP consensus sequence (RNP-CS), interacts with a subset of U6 snRNAs characterized by their relatively long 3'-oligoridylate tail. Binding occurs at this tail since the protein becomes cross-linked upon irradiation with UV light of nuclear extracts containing 3' end-labeled U6 snRNAs. Significantly, the hnRNP C protein, either purified from HeLa cells or produced as recombinant protein from *Escherichia coli*, induces the release of elongated U6 snRNAs from U4·U6 snRNPs. Two points must be underlined. First, the fact that the hnRNP C protein might bind to an oligouridylate tail comprising from 8 to 12 residues agrees with previously published data that the shortest uridine oligoribonucleotide that binds efficiently to the hnRNP C protein is r(U)₈ (32). Second, the disruption of U4·U6 snRNPs seems to be specific of the hnRNP C protein since a mutated protein containing the RNP-CS domain alone, as well as other factors with RNP-CS (U2AF, SF2·ASF) failed to disrupt U4·U6 snRNPs.

Since U4 and U6 snRNAs become separated within the spliceosome before the first step of splicing takes place (33), and since the hnRNP C protein is supposed to be involved in splicing (14), it is tempting to hypothesize that U4·U6 snRNP disruption induced by the hnRNP C protein is what happens during splicing. Obviously, such a scenario implies that U6 snRNA functions in splicing with an elongatable 3'-OH end.

Although most of the U6 snRNAs in mammalian cells have a 2',3'-cyclic phosphate end (12), the presence of U6 snRNAs with 3'-OH end within the spliceosome is indubitable. Indeed, the five snRNAs involved in splicing are 3' end-labeled by cytidine 3',5'-bisphosphate when extracted from mammalian affinity-selected spliceosomes (34). Also, we have previously obtained evidence that elongated U6 snRNAs are present within the spliceosome and that a 2',3'-cyclic phosphate at the end of U6 snRNA is a consequence of splicing instead of being a requirement for it (13). Finally, it is known that in organisms U6 snRNAs consist of forms with different 3' end groups (12). For example, all U6 snRNAs have 3'-OH end in *T. brucei*, while in man and other mammals the >p, -OH ratio is 9:1.

A U6 snRNA-hnRNP C protein interaction functional in splicing raises the question of how elongated U6 snRNAs are generated. They appear from shorter forms upon incubation of the extracts with an excess of UTP, as well as with ATP under splicing conditions and are present in all of the U6 snRNA-containing complexes (13). In fact, we can now add that preexisting elongated U6 snRNAs exist as well and are assembled into multi-snRNP complexes. This was ascertained by the finding that some labeled U6 snRNAs with long uridylate tail have left their label when subjected to β -elimination (results not shown). We have discussed already the possibility that elongatable U6 snRNAs might be generated from molecules terminating by a 2',3'-cyclic phosphate also present in U4·U6 and U4·U5·U6 complexes (13). It remains to be understood why most of U6 snRNAs terminate by a 2',3'-cyclic phosphate end in mammals and several other species (12). It could impair U6 snRNA to be bound to the hnRNP C protein before the interaction is required. Similarly, once the splicing reaction is accomplished, it could preserve U6 snRNA from untimely association with the hnRNP C protein, which would lead to the lack of U4·U6 snRNA interaction, therefore leaving U6 snRNA unavailable for a new round of splicing.

The hypothesis of a U6 snRNA-hnRNP C protein interaction related to the splicing mechanism is also compatible with the recent proposal that the hnRNP C protein, as well as other hnRNP proteins, have RNA chaperone activity (Ref. 35 and, for a review, see Ref. 36). It is thought that these proteins promote association or dissociation of trans-acting factors in modulating the pre-mRNA conformation. It is therefore tempting to speculate that the hnRNP C protein acts similarly on the U6 snRNA conformation to disrupt U4·U6 snRNP and promote U6-U2 base pairing. At this stage, it is interesting to compare the annealing and the disruption of U4·U6 snRNAs in the yeast system and our proposal of a spliceosomal U6 snRNA-hnRNP C protein interaction occurring in mammals. In yeast, it has been proposed that PRP24, a U6-specific binding protein with RNP-CS, directs stabilization of U6 snRNP in a free form and promotes reannealing of U6 with U4 through the formation of a transient intermediate PRP24·U4·U6 (37). At first glance, this is more compatible with hnRNP C protein having an annealing activity than inducing U4·U6 snRNP disruption. However, it has been proposed that PRP24 could have another role in the U4·U6 cycle in concert with PRP28, a protein of the DEAD box family (38, 39). Once disruption of base-paired U4·U6 has occurred, PRP24 could serve to stabilize the unwound form of U6 snRNA (40). This seems to be very similar to what we propose for the function of the U6 snRNA-bound hnRNP C protein. If the hnRNP C protein bound to U6 snRNA has in mammals the same stabilizing function as the PRP24 in yeast, what could be the mammalian equivalent of PRP28? Keeping in mind that ATP is not required to obtain hnRNP C protein-induced disruption of U4·U6 snRNPs, one can envisage at least two possibilities. Oligomerization of hnRNP C isoforms could lead to a

complex having both PRP24 and PRP28-like activities. As a matter of fact, it is known that the hnRNP C protein is part of a tetrameric structure containing three C1 and one C2 isoforms (41). Alternatively, one of the specific U4-U6 snRNP proteins (42) could have PRP28-like activity, becoming functional only when the hnRNP C protein has bound to the oligouridylate tail.

Acknowledgments—We thank D. Portman and G. Dreyfuss for providing recombinant hnRNP C protein and K94 mutant and helpful comments on the manuscript.

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