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U4 Small Nuclear RNA Dissociates from a Yeast Spliceosome and Does Not Participate in the Subsequent Splicing Reaction

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U4 and U6 small nuclear RNAs reside in a single ribonucleoprotein particle, and both are required for pre-mRNA splicing. The U4/U6 and U5 small nuclear ribonucleoproteins join U1 and U2 on the pre-mRNA during spliceosome assembly. Binding of U4 is then destabilized prior to or concomitant with the 5′ cleavage-ligation. In order to test the role of U4 RNA, we isolated a functional spliceosome by using extracts prepared from yeast cells carrying a temperature-sensitive allele of *prp2* (*rna2*). The isolated *prp2*Δ spliceosome contains U2, U5, U6, and possibly also U1 and can be activated to splice the bound pre-mRNA. U4 RNA does not associate with the isolated spliceosomes and is shown not to be involved in the subsequent cleavage-ligation reactions. These results are consistent with the hypothesis that the role of U4 in pre-mRNA splicing is to deliver U6 to the spliceosome.

Splicing of introns from nuclear pre-mRNAs occurs by two cleavage-ligation (transesterification) reactions. The first reaction is a cleavage at the 5′ splice site and the formation of a branched intron-exon 2 lariat molecule. The second reaction involves cleavage at the 3′ splice site and ligation of the exons. For splicing to occur, the sequence-conserved regions in the intron (the branch site and the 5′ and 3′ splice sites) must be recognized and brought in close proximity. The interaction between splice sites is achieved through an ordered assembly of four small nuclear ribonucleoproteins (snRNPs) (U1, U2, U5, and U4/U6) and protein factors on the pre-mRNA to form the spliceosome where splicing occurs (for reviews, see references 14, 21, 23, 28, 31, and 33).

U4 and U6 RNAs reside in a single ribonucleoprotein particle (8, 16), and both are required for splicing in mammals and in yeast cells (3, 5, 9, 32). During spliceosome assembly, U1 and U2 snRNPs bind to pre-mRNA at the early stages (27, 30). The presplicing complex is converted to the spliceosome after the entry of U4/U6 and U5 snRNPs (12, 17, 24). Interestingly, prior to or concomitant with the 5′ cleavage reaction, U4 RNA is no longer detected in the spliceosome when analyzed by native gel electrophoresis (12, 17, 24). However, it was suggested that U4 may not actually be released because a spliceosome containing spliced products can be affinity selected by using oligonucleotides complementary to U4 (6). It is possible that U4 is lost under the conditions of gel electrophoresis, which might indicate that the binding of U4 is destabilized during the assembly of the spliceosome. Nonetheless, it is still not clear whether U4 is involved only in the assembly of the spliceosome or whether it also has a catalytic function.

One way to determine whether U4 RNA is present during the catalytic phase of splicing is to isolate a spliceosome prior to transesterification and to test whether U4 is both present and required for the splicing reaction. We took advantage of a yeast temperature-sensitive mutant, *prp2*, which is defective in pre-mRNA splicing (22, 26). Heat-inactivated *prp2* mutant extracts cannot catalyze the cleavage-ligation reaction but will accumulate unspliced pre-mRNA in a spliceosome (19, 22). This *prp2*Δ spliceosome is functional, since it can be activated to splice if supplemented with splicing factors and ATP. At least two factors (PRP2 and bn) are required for the first cleavage-ligation. Additional factors are required for the second reaction (14, 19). The fact that a spliceosome can be activated implies that all *trans*-acting splicing factors involved in the transesterification reaction are present either in the spliceosome fraction or in the complementing fraction. The absence of a component (e.g., a small nuclear RNA [snRNA]) from both fractions indicates either that the component is not directly involved in the transesterification reaction or that its activity is required only for assembly of the spliceosome.

Upon analyzing the snRNA content and the functionality of the isolated *prp2*Δ spliceosome, we have found that U4 RNA is dissociated from the spliceosome prior to the first cleavage-ligation. Furthermore, the subsequent splicing reaction does not require U4, since it is absent in purified complementing fractions.

MATERIALS AND METHODS

Extract preparation and manipulations. Preparation of yeast splicing extracts was carried out as previously described (20, 22) from a wild-type strain, EJ101 (20), and two temperature-sensitive strains carrying the same *prp2-1* mutant allele, SS304 (22) and CRL2101 (α *prp2-1 ade2 his3 lys2-801 ura3* [25a]). Mutant extracts were heat inactivated essentially as described elsewhere (19). Routinely, 2 μl of 200 mM $MgCl_2$ and 2 μl of 2 M KPO_4, pH 7.3, were added to 196 μl of extracts and the mixture was incubated at 37°C for 30 min. The heat-inactivated extracts can be stored at −80°C without a significant loss of spliceosome formation activity. Micrococcal nuclease digestion of wild-type extracts was carried out essentially as described by Cheng and Abelson (11). Typically, 4 μl of micrococcal nuclease (Boehringer Mannheim; 22 U/μl) and 1 μl of 20 mM $CaCl_2$ were added to 15 μl of extract and the mixture was incubated at 30°C for 30 min. The reaction was stopped by adding 2 μl of 50 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), and the mixture was chilled on ice. The micrococcal nuclease-treated extracts were prepared fresh each time before use for complementation.

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Pre-mRNA substrate preparation. Pre-mRNA for the in vitro splicing reaction was prepared by in vitro transcription of the SP6-actin plasmid by using SP6 RNA polymerase (Promega or Ambion) (19). Water was used in place of [^{32}P]UTP when nonradioactive pre-mRNA was prepared. The SP6-actin plasmid, previously referred to as Proteus6-actin (20), has a single intron with the two flanking exons from the yeast actin gene inserted in an SP6 expression vector. The pre-mRNA in the SP6 transcription mixture was either purified through a Bio-Gel P-6 (Bio-Rad) column equilibrated with 40 mM KPO_4, pH 7.3, or used directly without purification (typically, 1 μl of transcription mixture was used in a 50-μl splicing reaction). In some cases, the DNA template in the transcription mixture was digested with RNase-free DNase (Ambion) at 0.2 U/μl of transcription reaction mixture for 10 min at 37°C, and RNA was purified by phenol extraction and ethanol precipitation (20).

Splicing reactions and spliceosome isolation. Typically, a 50-μl splicing reaction was carried out as described elsewhere (19) at 23°C for 30 min and the mixture was chilled on ice. After this, 150 μl of buffer GT (40 mM KPO_4, pH 7.3, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.05% Triton X-100) was added and gently mixed. The mixture was layered on a glycerol gradient made from 15 to 27% glycerol in buffer GT. Sedimentation was carried out in a Beckman SW55 rotor at 50,000 rpm for 2 h at 2°C, and 200-μl fractions were collected from the bottom of the centrifuge tube (19). The radioactivity of the gradient fractions was counted by Cerenkov radiation measurement in a Beckman LS 5000TD. The fractions were stored at −80°C.

Northern (RNA) blotting and hybridizations. RNA was isolated from gradient fractions (100 μl) by the addition of 200 μl of stop buffer (50 mM sodium acetate, pH 5.3, 1 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], 25 μg of glycogen [as a carrier] per ml, 10 μg of proteinase K [Boehringer Mannheim] per ml) and was incubated briefly at room temperature. The mixture was then extracted with 300 μl of phenol-chloroform and then ethanol precipitated (20). The pellet was resuspended and separated in a denaturing 5% polyacrylamide (29:1, acrylamide-bisacrylamide) gel and electroblotted onto a GeneScreen Plus membrane (NEN/DuPont) essentially as described previously (12). Before blotting, the gel was washed at room temperature in 25 mM $NaPO_4$ buffer (pH 6.5) containing 0.5% SDS for 5 min and was rinsed twice with the same buffer without SDS. Washing the gel with SDS-containing buffer and maintaining a temperature at 4°C during electrotransfer enhance the blotting efficiency and uniformity. In the case of separating RNA in formaldehyde-agarose gels (29), the RNA pellet was resuspended in water and adjusted to 2.2 M formaldehyde and 50% formamide in MOPS buffer (20 mM MOPS [3-(*N*-morpholino)propanesulfonic acid], 5 mM sodium acetate, 1 mM EDTA, pH 7.0) in a total volume of 10 μl. The RNA sample was heated at 65°C for 5 min and chilled on ice. Two microliters of a 30% glycerol solution containing tracking dyes was then added, and the mixture was applied to a 2% agarose gel in 2.2 M formaldehyde-MOPS buffer. After electrophoresis, the gel was washed in water for 30 min and was equilibrated in 20× SSC (i.e., 3 M sodium chloride plus 0.3 M sodium citrate). RNA was transferred onto a Biotrans nylon membrane (ICN) by using a PosiBlot pressure blotter (Stratagene) at 80 mm Hg (ca. 11 kPa) for 4 h. After electroblotting or pressure blotting, the membrane-bound RNA was UV irradiated and hybridized with ^{32}P-labeled plasmid DNA prepared by random primer extension as described by Cheng and Abelson (12). The U1, U2, U4, and U5 plasmids (12) and the U6 plasmid, pT7U6 (13), were obtained from J. Abelson's laboratory.

Spliceosome conversion assay. The gradient-isolated *prp2*Δ spliceosome was incubated in a 20-μl reaction mixture containing 70% (vol/vol) gradient fractions, 5% (vol/vol) splicing extracts or 10% (vol/vol) each of the two partially purified splicing factors, 2 mM ATP, and 5 mM additional $MgCl_2$ at 23°C for 30 min. The reaction was stopped by adding 280 μl of the stop buffer and was followed by phenol extraction and ethanol precipitation. ^{32}P-labeled RNA was separated in a denaturing 7.5% polyacrylamide gel (19). Nonradioactive RNA was separated in a 2% agarose gel containing formaldehyde, transferred onto a membrane, and hybridized by using a ^{32}P-labeled actin DNA probe as described above.

Quantitation of autoradiograms. Gels or membrane blots were exposed to Kodak XAR5 films at −80°C without an intensifying screen for different lengths of time. The films were developed and scanned with a Bio-Rad video densitometer model 620, and the data were analyzed according to the instructions of the manufacturer.

Purification of the PRP2 and bn proteins. Details of the purification procedure for the two splicing factors, PRP2 and bn, will be described elsewhere (16a). The PRP2 protein was purified to greater than 95% purity from a yeast strain (RL92/pJDB207-RN2) overproducing the protein (18, 19). Briefly, splicing extracts were subjected to DEAE-Sepharose and carboxymethyl-Sepharose chromatography, ammonium sulfate precipitation, and gel filtration chromatography. The bn factor was purified from wild-type splicing extracts. The extract was incubated in a 100°C water bath for 5 min (bn is heat stable); after centrifugation, the supernatant was subjected to DEAE, phosphocellulose, and hydroxyapatite chromatography. The purity of bn has not yet been established.

RESULTS

In order to test whether U4 RNA is involved in the catalytic reaction of pre-mRNA splicing, a spliceosome containing unspliced pre-mRNA was isolated from *prp2* mutant extracts by glycerol gradient sedimentation. We then analyzed the snRNA content, especially U4, in gradient fractions containing spliceosomes by Northern hybridization. Finally, we assayed whether a gradient-isolated spliceosome lacking U4 RNA could still splice when supplemented with protein factors free of U4.

The isolated *prp2*Δ spliceosome is lacking U4 snRNA. When ^{32}P-labeled pre-mRNA was incubated with heat-inactivated *prp2* mutant extracts, a fast-sedimenting complex containing unspliced pre-mRNA was identified in a glycerol gradient (Fig. 1). The gradient fractions 5, 6, and 7 contain most of the spliceosomes and will be referred to hereafter as the spliceosome fractions. The isolated *prp2*Δ spliceosome can be activated to splice the associated pre-mRNA when incubated with ATP and complementing extracts in a spliceosome conversion assay (19) (also see below). We reasoned that spliceosome-bound snRNAs should be found in these spliceosome fractions, and they would be absent in the corresponding gradient fractions if the spliceosome is not formed when pre-mRNA is omitted. Therefore, we carried out splicing reactions using heat-inactivated *prp2* mutant extracts, with or without the addition of pre-mRNA. The reaction mixtures were then sedimented through glycerol gradients in order to analyze the association of snRNAs with the spliceosome. The pre-mRNA used was not radioactively

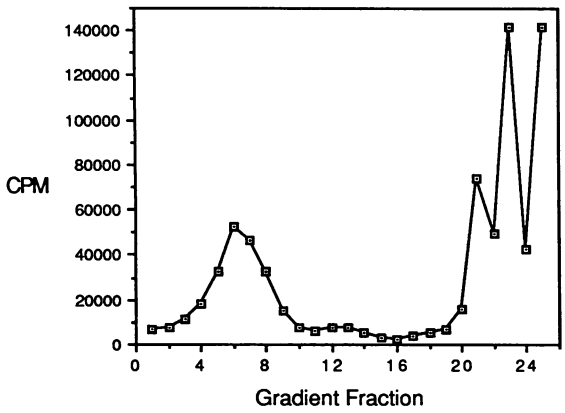

FIG. 1. Glycerol gradient sedimentation analysis of spliceosome formation in *prp2* mutant extracts. ^{32}P-labeled pre-mRNA was synthesized in vitro and purified through a Bio-Gel column. A 50-μl reaction was carried out by incubating the radioactive pre-mRNA in a heat-inactivated mutant SS304 extract under splicing conditions. After spliceosome formation, the reaction mixture was diluted and sedimented through a glycerol gradient. Fractions (200 μl each) were collected from the bottom of the gradient (starting with fraction 1), and radioactivity was determined by Cerenkov radiation measurement as counts per minute. Fraction 6 is the peak fraction of spliceosome-containing unspliced pre-mRNA.

labeled, so that the snRNA content in each gradient fraction could be analyzed by Northern hybridization. We separated the RNA isolated from individual gradient fractions by electrophoresis in a denaturing acrylamide gel. After immobilization, the RNA was hybridized sequentially with ^{32}P-labeled probes corresponding to U2, U1 plus U5, U4, and U6. The sequential hybridization is employed to establish the snRNA identity of individual hybridized bands. Figure 2 shows the autoradiogram after hybridization with all five probes.

In the splicing reaction containing pre-mRNA, fractions 5 and 7 were shown to contain U2, some U1, both $U5_L$ and $U5_S$, and U6 RNAs (Fig. 2A). However, when spliceosome assembly was prevented by the omission of pre-mRNA, these fractions contained low levels of U1 and U2 snRNPs. U4, U5, and U6 snRNPs were not detected (Fig. 2B). These results indicate that most of the U2, U5, and U6, and possibly some U1, present in fractions 5, 6, and 7 (Fig. 2A) is associated with the spliceosome. The snRNAs found near the top of the gradient (fractions 13 to 19) from either reaction may represent individual snRNPs or complexes containing more than one snRNP. Furthermore, it appears that virtually all of the U2 snRNPs in the extract sedimented to the spliceosome fractions (Fig. 2A). We have not done experiments to test whether all of the U2 snRNPs were assembled into spliceosomes in these fractions. Nor have we compared the levels of U2 with those of pre-mRNA.

U4 RNA was not detected in gradient fractions 1 to 11 from the splicing reaction containing pre-mRNA (Fig. 2A). All of the U4 RNAs appeared to sediment near the top of the gradient (fractions 13 to 19, Fig. 2A) and were not associated with the spliceosome. The intensities of the bands corresponding to snRNAs are comparable, indicating that all snRNAs on the membrane hybridized with the ^{32}P-labeled probes with almost equal efficiency (Fig. 2). Thus, significant amounts of U4 RNA that was present in the spliceosome fractions would have been detected. In fact, the level of U4 RNA is unlikely to be greater than 5% of the level of U5 in the spliceosome fractions (Fig. 2A). These results indicate that U4 RNA dissociates from the *prp2Δ* spliceosome under the conditions of glycerol gradient sedimentation, as well as during gel electrophoresis (12).

In order to demonstrate that the dissociation of U4 RNA from the *prp2Δ* spliceosome is not unique to the particular extracts used as well as to compare the molar amounts of U4 RNA and pre-mRNA, we repeated the Northern analysis using extracts prepared from another *prp2-1* strain, CRL2101. Splicing reactions containing ^{32}P-labeled or nonradioactive pre-mRNAs were carried out in CRL2101 extracts, and the reaction mixtures were analyzed by glycerol gradient sedimentation as described above. As shown in Fig. 3A, the *prp2Δ* spliceosome was found mainly in fractions 5, 6, and 7. Therefore, it appears that the sedimentation rate of the *prp2Δ* spliceosome in the gradient is the same regardless of the source of the mutant extracts (Fig. 1). In order to quantitate the amounts of U4 RNA and pre-mRNA by Northern hybridization, we isolated RNA from the gradient fractions containing nonradioactive pre-mRNA. The RNA was separated in a formaldehyde-agarose gel and transferred onto a nylon membrane by applying positive pressure to the gel. We found that more RNA was transferred onto the membrane by using agarose gels and pressure blotting than was transferred by using polyacrylamide gels and electroblotting (data not shown). After immobilization, the RNA on the membrane was hybridized with the U4 probe followed by the pre-mRNA probe. Both ^{32}P-labeled probes were prepared at the same time under identical conditions. As shown in Fig. 3B, U4 RNA was not detected in the spliceosome fractions. However, upon a much longer exposure of the same membrane, a small amount of U4 RNA could be detected (Fig. 3C). The molar amount of U4 RNA is estimated to be less than 0.5% of that of pre-mRNA in the spliceosome fractions (Fig. 3B and C). Although it is not yet possible to quantitate the percentage of the pre-mRNAs actually assembled into the spliceosomes in these fractions, these results do suggest that the vast majority of the spliceosomes are lacking U4 RNA. Therefore, the dissociation of U4 RNA from the *prp2Δ* spliceosome appears to be strain independent.

Splicing reaction in the isolated spliceosome does not require U4 snRNA. To confirm that spliceosomes lacking U4 are functional, the *prp2Δ* spliceosome containing ^{32}P-labeled pre-mRNA in fractions 5 and 7 (Fig. 3A) was incubated with complementing extracts under spliceosome conversion conditions. Three samples were used to complement the spliceosome: a wild-type extract, a micrococcal nuclease-digested extract presumably lacking snRNAs, and a mixture of partially purified fractions containing the PRP2 and bn activities. After incubation, the reaction products were analyzed by electrophoresis in a denaturing polyacrylamide gel followed by autoradiography (Fig. 4). The amounts of spliced products produced from spliceosome conversion reactions using wild-type extracts or micrococcal nuclease-digested extracts are similar, indicating that a spliceosome lacking U4 RNA is functional. These data also suggest that no RNA other than those in the spliceosome fractions is likely to be required for the actual catalytic reaction. In order to confirm that additional RNA is not required, we complemented the spliceosome with partially purified fractions containing PRP2 and bn. These partially purified factors converted the pre-mRNA in the spliceosome to splicing intermediates with a small amount of lariat intron (Fig. 4).

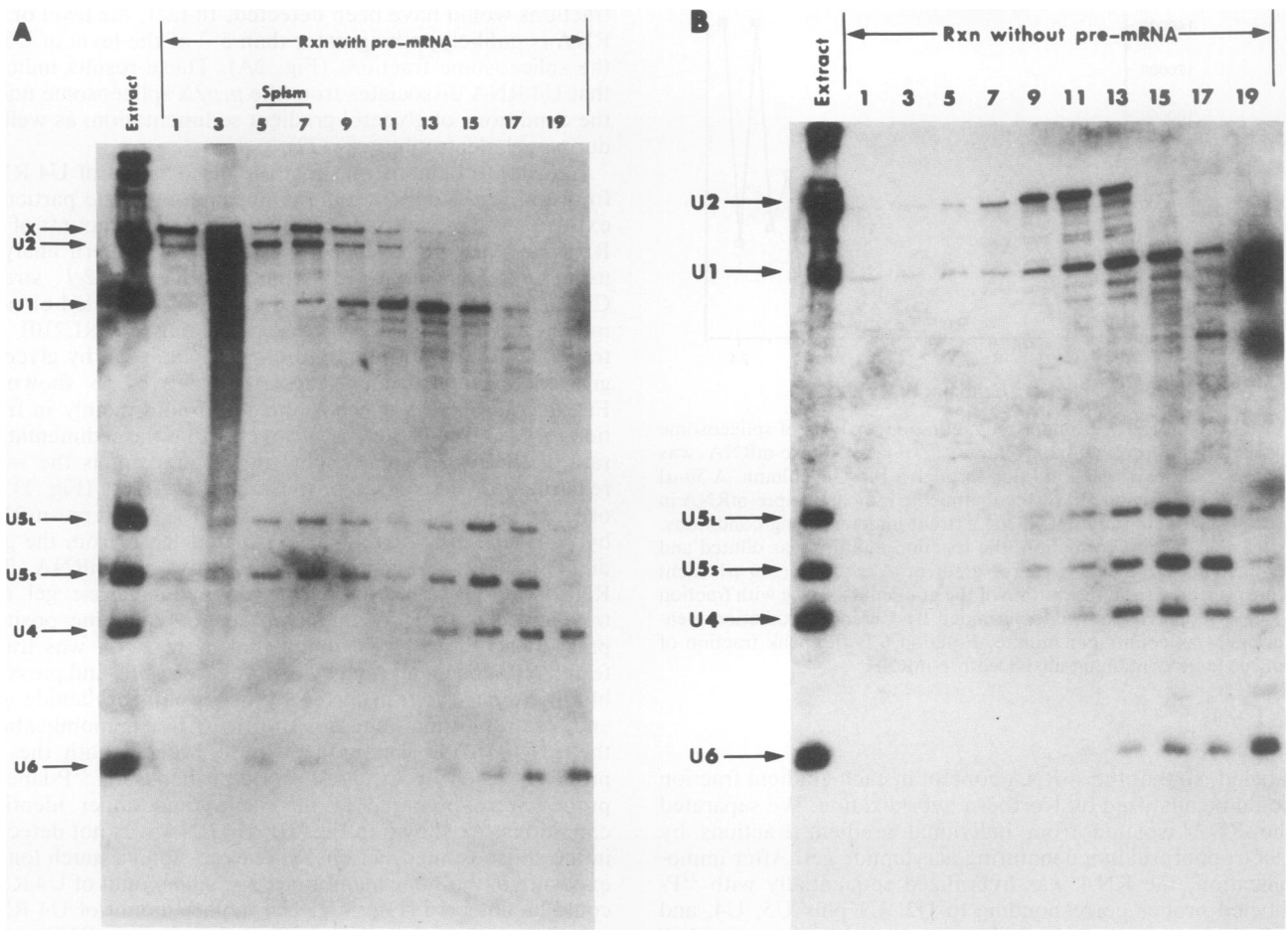

FIG. 2. Northern analysis of the snRNA content in gradient fractions from the splicing reaction with (A) or without (B) pre-mRNA. The splicing reaction and glycerol gradient sedimentation were identical to those described for Fig. 1 except that unpurified nonradioactive pre-mRNA or water was used in place of ^{32}P-labeled pre-mRNA. Total RNA from selected fractions was separated in a denaturing 5% acrylamide gel and then immobilized on a membrane. The RNA was probed with ^{32}P-labeled plasmid DNA corresponding to the sequence of U2 snRNA. After autoradiography, RNA on the membrane was rehybridized with a mixture of U1 and U5 probes, followed by the U4 probe, and then with U6. The autoradiograms shown are after hybridization with all five probes. Fraction numbers from each glycerol gradient are given at the top; snRNAs are identified at the left; band X was detected after probing the RNA from the "with pre-mRNA" gradient with U2 plasmid DNA. The nature of band X is not known; however, it was greatly diminished if the pre-mRNA was digested with DNase before being used in the splicing reaction (data not shown). For comparison, total RNA from a wild-type splicing extract is also shown. Splsm indicates the major gradient fractions containing the spliceosome.

This partial reaction is consistent with previous observations that extracts containing PRP2 and bn allow the 5′ cleavage-ligation to occur but may need additional factors to complete the reaction (19). We estimated that in all cases, 5 to 10% of the pre-mRNAs was converted to splicing intermediates or spliced products in fractions 5 and 7 (Fig. 4). The efficiency of spliceosome conversion indicates that at least 5% of the pre-mRNAs is in the functional spliceosomes in these gradient fractions. Similar results were obtained when the *prp2*Δ spliceosome containing nonradioactive pre-mRNA was subjected to the spliceosome conversion assay followed by Northern analysis (data not shown). The amount of the convertible pre-mRNAs (5 to 10%) is greater than 10 times the amount of pre-mRNAs which may associate with U4 (<0.5%) in the spliceosome fractions (cf. Fig. 3B and C and Fig. 4). By comparing the amounts of the convertible pre-mRNAs and the possibly U4-associated pre-mRNAs, we show that at least 90% of the isolated functional spliceosomes do not contain U4 RNA. Therefore, the very low level of U4 RNA present cannot be responsible for the splicing reaction observed in the spliceosome fractions.

In order to conclude that U4 RNA is not required for the catalytic activity of the spliceosome, it was necessary to show that there was no U4 RNA present in some of the complementing samples. Therefore, all of the samples used to complement the spliceosome in Fig. 4 were analyzed by Northern hybridization for their U4 RNA content (Fig. 3B and C). As expected, the full-length U4 RNA (160 nucleotides long) is present in the wild-type extract. After nuclease digestion, no full-length U4 RNA is seen, but a very small amount of a U4 fragment (about 40 nucleotides) is detected, suggesting that micrococcal nuclease degrades most but not all of the RNAs in the extract. It is not clear whether the remaining U4 fragment is active in the conversion reaction or which sequences of U4 RNA it contains. Nevertheless, conclusive results were obtained from the partially purified samples. No U4 RNA was detected in the purified fractions

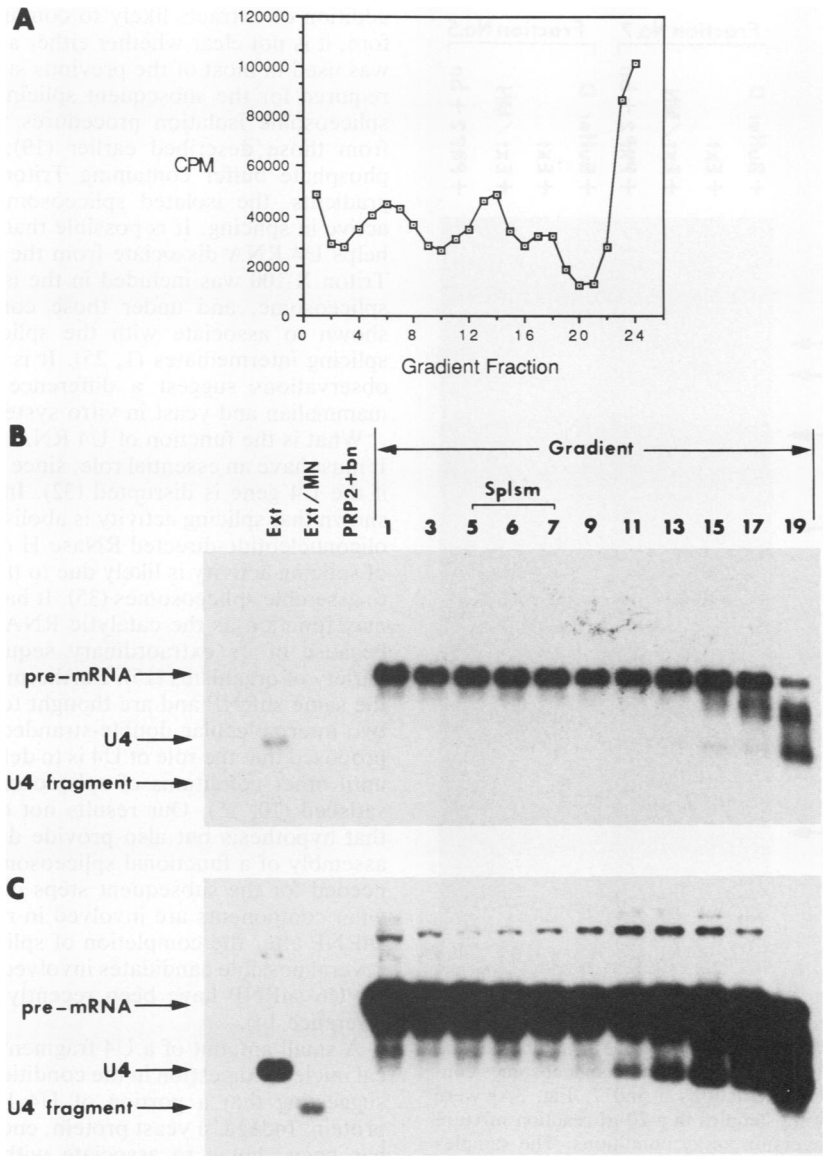

FIG. 3. Quantitation of U4 RNA by formaldehyde agarose gel and Northern hybridization. The transcription reaction mixture containing ^{32}P-labeled or nonradioactive pre-mRNA was digested with RNase-free DNase and used for splicing without further purification. The mutant extract used was prepared from CRL2101. The splicing reaction and glycerol gradient sedimentation were carried out as described for Fig. 1. (A) Gradient profile of the splicing reaction containing ^{32}P-labeled pre-mRNA. (B) Northern analysis of the amount of pre-mRNA and U4 RNA in the gradient fractions and various samples used in the spliceosome conversion assay. RNA was isolated from 100-μl gradient fractions (numbered at the top; Splsm indicates the spliceosome fractions) and samples used to complement the spliceosome (Ext, wild-type extracts [1 μl]; Ext/MN, micrococcal nuclease-treated extracts [1.5 μl]; PRP2+bn, purified factors [2 μl each]). After electrophoresis in a 2% agarose gel containing formaldehyde, RNA was transferred onto membrane and hybridized with the U4 probe followed by the actin DNA probe. The blot was exposed to X-ray film without an intensifying screen for 18 h. The hybridized RNAs are identified at the left. (C) Autoradiogram obtained by exposing the blot in panel B for 36 h with two Lightning Plus intensifying screens (DuPont).

containing PRP2 and bn even after a long exposure of the membrane (Fig. 3C). In fact, the amount of U4 RNA possibly present in the purified protein samples is much lower than that in the spliceosome fractions. It is not surprising that both samples are free of U4, for they were isolated through many purification steps. Since the *prp2*Δ spliceosome lacking U4 RNA can be activated at least to carry out the first transesterification by protein factors free of U4, we conclude that U4 RNA is not required for the splicing reaction phase. The essential role of U4 must lie, therefore, in spliceosome assembly.

DISCUSSION

We have utilized *prp2* mutant extracts and glycerol gradient sedimentation to isolate a spliceosome lacking U4 RNA. These spliceosomes can be complemented to carry out at least the first catalytic reaction in pre-mRNA splicing by the addition of extracts or partially purified factors that are demonstrably free of U4 RNA. Therefore, U4 RNA, while possibly loosely bound to the spliceosome, is not required for the catalytic reaction itself. This report directly tests the involvement of an snRNA in the catalytic activity of the

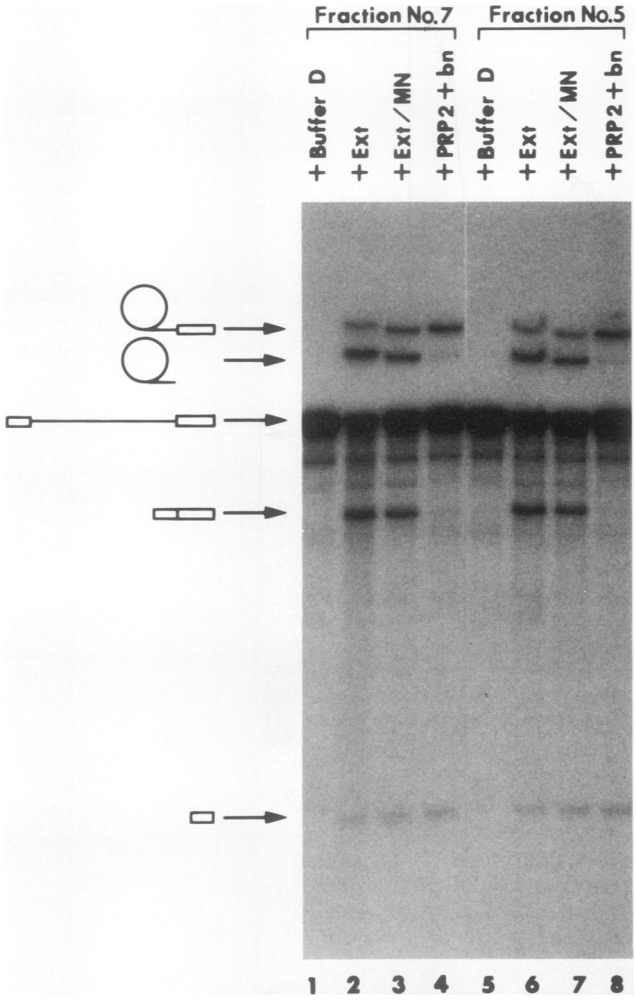

FIG. 4. Spliceosome conversion assay of the gradient-isolated spliceosomes lacking U4 RNA. Gradient-isolated spliceosomes containing ^{32}P-labeled pre-mRNA (fractions 5 and 7, Fig. 3A) were incubated with complementing samples in a 20-μl reaction mixture under the spliceosome conversion assay conditions. The samples used to complement were buffer D (the dialysis buffer used in the last step of extract preparation), a wild-type extract (Ext), a micrococcal nuclease-treated extract (Ext/MN), and the purified splicing factors (PRP2+bn). A schematic presentation of pre-mRNA, intermediates, and products is shown at the left. In descending order, these are intron-exon 2, lariat intron, pre-mRNA, mature mRNA, and exon 1. Lanes are also numbered at the bottom of the figure.

spliceosome. The tests result from the ability to isolate and analyze the spliceosome and to perform splicing reactions using isolated spliceosomes supplemented with purified factors. This combination should allow us to analyze further the function of spliceosomal components or complementing factors and to study the interactions between them.

The *prp2*Δ spliceosome was previously thought to be equivalent to a wild-type pre-mRNA-containing spliceosome, called the A1 complex (12). It has also been shown that U4 RNA was not found in the A1 complex as analyzed by gel electrophoresis and Northern hybridization. However, the snRNA content of the spliceosomes isolated from glycerol gradients was not analyzed. Furthermore, the functionality of the isolated *prp2*Δ spliceosome was tested by the addition of extracts likely to contain RNA (12, 19). Therefore, it is not clear whether either a spliceosome lacking U4 was used in most of the previous studies or U4 RNA is still required for the subsequent splicing reaction (12, 19). The spliceosome isolation procedures used here are modified from those described earlier (19); by using a potassium phosphate buffer containing Triton X-100 in the glycerol gradients, the isolated spliceosome is found to be more active in splicing. It is possible that the use of Triton X-100 helps U4 RNA dissociate from the spliceosome. However, Triton X-100 was included in the isolation of a mammalian spliceosome, and under those conditions, U4 RNA was shown to associate with the spliceosome containing the splicing intermediates (1, 25). It is not clear whether these observations suggest a difference of U4 binding in the mammalian and yeast in vitro systems.

What is the function of U4 RNA in the splicing pathway? It must have an essential role, since yeast cells are not viable if the U4 gene is disrupted (32). In vitro studies have also shown that splicing activity is abolished if U4 is degraded by oligonucleotide-directed RNase H digestion (3, 5). The loss of splicing activity is likely due to the inability of the extract to assemble spliceosomes (35). It has been proposed that U6 may function as the catalytic RNA in the splicing reaction because of its extraordinary sequence conservation in a variety of organisms (15). Furthermore, U4 and U6 reside in the same snRNP and are thought to be base paired through two intermolecular double-stranded regions (8, 16). It was proposed that the role of U4 is to deliver and to sequester U6 until other conditions of spliceosome assembly have been satisfied (10, 15). Our results not only are consistent with that hypothesis but also provide direct evidence that after assembly of a functional spliceosome U4 RNA is no longer needed for the subsequent steps of splicing. It is not clear what components are involved in reassembly of the U4/U6 snRNP after the completion of splicing reaction. However, several possible candidates involved in the reassembly of the U4/U6 snRNP have been recently suggested (reviewed in reference 14).

A small amount of a U4 fragment survived the micrococcal nuclease digestion in the conditions used (Fig. 3B and C), suggesting that a portion of U4 RNA may be bound by protein. Indeed, a yeast protein, encoded by the *PRP4* gene, has been shown to associate with the U4/U6 snRNP; in addition, heat inactivation of *prp4* mutant extracts (19) or anti-PRP4 antibodies (2, 4) inhibit pre-mRNA splicing and spliceosome assembly in vitro. Furthermore, the 5′ portion of U4 RNA is shown to be bound by the PRP4 protein and is resistant to nuclease digestion in vitro (34). Regions of U4 required for PRP4 protein binding and snRNP interactions have also been identified in vivo through construction of U4 with a deletion or a chimeric yeast-trypanosome U4 RNA (7). It will be interesting to know whether PRP4 protein dissociates together with U4 RNA from the spliceosome or remains bound after U4 has left.

It may be noteworthy that U1 RNA is underrepresented in the spliceosome. There is a relatively smaller amount of U1 RNA than U2, U5, or U6 RNA in the gradient fractions containing spliceosomes (Fig. 2A). Moreover, the amount of U1 RNA does not decrease in the corresponding fractions when pre-mRNA is omitted, whereas the amounts of U2, U4, U5, and U6 RNAs are greatly diminished or undetectable under the same conditions. In fact, sedimentation of U1 in glycerol gradients appears very similar with or without spliceosome formation (cf. Fig. 2A and B). It is possible that the small amount of U1 sedimenting in the spliceosome

region is not an integral part of the spliceosome. A loose interaction of U1 with the active spliceosome has been described previously, and it has been shown that U1 can be lost under stringent conditions (35). However, U1 has also been reported as an integral spliceosomal component (1, 25). We are currently isolating functional spliceosomes under conditions under which U1 may be dissociated and quantitating the amount of U1 RNA present. This would allow a direct test of the involvement of U1 snRNA in the splicing reaction that takes place in the spliceosome.

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