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Mammalian U6 Small Nuclear RNA Undergoes 3′ End Modifications within the Spliceosome

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Mammalian U6 small nuclear RNA (snRNA) is heterogeneous with respect to the number of 3′ terminal U residues. The major form terminates with five U residues and a 2′,3′ cyclic phosphate. Because of the presence in HeLa cell nuclear extracts of a terminal uridylyl transferase, a minor form of U6 snRNA is elongated, producing multiple species containing up to 12 U residues. In this study we have used glycerol gradients to demonstrate that these U6 snRNA forms are assembled into U6 ribonucleoprotein (RNP), U4/U6 snRNPs, and U4/U5/U6 tri-snRNP complexes. Furthermore, glycerol gradients combined with affinity selection of biotinylated pre-mRNAs led us to show that elongated forms of U6 snRNAs enter the spliceosome and that some of these become shortened with time to a single species having the same characteristics as the major form of U6 snRNA present in mammalian nuclear extracts. We propose that this elongation-shortening process is related to the function of U6 snRNA in mammalian pre-mRNA splicing.

U6 small nuclear RNA (snRNA) is one of the five evolutionarily conserved snRNAs (U1, U2, U4, U5, and U6 snRNAs) packaged in small nuclear ribonucleoproteins (snRNPs) that are essential components of the eukaryotic splicing apparatus called the spliceosome (for a review, see references 4, 25, 28, and 37). There are several features distinguishing U6 from the other snRNAs. It is synthesized by RNA polymerase III instead of polymerase II. It lacks the 2,2′,7-trimethyl guanosine 5′ cap and the binding site for the core (or Sm) proteins found in the other snRNAs (for a review, see reference 10). It occurs in the form of different snRNP complexes. In the U6 snRNP, the 3′ terminal uridylate tract of a fraction of U6 snRNA is bound by La protein and is therefore precipitable by anti-La antibodies (41). In the form of U4/U6 snRNP, it is base paired to U4 snRNA, which makes it precipitable by anti-Sm antibody. Finally, the form in which U6 snRNA enters the spliceosome is the U4/U6/U5 tri-snRNP complex (2, 3, 8, 22, 24, 27). There is also recent evidence that U6 snRNA may play a catalytic role in the splicing reaction. This comes from the finding that several point mutations result in splicing inhibition (14, 30) and the discovery that U6 snRNA genes from certain yeast species contain an intron located in a region crucial for splicing and whose insertion could result from an aberrant splicing event (34, 38, 39).

Quite interestingly, earlier (33, 35) and very recent (29, 41) studies have led to the conclusion that the steady-state population of U6 snRNA is heterogeneous in size mainly because it is subject to 3′ end modifications. These involve UMP addition and the formation of a 2′,3′ cyclic phosphate (>p). The predominant form of human and mouse U6 snRNA terminates with five Us and a 2′,3′ cyclic phosphate group (29), but a substantial fraction (about 10%) has an oligouridylate stretch of variable size and terminates with a 3′ hydroxyl group. The two forms are precipitable by anti-Sm antibodies, but only the minor ones are precipitated by anti-La antibodies (41).

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In this work, we were interested in determining whether the minor forms of U6 snRNA are used in splicing. To follow them, they were labeled with [α-^{32}P]UTP because of the presence in HeLa cell splicing extracts of a U6-specific UMP adding activity, most likely the same as those previously found in other cells (20, 33). We demonstrate that elongated forms of U6 snRNA are present into the U4/U6 snRNP, the U4/U5/U6 tri-snRNP complex, and finally, the spliceosome. More importantly, during splicing some of these elongated forms become shortened down to a single RNA species having the same gel mobility as the major form of U6 snRNA and carrying a 2′,3′ cyclic phosphate at its 3′ end.

MATERIALS AND METHODS

Materials. SP6 RNA polymerase, RNasin, nuclease P_1, and restriction enzymes were from Promega Biotec, the $M^7G5'pppG5'$ cap and sequencing kit was from Pharmacia LKB Biotechnology Inc., ^{32}P-labeled ribonucleotide triphosphates were from Amersham Corp., and streptavidin-polyester beads (Epicon) were from Pandex Laboratories Inc. The antibodies used were the mouse monoclonal anti-Sm (7.13) from S. Hoch (Agouron Institute, La Jolla, Calif.) and a patient serum of SSb specificity (anti-La antibodies). All other chemicals were of analytical grade.

Extracts. HeLa cell nuclear extracts for in vitro splicing reactions were prepared by the method of Dignam et al. (12), except that triethanolamine buffer was used instead of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES). The DEAE-Sepharose-cleared extract was adapted from that of Hinterberger et al. (19) as follows. The snRNPs contained in the flowthrough from the first DEAE-Sepharose column (DEAE-Sepharose 1) were concentrated four times in microconcentrators (Centricon 30; Amicon) and then dialyzed for 3 h against two changes of 500 ml of Dignam's buffer D containing 1 mM $MgCl_2$. The U4/U6-depleted extract was a generous gift from A. Bindereif (Max-Planck-Institut, Berlin, Germany). It was free of U4 and U6 snRNAs as judged from Northern (RNA) blot analysis (not shown). The partially purified terminal uridylyl transferase (TUTase) was obtained

as follows. Nuclear extract was applied to a heparin-Sepharose column preequilibrated in buffer D. After washing, adsorbed material was step eluted by 600 mM KCl, dialyzed against buffer D containing 50 mM KCl, and then loaded onto a DEAE-cellulose column equilibrated in the same buffer. TUTase activity was step eluted at 250 mM KCl, precipitated by ammonium sulfate, and then dissolved in buffer D before being applied to a DNA-agarose column. TUTase activity was present in the flowthrough of this column. The final step was the fractionation of this material on a 5 to 20% sucrose gradient made in buffer D without glycerol. Gradients were centrifuged in a SW 40 Beckman rotor for 20 h at 40,000 rpm and 4°C. A total of 20 fractions were collected from the top of the tube. Those containing TUTase activity were pooled and used for the U6 snRNP labeling. At all steps of the purification procedure, TUTase activity was detected as follows: fractions (2 to 3 μg of protein) were incubated for 30 min at 30°C in 10 μl of Dignam buffer D containing 1 μg of brewer's yeast 5S RNA as a substrate and 10 μCi of [α-^{32}P]UTP. The samples were then extracted with phenol-chloroform (1:1), ethanol precipitated, and electrophoresed in a 10% polyacrylamide–8 M urea gel. Labeled 5S RNA was revealed by autoradiography.

U6 snRNA labeling and sequencing. Endogenous U6 snRNA species were labeled by incubating 100 μl of nuclear extract in the presence of 100 μCi of [α-^{32}P]UTP for 30 min at 30°C. The reaction was stopped by the addition of 100 μl of 2× proteinase K buffer (Tris-HCl [pH 7.8], 50 mM EDTA, 0.5% sodium dodecyl sulfate [SDS]). Digestion with proteinase K (4 mg/ml) was for 30 min at 40°C. Upon phenol-chloroform extraction and electrophoresis in a 10% polyacrylamide–8 M urea gel, the labeled RNAs were excised from the gel and eluted overnight at 37°C in 0.8 M ammonium acetate containing 0.1% SDS and 0.1 mM EDTA. The RNAs were recovered by ethanol precipitation in the presence of 10 μg of carrier tRNA and then washed twice with 70% ethanol before being dried. In order to generate RNAs with a 3′ phosphorylated end, the last nucleoside was removed by β-elimination. This was performed by resuspending dried RNAs in 50 μl of sodium periodate (0.15 mg/ml in 15 mM sodium acetate [pH 4.75]) and incubating them in the dark at 0°C for 30 min. After ethanol precipitation, 50 μl of aniline was added and the RNAs were further incubated in the dark at 65°C for 20 min. Sequencing was performed according to the manufacturer's recommendations.

In vitro transcription and splicing. A total of 1 μg of adenovirus pSP62Δi1 and pSP62Δi1Δe1 21 plasmids (15) linearized with *Sca*I was used as templates for the SP6 RNA polymerase-directed in vitro synthesis of either cold or labeled RNAs. In the latter case, transcription reactions were performed in the presence of 100 μCi of [α-^{32}P]UTP as previously described (40). Affinity selection of spliceosomes required the use of biotinylated RNAs. To this aim, the above transcripts were biotinylated at both ends as follows. A total of 10 μg of RNA in 30 μl of water was submitted to periodate oxidation for 30 min in the dark at 0°C with 1 μl of a solution containing 10 mg of sodium periodate (pH 4.75) per ml. A total of 23 μl of a solution containing 10 mg of biotin aminocaproylhydrazide per ml was immediately added, and another 23 μl of a solution containing 6.3 mg of cyanoborohydrate per ml dissolved in 200 mM potassium phosphate (pH 8.0) was added next. Incubation was at room temperature for 45 min. The volume was then raised to 400 μl with water, and biotinylated RNAs were finally purified by three successive ethanol precipitations. Standard reaction mixtures under splicing conditions contained, in a volume of 100 μl, 2 mM ATP, 20 mM creatine phosphate, 2 mM $MgCl_2$, 100 μCi of [α-^{32}P]UTP, 40 μl of nuclear extract, and 1 μg of pre-mRNA (unless otherwise stated). Reactions were run at 30°C and then loaded onto glycerol gradients. Of course, [α-^{32}P]UTP was omitted in the reaction mixtures containing ^{32}P-labeled pre-mRNA.

Glycerol gradient centrifugation and affinity selection of biotinylated material. The above reaction mixtures were fractionated by centrifugation in glycerol gradients as previously described (22). In brief, 100-μl reaction mixtures were loaded onto 4-ml 10 to 30% gradients made in 50 mM Tris-glycine buffer (pH 8.8). Centrifugation was in an SW 60 Beckman rotor at 41,000 rpm for 3 h at 4°C. A total of 18 fractions of 220 μl were recovered from the top. The RNAs present in each fraction were analyzed directly, after proteinase K treatment, or after affinity selection on streptavidin-polyester beads. Before addition of streptavidin, each fraction was mixed to 1 volume of 50 mM Tris-glycine buffer and then incubated at 4°C for 30 min. Polyester beads containing adsorbed material were recovered by 1 min of centrifugation in a microfuge and washed four times with 1 ml of 50 mM Tris-glycine buffer. The RNAs were extracted and then analyzed in a 10% polyacrylamide–8 M urea gel.

To separate free U6 and U4/U6 snRNPs from the cleared extract (Fig. 3), the same 10 to 30% glycerol gradients were used, except that centrifugation was in a Beckman SW 40 rotor at 29,000 rpm for 18 h at 4°C. Fractions (500 μl) were collected from the top, and aliquots were incubated with the TUTase-enriched fraction in the presence of 10 μCi of [α-^{32}P]UTP at 30°C for 30 min in order to label the U6 snRNA with 3′-OH ends.

Immunoprecipitations. A total of 100 μl of glycerol gradient fractions (see Fig. 3A and C) was added to 50 μl of either anti-La or anti-Sm antibodies bound to protein A-Sepharose and incubated for 1 h at 4°C. After four washes with 1 ml of NET2 buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Nonidet P-40, 0.5 mM dithiothreitol), bound material was submitted to proteinase K digestion. Released RNAs were extracted, separated by electrophoresis in 10% polyacrylamide-urea gels in Tris-borate-EDTA (TBE) buffer, and finally detected by autoradiography.

Northern hybridizations. RNAs present in each fraction of the glycerol gradients were electroblotted from denaturing gels to Hybond membranes under the same conditions as described by Blencowe et al. (5). The membranes were UV treated for 30 s, baked, and then prehybridized for 2 h (9). DNA probes complementary to U4 (nucleotides 65 to 85), U6 (nucleotides 77 to 95), and U5 (nucleotides 69 to 87) snRNAs were synthesized and end labeled with T4 polynucleotide kinase in the presence of [α-^{32}P]ATP. Hybridizations were in the same buffer at 42°C. One wash of 5 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and then three washes of 15 min each in 1× SSC–0.1% SDS were performed at room temperature before autoradiography.

RNase P_1 digestion and thin-layer chromatography. Elongated and shortened labeled U6 snRNA forms were eluted from the gel and digested with P_1 exonuclease either directly or after acidic treatment. In this latter case, eluted RNA was resuspended in 10 μl of water, and then 1 μl of 0.1 N HCl was added. The sample was left at room temperature for 2 h before the RNA was recovered by ethanol precipitation. To the acid-treated and nontreated samples in 10 μl of 50 mM ammonium acetate (pH 5.3), 1 μg of nuclease P_1 was added and then incubation was for 1 h at 50°C. The digestion products were analyzed by thin-layer chromatography on a

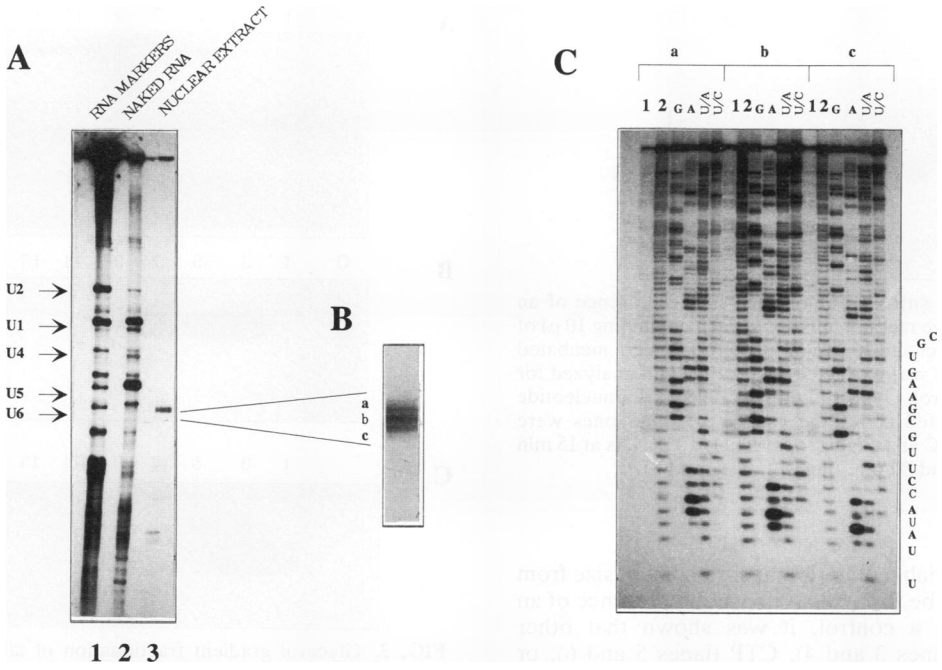

FIG. 1. Incubation of HeLa cells nuclear extract with [α-^{32}P]UTP leads to the 3′ end labeling of U6 snRNA. (A) Two separate assays have been carried out. In the first one, 5 μl of nuclear extract was incubated in the presence of 10 μCi of [α-^{32}P]UTP (lane 3). In the second one, the same amount of nuclear extract was deproteinized, and then the resulting RNAs were incubated as above after the addition of 4 μl of partially purified TUTase (lane 2). Lane 1, total RNAs from an in vivo ^{32}P-labeled nuclear extract used here as markers. (B) The same sample as in panel A (lane 3) was electrophoresed in a gel with better resolution. It appears that at least three closely spaced bands are labeled. (C) Bands denoted a to c in panel B were eluted from the gel, and the resulting RNAs were submitted to β-elimination and sequencing (see Materials and Methods). Lanes 1 and 2 refer to alkaline hydrolysis of species a to c without (lanes 1) or with (lanes 2) β-elimination. Since bands denoted a to c were very close, the sequence written on the right can be read only from c, which is the least contaminated with other end-labeled U6 snRNA.

DEAE-cellulose plate developed in a solution of 0.2 M ammonium formate, 9 M urea, and 1 mM Na_2-EDTA (31).

RESULTS

In vitro addition of UMP residues to the 3′ end of U6 snRNAs. As expected from earlier studies (20, 33), incubating HeLa cell nuclear extracts with [α-^{32}P]UTP results in the labeling of mainly one discrete RNA migrating to the same position as a U6 snRNA marker and some other minor species of smaller size (Fig. 1A, lane 3). None of the three other ribonucleoside triphosphates are incorporated into species of this size range. However, while nothing is labeled with [α-^{32}P]GTP, [α-^{32}P]CTP gives rise to an intense labeling of tRNAs, most likely because of the enzyme which adds C-C-A to the 3′ end of all tRNAs, and high-molecular-weight RNAs are labeled with [α-^{32}P]ATP (data not shown). Close inspection of the U6 snRNA region in the gel shown in Fig. 1A, lane 3, revealed a somewhat heterogeneous pattern. A gel with better resolution which revealed several close bands was therefore used (Fig. 1B). Those bands (labeled a, b, and c) were excised from the gel, and eluted RNAs were submitted to β-elimination in order to generate RNAs with 3′ phosphate ends before being sequenced (Fig. 1C). The sequence to the right of panel C can be read from that of band c and corresponds to the U6 snRNA sequence (23). Bands b and a show the same sequencing pattern as band c except that they have, respectively, one and two additional UMP residues. The results clearly show that a single U residue is added in all three of the examined species and that all have the U6 snRNA sequence. However, compared with the major form of U6 snRNA in HeLa nuclear extract, the three species have fewer U residues at their 3′ ends (from two to four instead of five). The former apparently is inefficiently labeled in the presence of [α-^{32}P]UTP. This is not surprising in the light of recent results demonstrating that the major form of U6 snRNA carries a 2′,3′ cyclic phosphate at its 3′ end (29).

An intriguing feature of the above results is the specificity of the labeling. The simple possibility that these heterogeneously sized forms of U6 snRNA are the only RNAs whose 3′ ends are accessible in the nuclear extract can be ruled out, since tRNAs are efficiently labeled with [α-^{32}P]CTP but not with [α-^{32}P]UTP. It is worth mentioning that many of the discrete RNAs present in the nuclear extract can be labeled at their 3′ ends when incubated after deproteinization in the presence of partially purified TUTase and [α-^{32}P]UTP (Fig. 1A, lane 2). However, it is clear that the abundance of these RNAs is not reflected by their labeling. For example, U1 and U5 snRNAs (identified by sequencing) are predominantly labeled, although they are not the only abundant RNAs in the extract.

Finally, we have investigated the possibility that more than one UMP residue might be added to U6 snRNP in the presence of an excess of UTP. Reaction mixtures were incubated for 30 min at 30°C in the presence of [α-^{32}P]UTP and then for a further 10 or 30 min after the addition of 1 mM cold UTP. Electrophoresis of extracted RNAs clearly shows that the initial labeling is distributed over eight slowly migrating U6 species (Fig. 2, lanes 1 and 2), confirming that

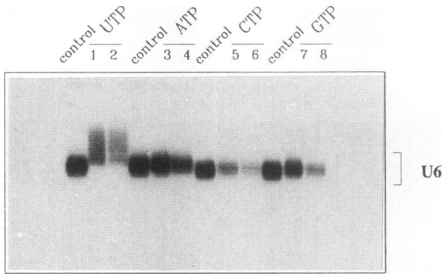

FIG. 2. Labeled U6 snRNA is elongated in the presence of an excess of cold UTP. Five reaction mixtures, each containing 10 μl of HeLa cell nuclear extract and 10 μCi of [α-^{32}P]UTP, were incubated for 30 min at 30°C. One was extracted at this time and analyzed for labeled RNAs as a control. Upon addition of one cold ribonucleotide triphosphate (as indicated above the lanes), the other ones were further incubated at 30°C and analyzed for labeled snRNAs at 15 min (lanes 1, 3, 5, and 7) and 30 min (lanes 2, 4, 6, and 8).

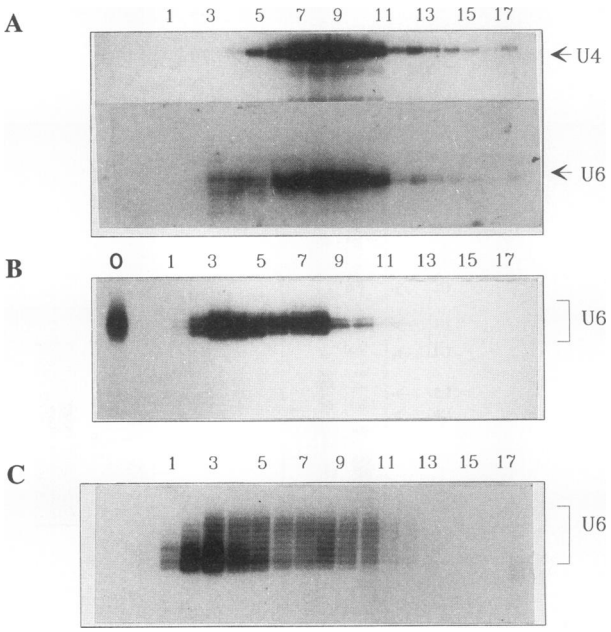

FIG. 3. Glycerol gradient fractionation of labeled U6 snRNAs from a DEAE-Sepharose-cleared extract (see Materials and Methods). (A) Northern blot hybridizations with U4 and U6 DNA oligonucleotide probes revealing that U6 snRNAs from these cleared extracts are distributed into free U6 and U4/U6 snRNPs upon centrifugation through 10 to 30% glycerol gradients. (B and C) Labeled (B) and labeled and then elongated (C) U6 snRNAs are distributed throughout the same gradients as in panel A run in parallel. To perform this, 100 μl of cleared extract was incubated in the presence of 100 μCi of [α-^{32}P]UTP for 30 min at 30°C before being layered on the gradient (B). (C) Cold UTP was added to a concentration of 1 mM after the extract was incubated with hot UTP, and then the reaction was continued for 15 min more before centrifugation. Centrifugation was from left to right, and numbers above the lanes refer to fractions. Lane 0 (B), labeled U6 snRNA before centrifugation. Electrophoresis was in 10% polyacrylamide-urea gels.

U6 snRNAs with variable oligo(U) tails, ranging in size from 6 to 12 residues, can be formed in vitro in the presence of an excess of UTP. As a control, it was shown that other nucleotides, ATP (lanes 3 and 4), CTP (lanes 5 and 6), or GTP (lanes 7 and 8), did not elongate the labeled U6 species which rather tend to disappear because of exonucleolytic removal of UMP residues. Interestingly, we have also found that naked U6 snRNA, as well as an SP6-U6 snRNA transcript, can be 3′ end labeled upon incubation with partially purified TUTase but not elongated, suggesting that the elongation process requires an RNP structure (data not shown).

The heterogeneously sized forms of U6 snRNA are packaged into U4/U6 snRNP. To assess the functionality of these forms of U6 snRNA that are substrates for the TUTase activity, we have investigated their occurrence in the well-characterized U4/U6 snRNP in which U4 and U6 snRNAs are joined by intermolecular base pairing. To this aim, we have used a concentrated nuclear extract which has been cleared by DEAE-Sepharose chromatography by the procedure described by Hinterberger et al. (19) instead of a splicing extract as in the above assays. Although not active in splicing itself, such an extract contains functional snRNPs, since it can complement a micrococcal nuclease-digested splicing extract (not shown). Moreover, most of its U4 and U6 snRNAs content is in the form of U4/U6 snRNP, and finally, it leads to exactly the same results as those shown in Fig. 1, implying that it contains both the TUTase activity and the heterogeneous forms of U6 snRNA.

In the following experiment, 150 μl of cleared nuclear extract was centrifuged through a 10 to 30% glycerol gradient. To analyze the U4 and U6 snRNA distribution throughout the gradient, the RNAs from each fraction were extracted, separated by electrophoresis, transferred to a nylon membrane, and hybridized with DNA oligonucleotide probes complementary to U4 and U6 snRNAs. As expected, the Northern blots in Fig. 3A show that the majority of U4 and U6 snRNAs comigrate exactly (lanes 6 to 11). However, it can be seen that some of the U6 molecules have sedimented free of U4 (Fig. 3A, lanes 2 to 4). To investigate how the U6 species that are substrates for the TUTase activity are distributed, cleared extracts were incubated with [α-^{32}P]UTP and then fractionated on parallel gradients either directly (Fig. 3B) or after a chase with cold UTP (Fig. 3C). Subsequent electrophoresis shows that labeled U6 snRNA, elongated or not, migrates at two positions, one most likely as free U6 snRNP (lanes 2 to 4) and the other one as U4/U6 snRNP (lanes 6 to 11). One can note, however, that more labeled U6 is in the free form. As the above assay does not allow for distinguishing between preexisting and newly assembled U4/U6 snRNP during incubation of the cleared extract with [α-^{32}P]UTP, we have performed a parallel experiment in which each fraction of the gradient corresponding to Fig. 3A was incubated in the presence of partially purified TUTase and [α-^{32}P]UTP. Once again, labeled U6 snRNA was distributed into two peaks, exhibiting a pattern quite similar to that shown in Fig. 3B (data not shown).

To further confirm that labeled U6 snRNA was present in free U6 as well as U4/U6 snRNP, we have submitted glycerol gradient fractions exhibiting U6 labeling to immunoprecipitation with antibodies of La and Sm specificities. Again we saw two kinds of labeled U6 snRNA. Because of its association with the La protein, the first one, located in the first fractions, was immunoprecipitated by anti-La but not by anti-Sm antibodies. In contrast, the second one was anti-Sm but not anti-La precipitable, therefore confirming that it belongs to the U4/U6 snRNP (results not shown). It is worth mentioning that in all these experiments, especially

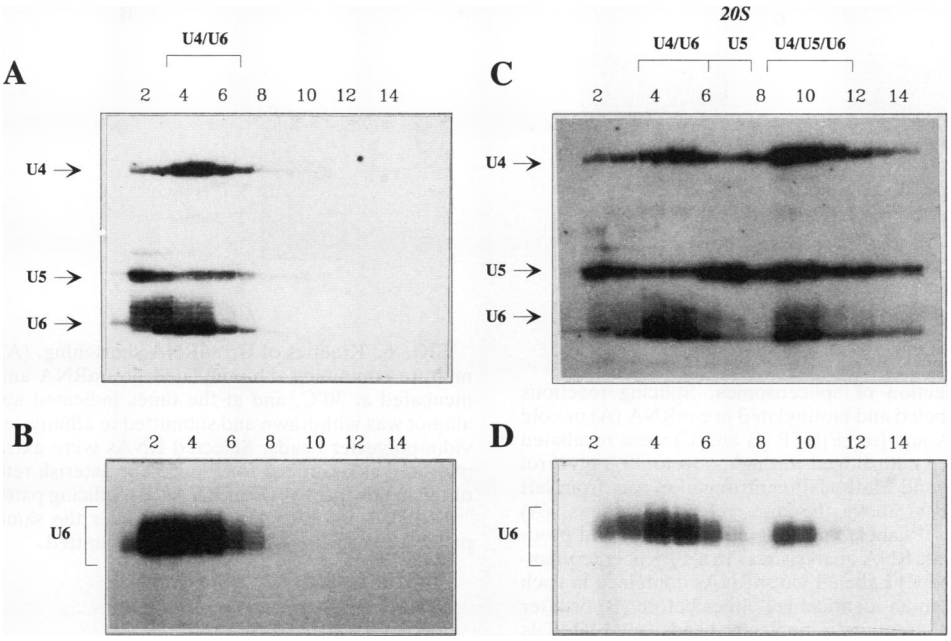

FIG. 4. Glycerol gradient fractionation of labeled U6 snRNAs from a U4/U6-depleted extract complemented with DEAE-Sepharose-cleared nuclear extract. Conditions for centrifugation allowing separation of the U4/U6 snRNP from the U4/U5/U6 tri-snRNP complex were as described in Materials and Methods. A total of 100 μl of cleared extract was incubated in the presence of 100 μCi of UTP and 1 mM cold UTP for 30 min at 30°C and then layered on the gradient. Labeled U6 snRNAs were revealed by autoradiography of a 10% polyacrylamide-urea gel (exposure time was 14 h at −80°C with a screen with Kodak films) (B). RNAs from this same gel were then electroblotted and hybridized to DNA probes complementary to U4, U5, and U6 snRNAs (A). The exposure time for the autoradiography was half an hour at −80°C with a screen. (D) A total of 20 μl of cleared extract was used to complement 20 μl of U4/U6-depleted extract. After 30 min of incubation at 30°C under splicing conditions and in the presence of 100 μCi of [α-^{32}P]UTP to label U6 snRNA, the reaction was layered on the gradient. Upon centrifugation as described above and fractionation, RNAs were extracted and subjected to electrophoresis. (C) Northern blots. The RNAs present in panel D were electroblotted and hybridized with DNA probes complementary to U4, U5, and U6 snRNAs. The autoradiography conditions for panels C and D were the same as in panels A and B, respectively.

those showing anti-Sm precipitates, labeled U6 snRNAs seems to be of a larger size when associated with U4 snRNA compared with the anti-La precipitable species, suggesting that a minimum length of the U stretch is required for U6 snRNA to be associated with U4. However, the U6 snRNA forms having the longest poly(U) tails are not quantitatively immunoprecipitated by anti-Sm antibodies.

Assembly of elongated U6 snRNA into U4/U5/U6 tri-snRNP complex. Conditions for glycerol gradient centrifugation allowing for separation of the U4/U5/U6 tri-snRNP complex from free U4/U6 and U5 snRNPs (22) have been used here. In order to favor the assembly of U4/U5/U6 complex, we have devised a complementation assay in which a U4/U6-depleted nuclear extract was complemented with the snRNP-rich cleared extract described above. As a matter of fact, most of the U6 snRNA content of the cleared extract is in the form of U4/U6 snRNP (Fig. 3A), and by definition, the U4/U6-depleted nuclear extract cannot contain the tri-snRNP complex. Since splicing is efficient in such a complementation assay, a functional U4/U5/U6 snRNP complex is most likely assembled from U4/U6 and U5 snRNPs, respectively, belonging to the cleared extract and the U4/U6-depleted nuclear extract. As a first experiment, the cleared extract alone was incubated under splicing conditions in the presence of [α-^{32}P]UTP and 1 mM cold UTP and then subjected to sedimentation through a glycerol gradient. It is apparent from Fig. 4B that the labeled and elongated U6 snRNAs are distributed over many fractions of the gradient corresponding to free U6 and U4/U6 snRNPs. Northern blot analysis of the gradient fractions with U4, U5, and U6 probes has confirmed that the cleared extract contains neither the U4/U5/U6 complex nor the previously described 20S U5 snRNP (1). The analyses also have revealed that a substantial fraction of U6 snRNA migrates more slowly than the mature U6 (29) and therefore has more than five U residues at its 3′ end in both free U6 (Fig. 4A, lanes 2 and 3) and U4/U6 snRNPs (Fig. 4A, lanes 4 to 6). As a second experiment, the U4/U6-depleted nuclear extract complemented with the cleared extract was incubated under splicing conditions in the presence of [α-^{32}P]UTP but in the absence of cold UTP. Northern blot analysis shown in Fig. 4C clearly shows that at least half of the U4/U6 snRNPs from the cleared extract now sediments as a 25S multi-snRNP complex (lanes 9 to 12) in which elongated forms of U6 snRNA are present. It is also quite clear that labeled and elongated U6 snRNA forms a peak sedimenting at the position of the U4/U5/U6 tri-snRNP complex (Fig. 4D, lanes 9 to 12). However, the possibility that U6 snRNA elongation might be a requirement for the formation of the tri-snRNP complex can be ruled out, since most of the U6 snRNA sedimenting at the 25S position has the expected size for the so-called mature U6 snRNA (Fig. 4C).

An interesting feature of these results is the assembly of elongated forms of U6 in all U6 snRNA-containing complexes, although the reactions were carried out in the absence of cold UTP. It therefore becomes evident that U6 snRNA is elongated under splicing conditions, i.e., in the presence of ATP and creatine phosphate. This is most likely

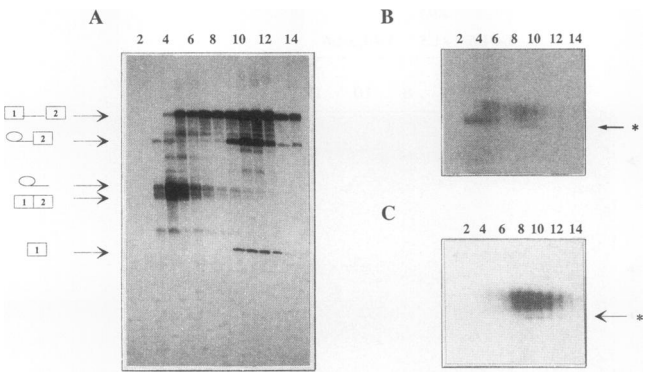

FIG. 5. Affinity selection of spliceosomes. Splicing reactions containing either ^{32}P-labeled and biotinylated pre-mRNA (A) or cold biotinylated pre-mRNA and [α-^{32}P]UTP (B and C) were incubated for 1 h at 30°C and then centrifuged through a 10 to 30% glycerol gradient (see Materials and Methods); centrifugation was from left to right, and the numbers above the lanes refer to fractions. (A) Typical distribution of ^{32}P-labeled splicing intermediates and products through the gradient. RNA analysis was in a 10% polyacrylamide–8 M urea gel. (B and C) Labeled U6 snRNAs contained in each fraction were analyzed in an identical gel either before (B) or after (C) affinity selection on streptavidin-polyester beads (see Materials and Methods). The asterisk refers to the position of the major form of U6 snRNA.

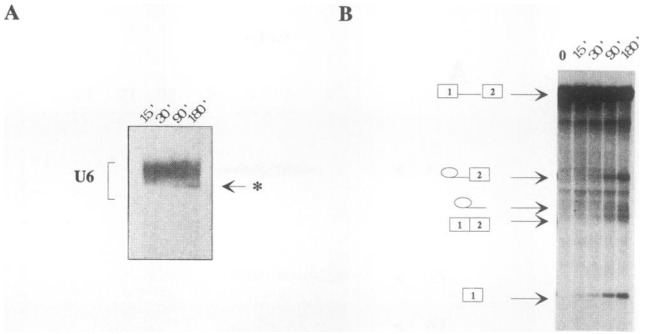

FIG. 6. Kinetics of U6 snRNA shortening. (A) Splicing reaction mixture containing a biotinylated pre-mRNA and [α-^{32}P]UTP was incubated at 30°C, and at the times indicated above the lanes, an aliquot was withdrawn and submitted to affinity selection on streptavidin-polyester beads. Selected RNAs were extracted and electrophoresed as described for Fig. 5. The asterisk refers to the position of the major form of U6 snRNA. (B) Splicing pattern of biotinylated ^{32}P-labeled pre-mRNA incubated under the same conditions as in panel A except that [α-^{32}P]UTP was omitted.

due to the synthesis of UTP in nuclear extracts in the presence of creatine phosphate. Of course, adding cold UTP to the complementation experiment presented above (Fig. 4C and D) did not change the U6 pattern obtained under splicing conditions (data not shown).

Elongated forms of U6 snRNA are assembled into the spliceosome. Sedimentation and affinity selection techniques were combined to determine whether elongated forms of U6 snRNA entered the spliceosome. An adenovirus pre-mRNA biotinylated at its 5′ and 3′ ends (see Materials and Methods) was incubated under splicing conditions in the presence of [α-^{32}P]UTP to label U6 snRNA. Upon fractionation of the reaction mixture on a 10 to 30% glycerol gradient and analysis of the distribution of labeled U6 snRNA, it appears that elongated forms are present in most if not all fractions, but a substantial amount sediments at the position of the spliceosomal peak (Fig. 5B). The position of the spliceosome has been determined by monitoring the distribution in a parallel gradient of intermediates and splicing products from a reaction mixture containing a ^{32}P-labeled and biotinylated pre-mRNA (Fig. 5A, lanes 9 to 12). It also shows that the splicing products are released from the spliceosome, in agreement with previous findings (22). To confirm that some labeled U6 molecules have effectively entered the spliceosome, all gradient fractions were submitted to affinity selection with streptavidin-polyester beads. After extensive washing, streptavidin-bound material was extracted and analyzed by electrophoresis in urea gels as described above (Fig. 5C). It is clear that only the forms of U6 snRNA present in fractions 8 to 13 are affinity selected, therefore demonstrating their presence in the spliceosome. Two control experiments were performed in parallel, one omitting pre-mRNA and the other one with nonbiotinylated pre-mRNA. No significant material was retained on the streptavidin-polyester beads in either of these two control experiments (not shown).

Elongated forms of U6 snRNA are shortened in the spliceosome to an RNA species having the same mobility as the major form of U6 snRNA and containing a 2′,3′ cyclic phosphate. Close inspection of the gel shown in Fig. 5C shows that among the affinity-selected species, one has the same mobility as the major form of U6 snRNA (the position of the major form of U6 snRNA was determined by Northern blot analysis of the same gel). Moreover, its presence in affinity-selected spliceosomes is observed only at times paralleling the appearance of splicing products (Fig. 6A and B). Further demonstration that this RNA form appears as a consequence of pre-mRNA splicing came from the observation that it is generated only when the reaction mixture contains a pre-mRNA. Indeed, splicing reaction mixtures incubated in the presence of [α-^{32}P]UTP either without the addition of pre-mRNA or containing the Δe1,Δi1 21 adenovirus pre-mRNA mutant from which spliceosomes are not assembled (15) led to the absence of the shortened form, while the elongated ones belonging to the U4/U6 and U4/U5/U6 complexes are normally present (Fig. 7, compare panels A and B with panel C).

Since the major form of U6 snRNA of many species has a 2′,3′ cyclic phosphate (>p) at its 3′ end, apparently derived from UMP residues that are added posttranscriptionally (29), we have investigated the possibility that the shortened form of U6 snRNA generated within the spliceosome could also have this same 3′ end modification. Therefore, the shortened U6 as well as another U6 snRNA among the elongated forms was eluted from the gel shown in Fig. 7C and digested with RNase P_1, and then the net charge of the digestion products was determined by DEAE thin-layer chromatography with a urea-NH_4 formate solvent system (Fig. 8). As expected from RNase P_1 digestion of a 3′ end-labeled U6 snRNA having a 3′-OH (or 3′-P) extremity, pU at the −2 position is generated from the elongated form (Fig. 8, lane 1). Two other products in addition to pU are generated from the shortened form (Fig. 8, lane 2), indicating that the latter has a 3′ end modification. One has a charge below −3, a net charge consistent with this spot being pU > p. The second one is at −4, which is the expected position for pUp. Since pUp carrying a 3′ monophosphate cannot be directly generated by nuclease P_1 digestion, we believe this pUp to have a 2′ monophosphate derived from the −3 product which would

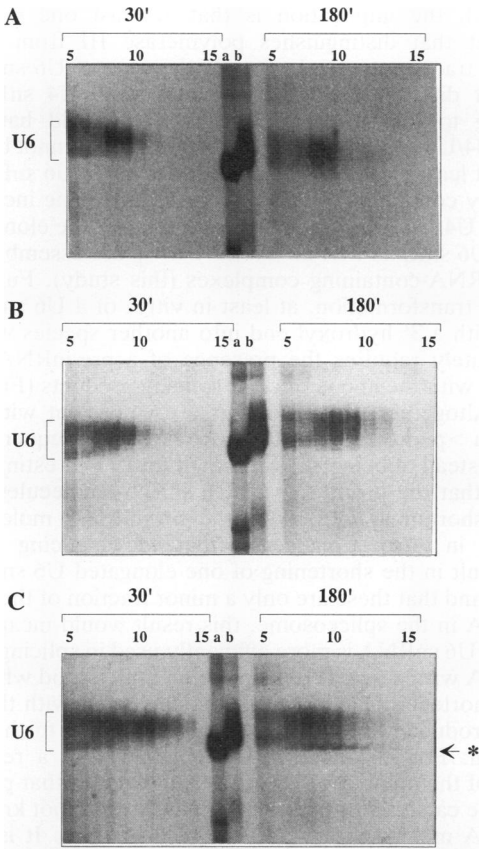

FIG. 7. Shortening of elongated U6 snRNA depends on spliceosome formation. Splicing reactions containing [α-^{32}P]UTP were incubated for 30 and 180 min either without pre-mRNA (A) or in the presence of either the pSP64Δe1Δi1.21 adenovirus mutant (B) or the pSP62Δi1 adenovirus pre-mRNA (C). All these reactions were centrifuged through 10 to 30% glycerol gradients, and then the fractions were extracted and analyzed for labeled U6 snRNAs. As in Fig. 5 and 6, the star refers to the position of the major form of U6 snRNA. Lanes a and b, control assays corresponding to labeled (a) and labeled and elongated (b) U6 snRNAs from nuclear extracts (see Fig. 1 and 2).

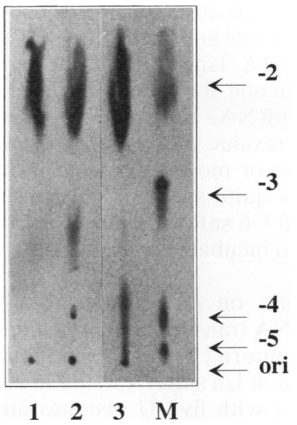

FIG. 8. The shortened U6 snRNA has a modified 3′ end. The shortened form of U6 snRNA seen in Fig. 7C as well as an elongated one were eluted from the gel and submitted to RNase P_1 digestion (lanes 2 and 1, respectively). Lane 3, RNase P_1 digestion products after the shortened form of U6 snRNA was submitted to an acidic treatment (see Materials and Methods). The net charge of the digestion products was determined by thin-layer chromatography on cellulose MN 300 DEAE plates. A smear instead of a spot is observed at the −4 position (lane 3). This is due to the presence of salt in the sample as a result of the acidic treatment. Lane M, markers obtained by RNase A digestion of a ^{32}P-labeled SP6 transcript.

be resistant to the 3′ phosphatase activity of nuclease P_1. To directly test this point, the shortened U6 snRNA was submitted to an acidic treatment, known to convert a 2′,3′ cyclic phosphate to a 2′ or 3′ phosphomonoester (6), before being digested by RNase P_1. As expected, the DEAE thin-layer chromatography now shows that the −3 spot has disappeared while the one at −4 is still represented by a small amount of resistant material (Fig. 8, lane 3). Thus, we conclude from these analyses that the spliceosomal U6 snRNA bears a >p at its 3′ end whose formation seems to be coupled to an event arising during splicing.

Fraction of elongated U6 snRNA that undergoes shortening during splicing. The labeled U6 snRNA fraction that undergoes shortening represents only a small fraction of the total labeled and elongated U6 snRNA (Fig. 7C). To quantitate this fraction, we compared the radioactivity in the shortened U6 snRNA with that in the total labeled U6 snRNA fraction of affinity-selected spliceosomes after 180 min of incubation. We found that the shortened U6 snRNA represents 1/10 of all labeled U6. In order to calculate the molar fraction of elongated U6 snRNA that undergoes shortening during splicing, it is necessary to know the total amount of elongated U6 snRNA present in the spliceosome. This was estimated from affinity-selected spliceosome by using both an ethidium bromide-stained gel, in which only the major form of U6 snRNA was seen, and Northern blot analysis, which gives the relative amount of the elongated form (data not shown). Densitometry scanning of Northern blot autoradiographs (corresponding to various exposure times) has revealed that the relative amount of the elongated form represents 1% of the total affinity-selected U6 snRNA. Under the splicing condition used in this study, we estimate that 1.3 pmol of U6 snRNA assembles into the spliceosome but only 1.3×10^{-3} pmol of elongated U6 snRNA has undergone a shortening process. Although this amount of shortened U6 snRNA is very low compared with the total amount of U6 snRNA in the spliceosome, it is only 10 times less than the calculated amount of pre-mRNA that is spliced under the same conditions.

DISCUSSION

Because of the presence of an enzyme specifically adding uridylic acid residues to the 3′ end of U6 snRNA in HeLa cell nuclear extracts, we have demonstrated that differentially elongated forms of U6 snRNA exist in all U6 snRNA-containing RNP complexes, namely, free U6, U4/U6, and U4/U5/U6 snRNPs. Quite importantly, these elongated forms enter the spliceosome, and some are subsequently shortened to a single species that has exactly the same characteristics as the major form of U6 snRNA present in mammalian nuclear extracts (29). Although the present data do not provide information about the mechanism by which U residues are removed from the spliceosomal U6 snRNA, we believe the discovery of such an event to be an important clue towards understanding how U6 snRNA functions in pre-mRNA splicing.

What determines U6 snRNA elongation and shortening?

Although most of the discrete RNA species present in nuclear extracts become substrates for TUTase activity once deproteinized (Fig. 1A, lane 2), their packaging into an RNP structure is detrimental for 3′ end modifications, except in the case of U6 snRNA. Furthermore, the latter receives more than one U residue only when present in RNP form, suggesting that one or more associated proteins render the elongation process quite specific. This is supported by the finding that an SP6 U6 snRNA transcript can be labeled but not elongated when incubated with partially purified TUTase (data not shown).

It is known that, on one hand, RNA polymerase III generates U6 snRNA transcripts lacking the right number of UMP residues of mature U6 snRNA and, on the other hand, that the major form of U6 snRNA found in HeLa cell nuclear extracts terminates with five U residues and a 2′,3′ cyclic phosphate (29). It is therefore highly likely that the posttranscriptional maturation of U6 snRNA is ensured by a two-step process including addition of U residues and then shortening resulting in the formation of a 2′,3′ cyclic phosphate. That a TUTase is involved in the first step of the process is obvious from this and other works (20, 33). As for the second step, two possibilities can be considered. One could be that a nuclease producing 3′-terminal phosphates and then a cyclase catalyzing conversion to the 2′,3′ cyclic phosphodiester are involved. Such enzymes exist in metazoan cells. It has been suggested that they are responsible for the formation of the 5′ splicing intermediates of intron-containing tRNAs (13). Another possibility is that cleavage and cyclization come from an event involving RNA catalysis, as in the case of the cleavage products of hammerhead ribozymes (7). We cannot decide at present which of the above mechanisms is involved. However, our results clearly demonstrate that the modification occurs in the presence of a pre-mRNA, suggesting that it could be related to the function of U6 snRNA in pre-mRNA splicing.

Is U6 snRNA shortening a consequence of pre-mRNA splicing? The problem is to understand what kind of 3′ end the spliceosomal U6 snRNA exhibits when the splicing reaction begins. Does it have five Us and a cyclic phosphate or a longer oligouridylic stretch containing a 3′ hydroxyl end? Previous work has shown the presence in affinity-selected spliceosomes of a U6 snRNA form whose 3′ end can be labeled in vitro with pCp (18), which argues that it has a 3′ hydroxyl. Evidence that this U6 snRNA is heterogenous in size and has more than five U's was obtained here. In our hands, these elongated forms appear to be labeled with [α-^{32}P]UTP only after incubation of the nuclear extract either with an excess of UTP or under splicing conditions, i.e., in the presence of ATP and creatine phosphate. If elongated U6 snRNA is required for splicing, we should expect UTP to be an essential cofactor for splicing. Preliminary results from our laboratory show that this could indeed be the case, since splicing can efficiently occur in the absence of creatine phosphate, provided that ATP-containing nuclear extracts were supplied with UTP (results not shown). Indeed, knowing that efficient in vitro splicing of mammalian pre-mRNAs requires the presence of creatine phosphate, it seems likely that UTP is generated from UDP in the presence of creatine phosphate.

If only elongated U6 snRNA with a 3′ hydroxyl end functions in splicing, what could be the reason for having the majority of U6 snRNAs with a 2′,3′ cyclic phosphate end? Recent studies indicate that the formation of >p on the fifth terminal UMP residue prevents La binding to U6 snRNA (41). Although the meaning of this in molecular terms is not understood, the implication is that at least one nuclear component that distinguishes polymerase III from polymerase II transcripts can be lost in the case of U6 snRNA and might direct the latter to interact with U4 snRNA. According to this view, the cyclization would have to precede U4/U6 and U4/U5/U6 formation. This cannot be the case for at least two reasons: first, newly made U6 snRNAs exclusively containing 3′ hydroxyl groups become incorporated into U4/U6 complexes (41), and second, the elongated forms of U6 snRNA that do not have a >p are assembled in all U6 snRNA-containing complexes (this study). Furthermore, the transformation, at least in vitro, of a U6 snRNA species with a 3′ hydroxyl end into another species with a >p absolutely requires the presence of a pre-mRNA and correlates with the appearance of splicing products (Fig. 6A and B). Altogether, these findings are consistent with the idea that a >p at the end of U6 snRNA is a consequence of splicing instead of being a requirement for it. Our estimation indicates that the proportion of U6 snRNA molecules that undergo shortening is 1/10 of the pre-mRNA molecules processed in vitro. Considering that each splicing event would result in the shortening of one elongated U6 snRNA molecule and that these are only a minor fraction of the total U6 snRNA in the spliceosome, this result would mean that elongated U6 snRNA is more efficiently used in splicing than U6 snRNA with a >p. It remains to be understood why the level of shortened U6 snRNA is low compared with that of splicing products. This would certainly deserve further investigation. For instance, we do not yet have a reliable estimate of the number of U6 snRNA molecules that participate in the catalysis of pre-mRNA, since we do not know if U6 snRNA molecules are used more than once. It is also possible that U6 snRNA shortening and splicing are not coupled events. In this case, it is difficult to understand why a pre-mRNA is required for U6 snRNA shortening to occur.

The presence of a cyclic phosphate at the end of U6 snRNA may not guarantee the formation of an active conformation of the spliceosome. It could, however, ensure that U6 snRNA is protected against exonucleolytic degradation and binding of La protein, thereby preventing its loss after each splicing cycle. It is therefore not surprising to find that the majority of U6 snRNA has a 3′ >p end. This raises the question of how the >p is removed from the U6 snRNA to become functional in splicing.

How does the string of U residues at the 3′ end of spliceosomal U6 snRNA contribute to pre-mRNA splicing? The ability of U6 snRNA to be elongated in all of the U6 snRNA-containing complexes upon incubation of nuclear extracts under splicing conditions makes it very likely that the string of U residues has something to do with pre-mRNA splicing. Although more work will be required to demonstrate this point, interesting hypotheses can be formulated. Keeping in mind that U4 and U6 snRNAs are separated as a consequence of the splicing reaction (8, 22, 26, 32), U6 snRNA elongation could be a signal for this. In support of such a scenario, preliminary results in our laboratory have indicated that anti-Sm antibodies fail to quantitatively immunoprecipitate U6 snRNA forms with a long poly(U) tail from glycerol gradient fractions containing only U4/U6 snRNP complexes. Another possibility could agree with the recent demonstration that U2 and U6 snRNAs become interactive during splicing (11, 43). For example, the poly(U) tail at the end of U6 snRNA may transiently displace a U2 snRNP-associated factor, thus favoring contacts between the two snRNAs. The obvious protein candidate is U2AF, an auxiliary protein known to mediate U2 snRNP interaction with

the polypyrimidine tract of mammalian pre-mRNAs (36). Such a scenario may also account for other interactions occurring within the spliceosome. Considering that several factors, including U2AF, are supposed to be involved in the recognition of the polypyrimidine tract 3' splice site (16, 17, 21, 36, 40), alternative binding of U2AF to the poly(U) tail of U6 snRNA may render this region of the pre-mRNA accessible to either one of these factors. In this respect, it will be of interest to determine whether U2AF can interact with elongated U6 snRNA.

The findings presented in this paper and the recent results reporting the existence of U6 snRNAs with modified 3' ends support the idea that the modifications occurring at the 3' end of U6 snRNA are relevant to its function in pre-mRNA splicing. We expect that the use of reconstituted U4/U6 snRNPs (42) from isolated U4 snRNP and SP6 transcripts of U6 snRNAs to complement U4/U6-depleted extract will be useful in settling this problem.

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