In vitro reconstitution of snRNPs: a reconstituted U4/U6 snRNP participates in splicing complex formation

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We have reconstituted in vitro the four snRNPs known to be involved in pre-mRNA splicing: U1, U2, U5, and U4/6. Reconstitution involves adding either authentic or in vitro-synthesized snRNAs to extracts enriched in snRNP structural polypeptides. The reconstituted snRNPs have the same buoyant density and are immunoprecipitated by the same antibodies as authentic snRNPs. Thus, the polypeptide composition of reconstituted snRNPs is similar, if not identical, to that of authentic snRNPs. We show further that a reconstituted U4/U6 particle is fully functional in forming splicing complexes with pre-mRNA. As is the case for the authentic U4/U6 snRNP, the reconstituted U4 snRNP, but not the U6 snRNA, dissociates from the complex prior to formation of the mature spliceosome. The ability to reconstitute snRNPs and assay their activity in spliceosome formation should provide a powerful approach to study these particles.

[**Key Words:** Reconstitution; snRNPs; Sm-binding site; spliceosome]

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Eukaryotic cell nuclei contain four major small nuclear ribonucleoproteins (snRNPs): U1, U2, U4/U6, and U5. Each of these particles contains one (or, for the U4/U6 snRNP, two) small nuclear RNAs (snRNAs) and a characteristic set of proteins [for review, see Luhrmann 1988]. The B, B', D, E, F, and G snRNP structural polypeptides are common to all four major snRNPs and associate with a sequence called the Sm-binding site. One or more of these polypeptides contains epitopes recognized by the Sm class of antibodies (anti-Sm antibodies). The U1, U2 (and, perhaps, U5; Gerke and Steitz 1986; Tazi et al. 1986; Lossky et al. 1987) snRNPs, contain specific proteins that are present only in one snRNP, in addition to these common structural polypeptides.

The U4 and U6 snRNAs are present predominantly in a single particle, the U4/U6 snRNP (Bringmann et al. 1984; Hashimoto and Steitz 1984; Rinke et al. 1985). U4 snRNA contains the Sm-binding site, and therefore associates directly with the common snRNP structural polypeptides. U6 snRNA lacks an Sm-binding site, and its interaction with proteins may be only indirect, resulting from association with U4 snRNP by intermolecular base pairing (referred to as U6 snRNA).

A variety of studies in HeLa cell and yeast extracts have demonstrated that all four snRNPs are involved in a series of complex interactions leading to the formation of a complex (the spliceosome) in which pre-mRNA splicing occurs. During spliceosome assembly, snRNPs interact with the pre-mRNA substrate (Mount et al. 1983; Black et al. 1985; Chabot et al. 1985; Bindereif and

Green 1986, 1987; Grabowski and Sharp 1986; Konarska and Sharp 1986, 1987; Pikielny and Rosbash 1986; Pikielny et al. 1986; Chabot and Steitz 1987; Chen and Abelson 1987; Krämer 1987; Rymond et al. 1987; Ruskin et al. 1988), with each other (Chen and Abelson 1987; Konarska and Sharp 1987; Lossky et al. 1987), and with other splicing factors (Gerke and Steitz 1986; Tazi et al. 1986; Krämer 1988; Ruskin et al. 1988). The U1 and U2 snRNPs bind to the 5' splice site and branch point, respectively. Following both of these pre-mRNA–snRNP interactions, a preexisting (U4/U6, U5) snRNP particle is assembled into the splicing complex (Chen and Abelson 1987; Konarska and Sharp 1987), primarily through interactions with bound U1 and U2 snRNPs (Bindereif and Green 1987). Several studies suggest that U5 snRNP may also contact the 3' splice site region (Chabot et al. 1985; Gerke and Steitz 1986; Tazi et al. 1986). Subsequently, the U4 snRNP, but not the U6 snRNA, dissociates from the complex (at least from complexes stable to gel electrophoresis) prior to the first covalent modification of the pre-mRNA (Pikielny et al. 1986; Chen and Abelson 1987; Lamond et al. 1988).

To understand the detailed mechanism of pre-mRNA splicing, one must determine how specific snRNA regions and snRNP structural polypeptides contribute to splicing complex assembly and catalysis. In yeast, one successful *in vivo* approach has been to mutate snRNA genes and determine the effect of these mutations on splicing (Parker et al. 1987; Seraphin et al. 1987; Siliciano and Guthrie 1988). In mammalian cells, although elegantly employed in one case (Zhuang and Weiner 1986), this approach probably is not generally applicable. The *in vitro* reconstitution of snRNPs described in this

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paper should facilitate the analysis of structure and function of snRNPs.

Results

In vitro reconstitution of U1, U2, U4/U6, and U5 snRNPs

Our strategy for reconstituting snRNPs is to incubate snRNAs (either authentic or synthesized *in vitro* with SP6 or T7 RNA polymerase) in extracts that are enriched in snRNP structural polypeptides under conditions used for pre-mRNA splicing. Three extracts were tested for their ability to support snRNP reconstitution: nuclear extract, a cytoplasmic S100, and a 'DE53 extract'. Nuclear extract is obtained by salt extraction of HeLa cell nuclei, and S100 is the high-speed supernatant of a cytoplasmic fraction (Dignam et al. 1983). The DE53 extract is obtained by a modification of the procedure used for the dissociation of a signal recognition particle (SRP), an RNP involved in the translocation of proteins across membranes (Walter and Blobel 1983). These researchers found that when SRP is treated with high concentra-

tions of EDTA, it adopts a partially unfolded conformation. During subsequent chromatography on DE53, the proteins flow through, whereas the RNA is retained. We have found that EDTA treatment of nuclear extract similarly yields an essentially RNA-free 0.2 M potassium acetate flowthrough fraction (containing <5% the level of snRNAs present in nuclear extract; data not shown), which is enriched in snRNP polypeptides.

An initial criterion for reconstitution was the formation of particles with the correct buoyant density. Previous studies have shown that authentic snRNPs remain intact structurally (Lelay-Taha et al. 1986) and functionally (Ruskin et al. 1988) in Mg^{2+} -containing CsCl density gradients. The buoyant density of each snRNP is characteristic and a function of the specific protein/RNA ratio of that particle (naked RNA has a density of 1.7 g/ml; most proteins have a density of 1.35 g/ml or less; and snRNPs have intermediate densities, ranging from 1.35 to 1.45 g/ml; see Lelay-Taha et al. 1986). The panel labeled 'Endogenous' in Figure 1 shows the RNAs extracted from each of 10 fractions collected across a CsCl gradient separation of a HeLa cell nuclear extract.

Figure 1. Density profiles of endogenous and reconstituted snRNPs. Reconstitution reactions were initiated by incubating a mixture of ^{32}P -labeled RNAs (U1, U2, U4, U5, U6 snRNAs, tRNA and 5S RNA) with various extracts for 20 min at 30°C and for 10 min at 37°C. Each reconstitution mixture was then fractionated on a 1-ml CsCl gradient, 0.1 ml fractions were collected from the top, and the RNAs were purified and fractionated on a 7% polyacrylamide–7 M urea gel. The gel, corresponding to the reconstitution with nuclear extract, was stained with EtBr and visualized on a UV transilluminator (*top left*). The amount of ^{32}P -labeled RNAs added to these reactions is negligible (~1 ng), so that all species detected by UV fluorescence are endogenous. The same gel (nuclear extract), as well as two others corresponding to reconstitutions carried out in S100 or DE53 extract, are analyzed by autoradiography (as indicated). Note that the top of each gradient is at the left (fraction 1).

U1 and U5 snRNPs have the lowest density: U1 and U5 snRNAs peak in fractions 1 and 2. U2 snRNP has a higher density: U2 snRNA is present in fractions 3–7, with a maximal concentration in fraction 4. Although U4 and U6 snRNAs are both at maximal concentration in fraction 6, as expected from their presence in a single U4/U6 particle, one can clearly detect some U4 snRNA in fractions 2–4, and some U6 snRNA in fractions 9–10. Thus, a fraction of each of these snRNAs is present as U4 snRNP and as U6 snRNA. Naked RNAs, such as 5S and tRNAs, are at the bottom of the gradient.

U1, U2, U4, U5, and U6 snRNAs, 5S RNA, and tRNA were purified individually from nuclear RNA, 3' end-labeled (England et al. 1980), reconstituted under standard conditions (see Materials and methods) in each of the three extracts, and fractionated on CsCl density gradients. An examination of the density profiles shows that in all three extracts a fraction of U1, U2, and U5 snRNAs is incorporated into particles of the same density as their endogenous counterparts. This result suggests that the *in vitro*-reconstituted snRNPs are structurally similar to authentic snRNPs.

The efficiency of reconstitution differs among the various snRNAs. In all three extracts, a sizable fraction (20–40%) of U1 snRNA is reconstituted into a U1 snRNP. Titration experiments show that 100–300 ng of U1 snRNA can be reconstituted in a 0.25-ml reaction (data not shown). The fraction of U2, U4, and U5 snRNAs reconstituted into snRNPs is smaller than that of U1 snRNA, and reconstitution is more efficient in S100 and in the DE53 extract than it is in nuclear extract. U6 snRNA is incorporated efficiently into a U4/U6 particle in S100 and nuclear extracts, which both have high levels of endogenous snRNPs.

A comparison of the results in the various extracts reveals qualitative as well as quantitative differences, particularly with regard to the U4/U6 snRNP. In S100, a large fraction of both U4 and U6 snRNAs is present in fractions 5–7, corresponding to the density of authentic U4/U6 snRNP. In the DE53 extract, however, the peak of U4 snRNA is near the top of the gradient, as expected for U4 snRNP. U6 snRNA is found exclusively at the bottom of the gradient, as expected for naked U6 snRNA. The results in nuclear extract are qualitatively similar to those obtained in S100 but reconstitution is more efficient for U6 snRNA and is less efficient for U4 snRNP.

These results can be explained by the variation in the concentrations of endogenous U4 snRNP and U6 snRNA in the three extracts. The DE53 extract is deficient in U6 snRNA; therefore, U4 snRNA is reconstituted only into a U4 snRNP. The concentration of added U4 and U6 snRNAs is relatively low, therefore, formation of U4/U6 snRNP is inefficient. In nuclear extract or S100, which contain high concentrations of endogenous snRNPs, U4/U6 particles are formed by interaction of added U6 snRNA and endogenous U4 snRNP or by interaction of reconstituted U4 snRNP and endogenous U6 snRNA. Data supporting this interpretation are presented below.

Immunoprecipitation of reconstituted snRNPs

As an independent confirmation of the fidelity of the reconstitution reaction, the polypeptide composition of the snRNPs was analyzed by immunoprecipitation with antibodies directed against specific snRNP structural polypeptides (see Luhrmann 1988). We used three different antibodies: anti-Sm antibodies, which recognize proteins common to all snRNPs (see Luhrmann 1988), anti-(U1,U2) RNP antibodies, which recognize the U1-specific A and the U2-specific A' polypeptides (Habets et al. 1985), and a monoclonal antibody directed against the U1-specific 70-kD protein (Billings et al. 1982). A reconstitution reaction was carried out in the DE53 extract with end-labeled U1, U2, U4, and U5, and the products fractionated on a CsCl density gradient. The appropriate gradient fractions (3–6) were then pooled, dialyzed, and immunoprecipitated with various antibodies. snRNAs were isolated from the immunoprecipitates and detected on a denaturing gel (Fig. 2). Anti-Sm antibodies immunoprecipitate particles containing U1, U2, U4, and U5 snRNAs; the anti-70-kD antibody immunoprecipitates U1 snRNA exclusively, and the anti-(U1, U2) antibody immunoprecipitates both U1 and U2 snRNAs. Thus, each antibody recognizes the expected subset of reconstituted snRNPs, demonstrating that the unique snRNP polypeptides interact only with the appropriate snRNAs and reinforcing the notion that reconstituted particles are structurally similar to authentic snRNPs.

Reconstitution of a U4/U6 snRNP

As mentioned above, the results obtained with U4/U6 snRNP reconstitutions vary in the different extracts.

Figure 2. Immunoprecipitation of reconstituted snRNPs. A reconstitution reaction was carried out in a DE53 extract and a mixture of 3' ³²P-end-labeled U1, U2, U4, U5 snRNAs, tRNA, and 5S RNA and fractionated on a CsCl density gradient. The appropriate gradient fractions were then pooled and dialyzed, and equal aliquots were either deproteinized and directly loaded on a gel (total) or first immunoprecipitated with the indicated antibodies. (N.I.) Nonimmune human serum. The RNAs were then analyzed by denaturing gel electrophoresis followed by autoradiography. Immunoprecipitations in this experiment were not quantitative, the recovery of U1, e.g., varies with the antibody and is less <30%, probably because the antibodies were not in excess.

These differences can be explained by variations in the concentration of endogenous snRNPs. If endogenous snRNPs are present, as in S100 and in nuclear extract, reconstituted U4 snRNP will form a complex with endogenous U6 snRNA, and added U6 snRNA will form a complex with endogenous U4 snRNP. This would be consistent with the observation that in nuclear extract as well as in S100, U4 and U6 snRNAs are incorporated into a particle of the same density (that of a U4/U6 snRNP) following addition of only one of these snRNAs (data not shown). Also consistent with this explanation is the fact that when U6 snRNA, which lacks an Sm-binding site, is added to a reconstitution reaction in nuclear extract or S100, it becomes immunoprecipitable with anti-Sm antibodies (Fig. 3).

To demonstrate that the immunoprecipitability of U6 snRNA with anti-Sm antibody is due to its association with endogenous U4 snRNP, we tested the effect of targeted cleavage of endogenous U4 snRNA (Berget and Robberson 1986; Black et al. 1986). Extracts that had been previously subjected to oligonucleotide-directed RNase H cleavage of U4 snRNA were used for reconstitutions in the presence of an in vitro-synthesized U6 snRNA (SP6-U6; see Materials and methods). Only cleavage with U4-specific oligonucleotides results in the loss of immunoprecipitability with the anti-Sm antibody (Fig. 3).

In contrast to nuclear extract and S100, DE53 extract does not support formation of a U4/U6 snRNP. Rather, the U4 snRNA is reconstituted into a particle whose density is the same as that of U4 snRNP. If this is due to the low concentration of snRNPs, both endogenous and reconstituted, and not to the lack of some essential ac-

Figure 3. Exogenous U6 snRNA associates with endogenous U4 snRNP. Reconstitution reactions were carried out in the presence of ^{32}P -labeled SP6-U6 snRNA and nuclear extract or extracts that had been treated with various oligonucleotides, as indicated above each lane (see below). Each reaction was then immunoprecipitated with either anti-Sm or anti-(U1) RNP antibody, as indicated, and the resulting snRNAs analyzed on a denaturing polyacrylamide gel. Oligonucleotide-treated extracts were prepared by incubating 45 μl nuclear extract, 3 μl 12.5 mM ATP, 3 μl 80 mM MgCl_2 , 3 μl 0.5 M creatine phosphate, 3 μl RNasin at 8 U/ μl , and 3 μl oligonucleotide (1 mg/ml) for 60 min at 30°C. SP6-U6 snRNA and KCl (to a final concentration of 0.5 M KCl) were then added and a reconstitution reaction was allowed to proceed for 40 min at 42°C. The oligonucleotides used in this experiment have been described previously (Black and Steitz 1986).

tivity, it should be possible to increase the concentration of reconstituted particles to a level sufficient to support U4/U6 snRNP formation. In the experiment shown in Figure 4, in vitro-synthesized U4 and U6 snRNAs (SP6-U4 and SP6-U6 are both GpppG-capped RNAs; see Materials and methods) were added to a reconstitution reaction in DE53 extract at significantly higher levels (at least 10- to 50-fold) than U4 and U6 snRNAs in the experiment shown in Figure 1. Each of these three reactions was analyzed on a density gradient (Fig. 4A). When only SP6-U4 snRNA is added, it is reconstituted into a particle of low density. When only SP6-U6 snRNA is added, it remains mostly as naked RNA at the bottom of the gradient. However, when both snRNAs are added, a fraction of each sediments in fractions 5 and 6, which correspond to the density of a U4/U6 snRNP (note that this gradient is slightly different from the one shown in Fig. 1, and under these

Figure 4. Complete reconstitution of a U4/U6 snRNP. (A) Three reconstitution reactions were initiated in a DE53 extract with 30 ng ^{32}P -labeled SP6-U4, ^{32}P -labeled SP6-U6, or both RNAs and fractionated on 1-ml CsCl gradients. Fractions of 0.1 ml were collected from the top and deproteinized, and the snRNAs were resolved on a 7% polyacrylamide-7 M urea gel and detected by autoradiography. (B) The fractions indicated from the middle gradient were dialyzed against buffer D, 15 mM MgCl_2 , 1 mM DTT, immunoprecipitated with the antibodies indicated, and the snRNAs were purified and analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. (N.I.) Nonimmune human serum. The total lanes correspond to equal fractions that were not immunoprecipitated prior to deproteinization.

conditions endogenous U4/U6 is in fractions 5 and 6; data not shown). Next, immunoprecipitations were carried out on various gradient fractions. SP6-U4 snRNA is present in immunoprecipitates of fractions 2,3 and 5,6, corresponding to U4 and U4/U6 snRNPs, respectively, whereas SP6-U6 snRNA is only present in the immunoprecipitates of fractions 5,6, corresponding to the U4/U6 particle (Fig. 4B). Thus, in a DE53 extract, high concentrations of U6 snRNA and reconstituted U4 snRNP can lead to U4/U6 snRNP formation.

Reconstituted U4/U6 snRNPs are assembled into splicing complexes

The experiments described above demonstrate that reconstituted snRNPs are physically similar to authentic snRNPs. However, these experiments do not indicate whether the reconstituted snRNPs are functional. To test the function of these reconstituted particles, we examined their ability to assemble into stable splicing complexes upon addition of pre-mRNA. Such complexes can be resolved on nondenaturing gels (Konarska and Sharp 1986; Pikielny and Rosbash 1986; Pikielny et al. 1986; Chen and Abelson 1987; Konarska and Sharp 1987). Using the conditions described by Konarska and Sharp (1986), two complexes can be detected with 32 P-labeled adenovirus (Ad) pre-mRNA: The first complex to appear contains only U2 snRNP; the second complex contains U2, U4/U6, and U5 snRNPs. The formation of the U2 snRNP-containing complex requires only the branch point/3'-splice-site region, whereas formation of the other complex requires, a 5' splice site in addition (Konarska and Sharp 1986).

SP6-U4 snRNA labeled with 32 P or SP6-U6 snRNA labeled with 32 P was reconstituted into a U4/U6 snRNP. The U4/U6 snRNP was then fractionated on a CsCl gradient and added to a splicing reaction in nuclear extract. Upon addition of an unlabeled Ad pre-mRNA, the complexes were separated electrophoretically on a native gel (Fig. 5A,B). With both 32 P-labeled U4 snRNA and 32 P-labeled U6 snRNA, we observe the formation of a complex with a mobility identical to that of the (U2, U4/U6, U5) splicing complex. The 32 P-labeled reconstituted snRNP is diluted by the large amount of unlabeled U4/U6 snRNP in the nuclear extract. Thus, as expected only a small fraction of 32 P-labeled U4/U6 snRNP is incorporated into this pre-mRNA-containing complex, and most of the radioactivity is present in fast migrating complexes corresponding to free snRNPs. Formation of this pre-mRNA-containing complex requires ATP and does not occur with an RNA that lacks a 5' splice site (Fig. 5A,B; the large complexes observed in Fig. 5B are nonspecific aggregates, as they are not dependent on pre-mRNA and disappear following ATP addition).

The final step in formation of an active spliceosome is the dissociation of U4 snRNP (Pikielny et al. 1986; Chen and Abelson 1987; Lamond et al. 1988). For the Ad pre-mRNA substrate, this complex comigrates with the (U2, U4/U6, U5) complex in the standard gel systems (Konarska and Sharp 1986, 1987). A more recently devised gel

system in which a high concentration of EDTA is included allows for the separation of those two complexes (Zillman et al. 1988). Figure 5C shows a time course of complex formation using 32 P-labeled Ad pre-mRNA or a reconstituted U4/U6 snRNP (containing 32 P-labeled SP6-U4 snRNA or 32 P-labeled SP6-U6 snRNA) and unlabeled pre-mRNA. As expected, the pre-mRNA is present in three complexes: (U2), (U2, U4/U6, U5), and (U2, U6, U5), whereas SP6-U4 snRNP can be found only in the (U2, U4/U6, U5) complex and SP6-U6 is present in the latter two complexes. The reactions shown in Figure 5C were performed in the presence of only small amounts of pre-mRNA. Under these conditions, the complex containing the 32 P-labeled U4 snRNP disappears at late times in the reaction, consistent with it being an intermediate in spliceosome formation. When more pre-mRNA is added, a larger fraction of reconstituted U4 snRNP comigrates with the (U2, U4/U6, U5) complex. However, we do not observe disappearance of this complex presumably because it is produced continually (data not shown) under these conditions. We have never observed incorporation of reconstituted U4 snRNP in a complex comigrating with the (U2, U6, U5) complex. We conclude that the reconstituted U4/U6 snRNP forms the same pre-mRNA-containing complexes as does endogenous U4/U6 snRNP, and particularly that reconstituted U4 snRNP leaves the complex prior to formation of the active spliceosome.

Discussion

We have developed procedures to reconstitute *in vitro* the four snRNPs involved in pre-mRNA splicing. The source of snRNP polypeptides can be (1) a nuclear extract, (2) a cytoplasmic extract, or (3) a DE53 extract. The properties of each of these extracts in a reconstitution reaction is a function of their content of intact snRNPs and free snRNP structural polypeptides. snRNPs are assembled in the cytoplasm, which is enriched in free snRNP polypeptides (for review, see Mattaj 1988). Therefore, it is not surprising that a cytoplasmic extract supports higher levels of reconstitution than a nuclear extract. Cytoplasmic and nuclear extracts contain roughly equivalent concentrations of intact snRNPs, probably resulting from nuclear leakage during subcellular fractionation (Hernandez and Keller 1983; Krainer and Maniatis 1985; Ruskin et al. 1988). The DE53 and S100 extracts allow equally efficient reconstitution, the former offering the additional advantage of being largely devoid of endogenous snRNPs. The conditions used for pre-mRNA splicing are compatible with snRNP reconstitution. $MgCl_2$ is required, and although not absolutely necessary, we include ATP because it disrupts aggregates (data not shown).

Recently, two groups have reported the *in vitro* reconstitution of U1 snRNP using *Xenopus laevis* egg extracts (Hamm et al. 1987, 1988) or HeLa cell S100 (Patton et al. 1987; Patton and Pederson 1988). They have demonstrated that the reconstituted U1 snRNP contains most, if not all, of the polypeptides present in the authentic U1

snRNP. It is shown here that not only U1 snRNP, but also the other three snRNPs involved in splicing, can be reconstituted into particles that appear to be structurally identical to their endogenous counterparts.

A final test of the fidelity of reconstitution must be

that the snRNP obtained is functional. Here, it is demonstrated that U4 and U6 snRNAs can be reconstituted into a U4/U6 snRNP similar to the particle present in a nuclear extract. Such a reconstituted U4/U6 snRNP is incorporated into the same splicing complexes as its en-

ogenous counterpart. The reconstituted U4 snRNP, but not the U6 snRNA, is then released from this complex to form a mature spliceosome.

Similar experiments with reconstituted U2 and U5 snRNPs have been unsuccessful so far (C.W. Pikielny et al., unpubl.), suggesting that the reconstitution of these snRNPs is, in some way, incomplete. U1 snRNP is not a viable candidate for this approach because of the difficulty in detecting U1 snRNA in splicing complexes fractionated by gel electrophoresis (Konarska and Sharp 1986, 1987; Pikielny et al. 1986; Chen and Abelson 1987), presumably because U1 snRNP is dissociated by the conditions used (Bindereif and Green 1987).

There are several possible explanations for the apparent lack of function of reconstituted U2 and U5 snRNPs. These snRNPs may lack an essential but as yet unknown polypeptide, or their conformation could be aberrant, as has been shown to occur with reconstituted ribosomal subunits (Traub and Nomura 1969). Alternatively, the short extensions present at the 5' and 3' ends of the synthetic snRNAs and the lack of post-transcriptional modifications could render these reconstituted snRNPs inactive. In the case of U4/U6 snRNP, the evidence presented here suggests that synthetic snRNA derivatives can be reconstituted into a functional form. It will be of interest to test whether some modifications occur in the extract.

We have not yet been able to show that reconstituted snRNPs are able to support splicing in complementation experiments with extracts depleted of U4/U6 snRNPs by RNase H-mediated degradation. We believe this is due to the technical difficulties of these experiments and the inability to obtain sufficient amounts of reconstituted snRNPs in a purified and highly concentrated form. It is conceivable (but in our opinion unlikely), that the final U6 snRNA-containing complex is inactive even though its electrophoretic mobility on native gels is identical to that of the active spliceosome. Because the reconstituted U4 snRNP is released from the complex, it seems highly likely that it has performed all of its required functions in splicing. In addition to the pre-mRNA-containing complexes, preliminary evidence suggests that the reconstituted U4/U6 snRNP interacts with U5 snRNP to form a heparin-sensitive U4/U6/U5 complex (data not shown), which is a likely intermediate in the formation of the mature spliceosome (Konarska and Sharp 1987). The reconstitution and assay methods described here should allow us to analyze the role of specific U4 and U6 snRNA sequences in formation of complexes involved in splicing.

Materials and methods

Reconstitution extracts

Nuclear extract and S100 were prepared according to Dignam et al. (1983). DE53 extract was adapted from Walter and Blobel (1983), as follows. Before use, the DE53 resin was washed for 5 min in 4 M potassium acetate (pH 7.5), rinsed repeatedly with distilled water and finally, equilibrated in 0.2 M potassium acetate (pH 7.5), 10 mM EDTA, 20 mM HEPES (pH 7.0), 10% glyc-

erol, and 1 mM DTT (DE53 buffer). EDTA (0.5 M) and 4 M potassium acetate (pH 7.5) were mixed with 1.6 ml of nuclear extract in Buffer D (Dignam et al. 1983) to final concentrations (in 2 ml total volume) of 80 mM KCl, 120 mM potassium acetate, 10 mM EDTA, 16 mM HEPES (pH 7.0), and 16% glycerol. This was then added to 1 ml of packed DE53 resin and mixed gently. Then, the slurry was incubated for 10 min on ice and for 10 min at 37°C with occasional mixing. It was then loaded in a 15-ml Bio-Rad disposable polypropylene column. The flowthrough fraction was recovered and the resin was washed with an additional volume of DE53 buffer. Both fractions were pooled and dialyzed against buffer D. The level of residual snRNA in the extract was measured by 3' end labeling of extracted RNA and was found to be undetectable (<5% of the levels of U1 snRNA present in a nuclear extract).

RNAs

Endogenous snRNAs were purified by cutting the appropriate bands from a gel after fractionating nuclear RNA. The RNAs were eluted by incubation overnight in 2 × PK buffer [0.2 M Tris (pH 7.5), 25 mM EDTA, 0.3 M NaCl, 2% SDS] at 37°C followed by phenol extraction and ethanol precipitation. The 3' end labeling was done according to England et al. (1980). Synthetic versions of U4 and U6 snRNA derivatives were obtained by *in vitro* transcription (Melton et al. 1984) of SP6-U4 and SP6-U6, two plasmids containing an SP6 promoter upstream of the human U4 snRNA (Bark et al. 1986) and U6 snRNA (Kunkel et al. 1986) genes. All transcription reactions were carried out in the presence of GpppG, generating 5'-capped RNAs (Konarska and Sharp 1984). The sequence of the synthetic snRNAs deduced from the sequence of the DNA is SP6-U4: GpppGA/AGCU—U4-coding sequences—ACUG/AAUUUUU (*Dra*I runoff), SP6-U6 GpppGAA/GUG—U6-coding sequences—AUUUU/G (runoff at a *Bam*HI site obtained by *Bal*31 deletion of the 3'-flanking sequences; note that for this RNA, the final U is replaced by a G).

Reconstitution reactions, density gradients, immunoprecipitations, and RNP gels

The appropriate RNA was incubated in 60% extract (in buffer D, Dignam et al. 1983), 3.2 mM MgCl₂, 2 mM ATP, 20 mM creatine phosphate, and 1.6 U/ml RNasin. In addition and except in the case of U6 snRNA (see below), tRNA was added to a final concentration of 400 µg/ml. The mixture was then incubated for 20 min at 30°C and for 10 min at 37°C. In the case of reconstitutions with U6 snRNA, we found that the best conditions were incubation in a nuclear extract for 40 min at 42°C in the same buffer as above except for the addition of KCl to a final concentration of 0.5 M and the omission of tRNA. The amount of RNA used varied depending on the species, but we find that reconstitutions with DE 53 and S100 saturated at ~500 ng of RNA/ml. For density gradient fractionation, DTT and MgCl₂ were added to final concentrations of 1 mM and 15 mM, respectively. This reaction was then mixed in a ratio of 3 : 5 with buffer D with 15 mM MgCl₂, 1 mM DTT, and CsCl (density, 1.7), such that the final density was ~1.4. This reaction mixture was centrifuged in polycarbonate tubes for at least 10 hr at 90,000 rpm at 4°C in a Beckman TL-100 tabletop ultracentrifuge in TLA-100 (0.2-ml) or TLA-100-2 (1-ml) rotors (see figure legends). Fractions of one-tenth the total volume were collected with a pipetman from the top of the tube. Dialysis was performed on 40-µl volumes in a DiaCell 10PS microdialyzer for 2 hr at 4°C against buffer D. Immunoprecipitations were done according to Bindereif and Green (1986). RNP gels were performed

according to Ruskin et al. (1988) or Zillmann et al. (1988), as specified in the figure legends.

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