In vitro assembly of yeast U6 snRNP: a functional assay

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U6 small nuclear RNA (snRNA) is the most highly conserved spliceosomal RNA, and it has been postulated to have a fundamental role in pre-mRNA splicing. To elucidate this role, we developed an in vitro system for reconstituting the functional U6 small ribonucleoprotein (snRNP). Treating splicing extracts with an oligonucleotide complementary to the central domain of U6 snRNA leads to both RNase H cleavage of the endogenous U6 snRNA and loss of splicing activity. Yeast U6 RNA, synthesized in vitro using T7 RNA polymerase, is then added to the oligonucleotide-treated extract, and restoration of splicing activity is monitored by the subsequent addition of substrate pre-mRNA. Addition of full-length, unmodified T7U6 snRNA (113 nucleotides) to oligonucleotide-treated extracts restores splicing activity efficiently. Using U6 RNA transcripts truncated at their 3' ends, we show that large deletions (39 nucleotides) produce molecules that are unable to restore splicing activity in vitro and cannot interact with the endogenous U4 snRNA or form a mature spliceosome. Finally, we show that substitution of the invariant G81 with C within the T7U6 RNA abolishes its ability of restoring splicing activity. Although the U4/U6 snRNP forms correctly, mature spliceosomes do not assemble.

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Splicing of nuclear mRNA and group II mitochondrial RNAs follows a common two-step mechanism of cleavage–ligation reaction (for review, see Green 1986; Padgett et al. 1986). Because splicing of group II mitochondrial RNAs can proceed by itself in the absence of any cofactor, catalysis must be achieved by the formation of active sites comprised solely of RNA (Peebles et al. 1986; Van der Veen et al. 1986). In contrast, splicing of nuclear mRNA requires numerous RNAs, proteins, and complex protein–RNA interactions. At least five small nuclear RNAs (snRNAs), U1, U2, U5, U4, and U6, are required for nuclear mRNA splicing in eukaryotes. These RNAs are associated with protein and function in the form of ribonucleoprotein complexes (snRNPs) (Krämer et al. 1984; Black et al. 1985; Chabot et al. 1985; Krainer and Maniatis 1985; Berget and Robberson 1986; Black and Steitz 1986; for review, see Reddy and Busch 1988; Lührmann 1988). Splicing involves interaction of the pre-mRNA with snRNPs and many cellular factors (for review, see Sharp 1987; Maniatis and Reed 1987; Vijayraghavan and Abelson 1989) to form a large complex termed the spliceosome (Brody and Abelson 1985; Fren-dewey and Keller 1985; Grabowski et al. 1985). Some of the snRNPs interact directly with the pre-mRNA (Chabot and Steitz 1987; Steitz et al. 1988). The U1 snRNP is the first to bind, and it interacts with the 5' splice site and the branchpoint (Mount et al. 1983; Zhuang and Weiner 1986; Ruby and Abelson 1988). The U2 snRNP binds to the branchpoint (Parker et al. 1987),

and the U5 snRNP possibly binds to the 3' splice site (Gerke and Steitz 1986; Tazi et al. 1986). The U4 and U6 snRNAs are associated together in the same snRNP (Bringman et al. 1984; Hashimoto and Steitz 1984). Although the U4/U6 snRNP is required for splicing (Berget and Robberson 1986; Black and Steitz 1986), there is no evidence that it interacts directly with the precursor.

Splice site selection in nuclear pre-mRNA is dictated by short sequences at or near the splice junctions (Shapiro and Senapathy 1987). U-snRNPs have been proposed to be involved in properly aligning splice junctions in pre-mRNAs to confer the appropriate architecture for catalysis (Lerner et al. 1980; Rogers and Wall 1980), similar to that specified by the self-splicing type I and II intron sequences (see Cech 1986). Alternatively, the snRNP components of the spliceosome may be directly responsible for catalysis of the intron excision and/or exon ligation reactions, although no catalytic activities of snRNPs have been identified thus far.

The U6 snRNA is the most highly conserved spliceosomal snRNA (Das et al. 1987; Brow and Guthrie 1988); U6 snRNAs from rat, yeast, and plant are >70% homologous. The apparent constraints on both the size and the sequence of U6 argues for a central role in the splicing process (Guthrie and Patterson 1988). On the basis of conserved structural features, the U6 sequence has been divided into four domains (Brow and Guthrie 1988). The 5'-terminal domain (nucleotides 1–27) contains a hairpin structure highly variable in length among species, which is responsible for essentially all the differences in size of the U6 RNAs. The central domain

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(nucleotides 28–54) seems likely to be single-stranded, because of its RNase H susceptibility (Guthrie and Patterson 1988). The contiguous stretch of conserved sequences (nucleotides 55–80) in U6 has been proposed to base-pair with U4 to form two contiguous stems that lie within the most highly conserved segment of U6 (Bringmann et al. 1984; Hashimoto and Steitz 1984; Rinke et al. 1985; Das et al. 1987; Siliciano et al. 1987; Kiss et al. 1988; Zucker-Aprison et al. 1988; Mottram et al. 1989). Finally, the 3'-terminal domain is divergent in sequence but constant in length (nucleotides 81–112). It has been proposed that the association between U4 and U6 is a dynamic one and that stable U4/U6 interaction is disrupted during splicing in vitro (Cheng and Abelson 1987; Lamond et al. 1988). Interestingly, U6 sequences bound by U4 show markedly higher conservation than does the comparable region of U4, suggesting that this region of U6 may be involved in catalysis (Brow and Guthrie 1989). This hypothesis is supported by the discovery of an intron within the single-copy gene encoding U6 RNA in the fission yeast *Schizosaccharomyces pombe*, which is located precisely in the middle of the most highly conserved region of U6 (Tani and Ohshima 1989). The site of intron insertion may reflect the proximity of that part of U6 to the catalytic center of the spliceosome.

Assembly of the snRNAs with proteins to form snRNP particles has been studied in vivo and in vitro using immunological methods and has provided extensive data of their structure (for review, see Mattaj 1988, 1989; Parry et al. 1989). However, the current lack of functional assays for in vitro-assembled U-snRNPs complicates further progress. A functional analysis is a powerful ally of the structural studies to understand the complexities of the assembly process. By synthesizing U6 snRNA in vitro with T7 RNA polymerase and combining it with *Saccharomyces cerevisiae* extracts depleted of their endogenous U6 RNA, we have been able to assemble the synthetic transcript into a functional snRNP able to reconstitute 50–100% of splicing activity in vitro. The reconstituted U6 snRNP comigrates with the endogenous U6 snRNP on nondenaturing polyacrylamide gels, it is immunoprecipitated by the same antibodies and it participates in spliceosome formation. Thus, the newly formed particle is structurally identical to the endogenous U6 snRNP. Until now, only in vivo approaches have been used to determine the effects of snRNA mutations on splicing (Parker et al. 1987; Seraphin et al. 1988; Siliciano and Guthrie 1988), but no in vitro systems have been available for studying the function of U6 RNA mutations and analyzing the functions of U6 snRNP in splicing. The assay offers the advantage of flexibility and ease of manipulation for the study of the pre-mRNA splicing mechanism.

Results

U6 snRNA can be specifically degraded in yeast extract

To test whether the synthetic U6 RNA could assemble into a functional snRNP, we first had to inactivate the

endogenous yeast U6 snRNP. We tested the ability of the oligonucleotides b, d, and f (Fig. 1A), which are complementary to nucleotides 1–32, 33–81, and 82–112, respectively, in U6 snRNA, to direct RNase H cleavage of the target RNA in a yeast splicing extract. These oligonucleotides were also used to construct the synthetic U6 RNA gene (see Materials and methods).

Oligonucleotides b, d, and f, as well as the control oligonucleotide c (Fig. 1A), were incubated in yeast splicing extract for 30 min at 30°C. Then, RNA was analyzed by Northern blot, using labeled U6 and U4 oligonucleotides as probes. Figure 2A (lane 2) shows that the control oligonucleotide c has no effect. The effects of oligonucleotides b, d, and f are shown, respectively, in the presence and absence of ATP. Only oligonucleotide d caused complete cleavage of the U6 RNA in the extract (lanes 5 and 6), and this degradation was strongly ATP dependent (cf. lanes 5 and 6 with lanes 15 and 16). About 60% of the U6 RNA was truncated when incubated with 3 μ M oligonucleotide b, either in the presence (lane 3) or absence of ATP (lane 13). Oligonucleotide f, complementary to the 3'-terminal domain, caused almost 100% cleavage of the U6 RNA at 3 μ M, either in the presence or absence of ATP. Oligonucleotide d is complementary to both the central domain and the U4/U6 interaction domain. To determine whether the efficient cleavage produced by oligonucleotide d resulted exclusively from its complementarity to the central domain, we tested two shorter oligonucleotides: d1, which is complementary to nucleotides 28–54 of the central domain (Fig. 1B), and d2, which is complementary to nucleotides 55–80 of the U4/U6 interaction domain. Only oligonucleotide d1 (at 0.3 μ M) completely cleaved its target RNA, and the cleavage was strictly ATP dependent (Fig. 2A, cf. lane 10 with lane 20). The same effect has been observed previously with an oligonucleotide complementary to residues 43–60 of the HeLa U6 RNA. On binding ATP, the U6 particle may undergo a conformational change, exposing sequences that become accessible to RNase H cleavage (Black and Steitz 1986).

Cleavage of U6 RNA inhibits splicing in vitro

To test whether the cleavage of yeast U6 RNA inhibited splicing activity, the extract was incubated under splicing conditions, first with an oligonucleotide and then with pre-mRNA substrate. The extract preincubated without an oligonucleotide (Fig. 2B, lane 1) shows the expected reaction products and intermediates. Preincubation with c, b, or d2 oligonucleotides allowed similar quantities of spliced pre-mRNA to be produced. A reduced formation of spliced message, together with a slight accumulation of excised intron, could be seen in the samples treated with 3 μ M oligonucleotide f (lanes 7 and 17). In contrast, pretreatment with either d or d1 oligonucleotides resulted in the complete disappearance of spliced actin pre-mRNA (lanes 5 and 6; 9 and 10). When the preincubation was done in the absence of ATP (lanes 13–20), only oligonucleotides d and d1 prevented the appearance of both splicing intermediates and

Figure 1. (A) Oligonucleotides for T7 promoter/U6 snRNA gene construction. The six oligonucleotides—a (47 nucleotides), b (51 nucleotides), c (49 nucleotides), d (49 nucleotides), e (43 nucleotides), and f (39 nucleotides)—are shown as base-paired duplexes. The construct was cloned into the *EcoRI*/*Bam*HI sites of M13mp18 and subcloned into pUC18, as described in Materials and methods. (Start site) Start of the transcription. (+1) The first nucleotide in the snRNA U6 gene. The T7 promoter is overlined. The *Dra*I termination site is shown upstream from the *Bam*HI terminus. (B) Proposed secondary structure of the yeast U4/U6 snRNAs (Brow and Guthrie 1988; Guthrie and Patterson 1988). The sequence complementary to oligonucleotide d1 is underlined. Arrows indicate the 3' ends of the various runoff transcripts described in the text.

products but only at the highest concentration used (3.0 μ M, lanes 15 and 19). At the low amount of both oligonucleotides, splicing of the precursor was not inhibited (lanes 16 and 20). Because ATP was added during the splicing phase of the incubation, it appears that the oligonucleotides, themselves, were degraded during the preincubation.

Stability of oligonucleotides incubated in yeast extract

To prevent cleavage of the T7U6 RNA after its addition to the treated extract, it was important to determine that the oligonucleotides were degraded themselves during the incubation in yeast extract. The stability of oligonucleotide d was analyzed by incubating the 3'-terminal-labeled oligonucleotide in yeast extract under splicing conditions (Fig. 3, lanes 3 and 4). Within 30 min, <1% of intact oligonucleotide was detected (lane 4, 0.3 μ M oligonucleotide). Very high quantities of oligonucleotides, up to 3.0 μ M, were not completely degraded during the incubation time of 30 min at 30°C (data not shown). We found that the concentration of oligonucleotides d or d1 necessary and sufficient to induce complete cleavage of U6 snRNA varied among different extracts from 0.15 to 0.3 μ M (data not shown).

In vitro-synthesized U6 RNA restores splicing activity

We asked whether the synthetic U6 RNA was able to assemble into a snRNP when incubated in the yeast ex-

tract depleted of its endogenous counterpart and whether the newly generated particle was capable of mediating pre-mRNA splicing. Figure 4 shows a titration curve of unlabeled U6 RNA synthesized in vitro using T7 RNA polymerase added to the depleted extract at various concentrations (from 200 to 0.05 nM). After 7 min of preincubation at 23°C, splicing activity was assayed by the addition of labeled pre-mRNA splicing substrate (0.2 nM). Samples that had received in vitro-synthesized U6 RNA produced actin RNA that was efficiently spliced (lanes 3–12). Extracts that had been treated with oligonucleotide d1 and then with tRNA or in vitro-synthesized U4 RNA before being incubated with the substrate were found to contain only unspliced pre-mRNA (data not shown). Routinely, addition of 10 nM T7U6 RNA to oligonucleotide-treated extracts reconstitutes 50–100% of splicing activity of control extracts. This concentration is similar to the level of the endogenous U6 snRNA present in the extract; our standard splicing assay contains 4–8 nM endogenous U6 RNA (data not shown). Thus, the synthetic U6 RNA can restore splicing activity in vitro when present at similar levels to that of the endogenous U6 snRNA.

Formation of snRNPs with synthetic U6 RNA

We used immunoprecipitation experiments and nondenaturing gel analysis to determine whether the in vitro assembled U6 snRNP was not only functionally active but also structurally correct. After incubating the syn-

Figure 2. RNase H cleavage of U6 snRNA in yeast extract. Various oligonucleotides (0.3 or 3.0 μM; listed in Fig. 1A,B) were incubated with yeast extract in the presence or absence of exogenous ATP for 30 min at 30°C. (A) Digestion of U6 snRNA was monitored by Northern hybridization using labeled U6 and U4 probes. (Lane 1) No oligonucleotide was added. The lowest bands (arrow) are specific cleavage products of U6 RNA. (B) Splicing of the actin pre-mRNA substrate in oligonucleotide-treated extracts. After treatment of the extract with various oligonucleotides in the presence or absence of ATP, ATP was added at 2 mM (lanes 13–22), and a standard mRNA splicing reaction was carried out using actin pre-mRNA as substrate. The major reaction products and intermediates are labeled as follows: (IVS-E₂) Intron–exon 2; (IVS) the excised intron; (p) precursor RNA; (m) the spliced message; (E₁) the excised exon 1.

thetic U6 RNA in the depleted extract, the assembly mix was immunoprecipitated with anti-m₃G or anti-PRP4 antibodies. It was shown previously that anti-m₃G antibodies could immunoprecipitate U4 as a consequence of its trimethylguanosine cap structure at its 5' end (Bringmann et al. 1983). The endogenous U6 does not have a trimethylguanosine cap structure but can be immunoprecipitated by anti-m₃G antibodies because of its association with U4 (Bringman et al. 1984). It has also been shown (Banroques and Abelson 1989) that the 52-kD protein encoded by the PRP4 gene of *S. cerevisiae* is a U4/U6 snRNP protein. The RNA species immunoprecipitated (Fig. 5A) were quantitated by Northern blot analysis using labeled U4 and U6 oligonucleotides as probes. As expected, both antibodies immunoprecipitated the U4/U6 snRNP (lanes 1 and 2). When the extract was treated with oligonucleotide d₁, U6 was no longer immunoprecipitated (lanes 3 and 4). When synthetic U6 RNA was added to inactivated extracts, U6 was again immunoprecipitated. Synthetic U6 RNA efficiently associates with the endogenous U4 and it forms a structurally correct snRNP, which is immunoprecipitated by both antibodies (lanes 6–11). No immunoprecipitation could be detected with the pre-immune serum (lane 5).

In vitro-reconstituted U6 snRNPs were also analyzed by electrophoresis in a nondenaturing gel (in the absence of added precursor mRNA). Northern analysis was performed using U6 or U4 oligonucleotide as probes. Hybridization with the U6 probe revealed the position of the endogenous U6 snRNP in control extracts (Fig. 5B, lane 1). In whole-cell extract, the U6 snRNP exists in three complexes, termed III', III, and V (Cheng and Abelson 1987). In our analysis, the majority of U6 was

found in complex V, whereas the rest of it was detected in the slower migrating complexes III and III', which also contained U4. Complex V probably represents the free U6 snRNP (at least free of U4), because there was no detectable U4 in this band. After treatment with oligonucleotide d₁, the endogenous U6 snRNPs were destroyed, because U6 RNA was no longer detected in the blot. U4 could be seen in the same sample, but its mobility changed after the cleavage of U6 (lane 2, U4 probe). The complexes detected represent two new forms of U4.

Figure 3. Stability of oligonucleotides incubated in yeast extract. Oligonucleotide d (49-mer) was 3'-terminal labeled with 5'-[³²P]pCP and incubated for 30 min at 30°C in the presence (lanes 3 and 4) or absence (lane 2) of extract. Either 0.15 μM (lanes 1–3) or 0.3 μM (lane 4) oligonucleotide was used. After deproteinization, the oligonucleotide was analyzed on a 15% acrylamide–8 M urea gel.

Figure 4. In vitro-synthesized U6 RNA restores splicing activity. The yeast extract was not treated (lane 1) or treated for 30 min at 30°C with 0.3 μ M oligonucleotide d1 in the presence of 2 mM ATP (lanes 2–12). Various concentrations of unlabeled T7U6 transcript were added (lanes 3–12), and incubation was continued for 7 min at 23°C. A standard mRNA splicing reaction was carried out using 32 P-labeled actin pre-mRNA as substrate. RNAs were analyzed on an 8% acrylamide–8 M urea gel. The major reaction products and intermediates are labeled as follows: (IVS-E₂) Intron–exon 2; (IVS) the excised intron; (p) precursor RNA; (m) the spliced message; (E₁) the excised exon 1.

Note that it seems unlikely that the band comigrating with complex III represents complex III, because it does not hybridize with the U6 probe. When the synthetic U6 RNA was incubated in the depleted extract, we observed the formation of two complexes with mobilities identical to those of forms V and III. When U6 RNA was added at a concentration of 10 nM (lane 3), only a part of it was assembled into forms V and III, whereas the rest of the U6 RNA was present as a fast migrating smear, which may represent naked U6 RNA. At a lower concentration (1 nM), the transcript was completely incorporated into complex III instead (lane 4). This experiment shows that the T7U6 RNA assembles in an snRNP that has the same electrophoretic mobility of the endogenous U6 snRNP on nondenaturing gels.

Spliceosome formation in reconstituted extracts

It has been shown (Pikielny et al. 1986; Cheng and Abelson 1987) that at least four splicing-specific complexes can be resolved by electrophoresis on nondenaturing polyacrylamide gels after the assembly of the yeast spliceosome. Complex B, the first detectable intermediate to form (Cheng and Abelson 1987), contains

both pre-mRNA and the U2 snRNP. A large complex then forms (complex A2), which contains U4/U6 and U5 particles in addition. Subsequently, U4 leaves the complex (complex A1), and a mature spliceosome is obtained that contains pre-mRNA, intermediates, products, and U2, U5, and U6 snRNPs. In Figure 6A (lanes 4–6), the extract was preincubated with oligonucleotide d1, and after the addition of 32 P-labeled pre-mRNA, the complexes were electrophoretically separated on a nondenaturing gel. We observed that only complex B accumulated. An intact U6 snRNP is required for further assembly of the spliceosome. When 32 P-labeled, full-length U6 snRNA and then unlabeled actin pre-mRNA were added to the depleted extract (Fig. 6B, lanes 5 and 6), we observed the formation of two complexes with mobilities identical to that of A1 and A2 complexes. No complexes could be detected in the absence of pre-mRNA (lane 4). The controls (Fig. 6A, B, lanes 1–3) consisted of a standard splicing reaction in which the extract was preincubated with no oligonucleotide and the precursor was labeled with 32 P. Figure 6B, (lanes 7–12) shows spliceosome formation after reconstitution with U6 RNA transcripts truncated at their 3' ends. These were obtained by digesting the pT7U6 DNA template with *FokI* (causing it to lack 18 nucleotides of the 3'-terminal domain), with *EcoNI* [causing it to lack the 3'-terminal domain (32 nucleotides) and 7 nucleotides of the U4/U6 interaction domain], and with *BclII* [causing it to lack the 3'-terminal domain and the majority of the U4/U6 interaction domain (22 nucleotides); see Fig. 7A and 1B] respectively. The *FokI*/U6 transcript was incorporated into the same complexes as the full-length U6 RNA (although the complexes formed appeared to be less stable to gel electrophoresis). In contrast, the *EcoNI* and the *BclII* truncated transcripts were not found in either complex, suggesting that the deleted region(s) was required for the formation of complexes involved in splicing.

Identification of U6 sequences required for pre-mRNA splicing in vitro

We used the in vitro U6 reconstitution system to identify sequences within the U6 snRNA that are required for snRNP assembly and pre-mRNA splicing. In our first approach, we compared the splicing efficiency restored by several U6 RNA transcripts that were shorter or longer than the reference U6 molecule (113 nucleotides; Fig. 7A). The *BamHI*/U6 RNA containing eight extra nucleotides at the 3' end (Fig. 7B, lanes 6–8) gave a lower reconstitution of splicing activity. The *FokI*/U6 RNA also was incapable of complete recovery of splicing (lanes 9–11). Interestingly, with this transcript, the intron lariat accumulated slightly and there was a decrease in the level of the spliced mRNA (at 10 nM; lane 9). When the U6 molecule was shortened further (lanes 12–18), neither splicing intermediates nor products could be detected. To determine whether the inability to restore splicing resulted from the loss of an essential region of U6 or merely to the instability of the shorter

Figure 5. Formation of snRNPs with synthetic U6 RNA. (A) Immunoprecipitation of the synthetic U6 snRNP. The yeast extract was not treated (lanes 1 and 2) or treated with the oligonucleotide for 30 min at 30°C (lanes 3–11). The in vitro-transcribed unlabeled U6 RNA (T7U6) was added at a final concentration of 100 nM (lanes 6 and 9), 10 nM (lanes 5, 7, and 10), or 1 nM (lanes 8 and 11). After incubation at 23°C, samples were subjected to immunoprecipitation with anti-PRP4 antibodies (lanes 1, 3, 6–8), anti-m³G antibodies (lanes 2, 4, 8–11), or preimmune serum (lane 5). RNAs were recovered from immunocomplexes, separated by electrophoresis on a 5% acrylamide–8 M urea gel, and analyzed by Northern hybridization with U6 and U4 probes. The bands migrating between U4 and U6 and under U6 are degradation products of U4 and U6, respectively. (B) Analysis of the synthetic U6 snRNP on nondenaturing gel. After treatment of the extract with oligonucleotide d1 (lanes 2–4), T7U6 RNA was added and incubated for 7 min at 23°C (lanes 3 and 4). No oligonucleotide or T7U6 RNA was added in the control (lane 1). Samples were subjected to electrophoresis on a nondenaturing polyacrylamide gel followed by Northern blot analysis. The same blot was used for hybridization with U6 and U4 probes. The endogenous U6 was present in complexes III', III, and V; the endogenous U4 was present in complexes III' and III. The endogenous U6 was not detected after treatment of the extract with oligonucleotide d1, whereas U4 was found as two new bands. Complex III was reconstituted after addition to the extract of 1 nM T7U6 (lane 4). Both forms III and V could be seen in the samples that received 10 nM T7U6 (lane 3).

transcripts, reconstitution experiments were done using ³²P-labeled U6 RNA molecules. Figure 7C shows that all of the transcripts were stable when incubated in the yeast extract. Only the *FokI*/U6 transcript, after incubation in the extract, was degraded to give a second, shorter band (Fig. 7C, lane 4). These results demonstrate that large deletions of U6 RNA (39 nucleotides or more) produce molecules incapable of reconstituting splicing activity in vitro. The ability of shorter U6 transcripts to interact with the endogenous U4 snRNA was also examined by immunoprecipitation with anti-m³G antibodies (Fig. 7D). The *FokI*/U6 truncated transcript was coprecipitated with the endogenous U4 snRNA to nearly the same extent as the full-length U6 molecule (the shorter degradation product of the *FokI*/U6 transcript was also coprecipitated; lanes 4 and 5). In contrast, both *EcoNI* and *BclI* truncated transcripts were not coprecipitated with the U4 snRNA (lanes 6–9).

Mutational analysis of the 3'-terminal domain of U6 snRNA

To understand the importance of the 3'-terminal domain of U6 snRNA in splicing, we analyzed the contribution

of conserved nucleotides of this region of the efficiency and fidelity of reconstitution of pre-mRNA splicing in vitro.

We constructed three U6 variants of the 3'-terminal domain: M2C/U6, M2G/U6, and M3/U6, in which invariant nucleotides have been changed, respectively, from position 81 to 95, 82 to 95, and 96 to 108. The sequence changes in the mutants used in this work are shown in Figure 8A. When M2C/U6 was assayed for its ability to reconstitute splicing activity (Fig. 8B), it was inactive. In contrast, M3/U6 restored splicing of actin pre-mRNA efficiently. This result confirmed that obtained using the *FokI*/U6 truncated transcript in reconstitution experiments. The lack of the 3' half of the 3'-terminal domain of U6 snRNA did not dramatically affect restoration of splicing. Thus, all of the conserved nucleotides of this region can be changed with little effect in efficiency of reconstitution. In contrast, the lack of functionality of M2C/U6 appeared to contradict the finding reached by RNase H cleavage experiments. Extracts containing endogenous U6 RNA that was truncated (approximately at nucleotides 82–112) by oligonucleotide f-directed cleavage still spliced pre-mRNA. Thus, the presence of the invariant residue G81 within

Figure 6. (A) Cleavage of the endogenous U6 blocks the formation of an active spliceosome. The extract was not treated (lanes 1–3) or treated (lanes 4–6) with the oligonucleotide d1 for 30 min at 30°C. Then, samples were incubated 2, 15, and 30 min with ³²P-labeled actin substrate. The complexes were separated by electrophoresis on a nondenaturing polyacrylamide gel. (B) The synthetic U6 snRNP participates in spliceosome formation. Lanes 1–3 are the controls, as in A. After treatment with the oligonucleotide, reconstitution was carried out using U6 RNA transcripts of various lengths (see Fig. 7A), labeled with ³²P (lanes 4–12). After incubation at 23°C, unlabeled actin transcript (2 nM) was introduced (lanes 5–12) and incubated for 15 or 30 min at 23°C. The precursor was not added in the control (lane 4). The reactions were analyzed as in A. The nomenclature of the complexes is that proposed by Cheng and Abelson (1987).

the U6 RNA may be essential for splicing activity. To test this possibility, we constructed M2G/U6, which contains G instead of C at position 81 (Fig. 8A). The results of the reconstitution using M2G/U6 RNA are clear: Splicing is restored, although not at the wild-type level (Fig. 8B). G81 must have an important role in splicing. It probably acts as a contact point with other snRNAs and/or with factors of the spliceosome. Interestingly, M2C/U6 can efficiently base-pair with the endogenous U4, but it does not participate in spliceosome formation (data not shown). This indicates that the interaction of U6 with U4 is not the only element required for further assembly of the spliceosome.

Interesting differences between yeast and human U6

The primary structure of yeast and human U6 are sufficiently conserved in sequence to be detectable by hybridization with a mouse U6 probe (Siliciano et al. 1987). The yeast U6 (112 nucleotides) is 75% homologous to human U6 (106 nucleotides) over more than half its length, except for a short variable region at the 5' terminus that is responsible for all the differences in size of the U6 RNAs (Brow and Guthrie 1988). In light of the recent finding that human U1 and U2 snRNAs are capable of forming functional snRNPs with amphibian proteins (Pan and Prives 1988), it was interesting to

know whether the human U6 RNA could recover splicing activity in extracts depleted of yeast U6, associate with yeast splicing proteins, and interact with yeast U4. To test this possibility, human U6 RNA, synthesized in vitro using SP6 RNA polymerase (Pikielny et al. 1989), was added to the oligonucleotide-treated extract, and the restoration of splicing activity was monitored by the addition of actin pre-mRNA (Fig. 9A, lanes 6–8). Despite the strong conservation of sequence, the synthetic human U6 RNA was unable to restore splicing of actin pre-mRNA. One explanation for this result is that the human U6 RNA is assembled less efficiently into snRNP particles and is degraded in yeast extracts, either because it is intrinsically unstable or because un-assembled RNA is more accessible to nucleases. This, however, was not the case; ³²P-labeled human U6 RNA was completely stable when it was incubated in yeast extracts (data not shown). To test whether the strong conservation of the U4/U6 interaction domain of U6 (80% of identity) was sufficient to allow the formation of a hybrid U4/U6 snRNP (even if inactive), we performed immunoprecipitation experiments using anti-m₃G and anti-PRP4 antibodies. The results shown in Figure 9B (lanes 10–15) clearly established that human SP6U6 RNA does not interact with the endogenous yeast U4 and is unable to form a hybrid snRNP in vitro consisting of human U6 snRNA and yeast proteins.

Discussion

A requirement for the U6 snRNP in the splicing reaction has been demonstrated in mammalian cell extracts by site-directed cleavage of the U6 RNA with RNase H: HeLa cell extracts, in which portions of U6 snRNA have been selectively degraded, have a decreased efficiency of pre-mRNA splicing in vitro (Black and Steitz 1986). Recently, it has been demonstrated that the yeast U6 gene is essential in vivo (Brow and Guthrie 1988); however, the function(s) that the U6 snRNP plays in the splicing process is still unknown. Because its high degree of sequence conservation implies a fundamental role(s) in splicing, the experiments described here were undertaken as a first step toward understanding what this role(s) may be.

Accessible sequences in the U6 snRNP

We have shown that a number of oligonucleotides complementary to the yeast U6 RNA were able to direct RNase H cleavage of the U6 RNA, but only in one case was the cleavage efficient and complete. This oligonucleotide was complementary to 27 nucleotides of the central domain region, which is supposed to be, at least in part, single-stranded in mammals and in yeast (Black and Steitz 1986; Brow and Guthrie 1988) and which contains very highly conserved sequences. When ATP was absent during the RNase H degradation, the central domain of U6 was not accessible for cleavage by the enzyme. It became available when ATP was present, suggesting that this part of U6 has an important role. The

A

Figure 7. Identification of U6 sequences required for pre-mRNA splicing in vitro. (A) The positions of the restriction sites in the U6 gene cloned under the T7 promoter [(Bc)BamHI; (E) EcoNI; (F) FokI; (D) DraI; (Ba) BamHI] and the sizes of the corresponding RNA transcripts are shown in the schematic drawing. (B) Splicing reconstitution by truncated RNAs. Conditions for reconstitution are described in Methods. (C) Stability of the shorter U6 RNAs. Reconstitution reactions were carried out in the presence of 32 P-labeled T7U6 transcripts. RNAs were analyzed on 10% acrylamide–8 M urea gel. Lanes 6–8 show the U6 transcripts of various length not incubated in the yeast extract. The BamHI and the EcoNI U6 truncated transcripts are not shown but gave the same results. The major reaction products and intermediates are labeled as follows: (IVS = E₂) Intron–exon 2; (IVS) the excised intron; (p) precursor RNA; (m) the spliced message (E₁) the excised exon 1. (D) Immunoprecipitation of reconstituted U6 snRNPs was carried out with anti-m₃G antibodies using the same conditions described in Fig. 5A. Lane 1 (C) is the control extract. The bands migrating between U4 and U6 and under U6 are degradation products of U4 and U6, respectively.

particularly strong conservation of the 3' half of the central domain indicates that this region may be more evolutionary constrained by its participation in multiple interactions in the splicing process (Guthrie and Patterson 1988).

The cleavage patterns we obtained with the other oligonucleotides suggest additional U6 RNA structural features. First, in yeast, as in mammals, the 5'-terminal domain of U6 is only partially sensitive to RNase H degradation, even at the highest concentration used in this study. This is consistent with the hypothesis of others (Black and Steitz 1986) that this region constitutes a recognition site for a U6-binding protein. Other explana-

tions are that the sequence in this region may not be available for hybridization to the oligonucleotide because of its proposed hairpin structure (Brow and Guthrie 1988). Also, binding of oligonucleotide b may not occur efficiently because of its self-complementarity. It is interesting to note that the shorter form of U6 produced by this cleavage seems to be rather unstable. Second, the U4/U6 interaction domain of U6 appears to be a region particularly resistant to RNase H targeting, even in the presence of ATP. This contradicts the notion that portions of this region are sensitive to RNase H cleavage only in the presence of ATP (Black and Steitz 1986). In light of the recent suggestion that

Figure 8. Mutational analysis of the 3'-terminal domain of U6 snRNA. (A) The sequence of the 3'-terminal domain of yeast U6 snRNA is shown (wt/U6). (cons) Consensus line; uppercase letters are invariant residues; Y and R indicate an invariant pyrimidine and purine, respectively. Residues identical among all the species except one are indicated by lowercase letters; two or more differences are indicated by dots (Guthrie and Patterson 1988). The U6 point mutations used in this study are indicated in boldface type. The names of the mutants are indicated at left. (B) Splicing reconstitution of the different U6 RNA mutants indicated in (A). Conditions for reconstitution are described in Materials and methods. The major reaction products and intermediates are labeled as follows: (IVS-E₂) Intron-exon 2; (IVS) the excised intron; (p) precursor RNA; (m) the spliced message; (E₁) the excised exon 1.

U4 and U6 undergo cyclic dissociation during the course of the splicing reaction (Cheng and Abelson 1987; Lamond et al. 1988), we propose that this portion of U6, once the complex with U4 is disrupted, may be engaged in additional interactions (McPheeters et al. 1989). Finally, structures proposed for the 3'-terminal domain of mammalian U6 cannot be formed in yeast (Brow and Guthrie 1988). Nevertheless, this unstructured region also is quite resistant to the targeted cleavage caused by oligonucleotide f. Because extracts containing the shortened form of U6, lacking nearly all of the 3'-terminal domain, still splice pre-mRNA we conclude that nucleotides 82–112 are not absolutely required for splicing *in vitro*. However, our results suggest that they may be important for efficient splicing.

In vitro-reconstituted U6 snRNP is identical to the endogenous U6 snRNP

By synthesizing U6 RNA *in vitro* with T7 RNA polymerase and combining it with yeast extracts, we have demonstrated that the synthetic U6 RNA is fully functional in reconstituting 50–100% of splicing activity in extracts depleted of their endogenous U6 snRNA. After directing RNase H cleavage, the oligonucleotides are also destroyed, presumably by an endogenous deoxyribonuclease (DNase) activity. This important finding allows reconstitution without first incubating oligonucleotide-treated extracts with exogenous DNase I to remove oligonucleotides.

We examined the newly formed snRNP further and found that it is similar, if not identical, to the endogenous U6 snRNP present in yeast splicing extracts. Cheng and Abelson (1987) have observed that in the crude extract the endogenous U6 snRNP exists in sev-

eral complexes, all of which have been identified using synthetic U6 RNA molecule. Moreover, we used immunoprecipitation experiments to demonstrate that the reconstituted U6 snRNP is structurally similar to the authentic U6 snRNP. By using anti-m₃G antibodies, we have shown that the intermolecular base-pairing between the endogenous U4 RNA and the synthetic U6 RNA occurs efficiently. In addition, anti-PRP4 antibodies immunoprecipitate only U4 in an extract in which endogenous U6 RNA has been digested, but they coprecipitate both U4 and the T7U6 after its addition to the extract. This reinforces the notion that the reconstituted particles are physically similar to their endogenous counterparts.

Several forms of the spliceosome have been detected by electrophoresis of splicing mixtures in nondenaturing polyacrylamide gels in both mammalian and yeast systems (Konarska and Sharp 1986; Pikielny et al. 1986; Cheng and Abelson 1987). We found that cleavage of the endogenous U6 snRNA results in the accumulation of the earliest complex detectable in spliceosome assembly (complex B). Addition of the T7U6 transcript to the oligonucleotide-treated extract restores the ability of the extract to form the later complexes.

Functions of the 3'-terminal domain

To define what parts of the U6 snRNA are required in the splicing process, we compared reconstitution activities of several U6 RNA transcripts truncated at their 3' ends. This analysis revealed that U6 RNA molecules lacking 54 and 39 nucleotides are unable to restore splicing activity *in vitro*, to interact with the endogenous U4, and to participate in spliceosome formation. In contrast, deletion of the 18 3'-terminal nucleotides of

Figure 9. (A) Synthetic human U6 RNA does not restore in vitro splicing activity in yeast. Reconstitution of splicing was done using 100, 10, and 1 nM yeast T7/U6 RNA (lanes 3–5) or the same amounts of HeLa SP6/U6 RNA (lanes 6–8). The extract was not treated with the oligonucleotide (lane 1) or was treated but not incubated with yeast T7/U6 transcript (lane 2). (B) HeLa U6 RNA does not interact with the yeast U4 RNA. After depletion of endogenous U6 RNA, the extract was incubated at 23°C with yeast T7/U6 at 100 nM (lanes 4 and 7), 10 nM (lanes 3, 5, and 8), or 1 nM (lanes 6 and 9), or HeLa SP6/U6 RNA at 100 nM (lanes 10 and 13), 10 nM (lanes 11 and 14), or 1 nM (lanes 12 and 15). Then, the samples were subjected to immunoprecipitation with anti-PRP4, anti-m₃G or pre-immune sera, as indicated. The first two

C

lanes show the immunoprecipitation of control extracts with anti-PRP4 (lane 1) and anti-m₃G (lane 2) antibodies. (C) Comparison of the U4/U6 interaction domain of HeLa and yeast U6. Stems I and II refer to intermolecular helices formed with U4. (cons) Consensus line; uppercase letters are invariant residues; Y and R indicate an invariant pyrimidine or purine, respectively. Residues identical among all the species except one are indicated by a lowercase letter; two or more differences are indicated by dots (Guthrie and Patterson 1988). Base-pairing is signified with a dashed line; unpaired nucleotides are signified with a cross. Variable nucleotides between HeLa and yeast U6 are underlined. Sac/U4 and Sac/U6 are *S. cerevisiae* U4 and U6, respectively.

U6 RNA results in a decrease of these activities and also in a diminution in the level of spliced message and accumulation of intron lariet. This suggests that nucleotides from position 95 to 112 are important for efficient assembly and/or may also have a role of holding the excised 5' exon in the splicing complex after cleavage at the 5' splice site (Steitz et al. 1988). Nevertheless, these 18 terminal nucleotides are not absolutely required (Table 1). These results are confirmed by our oligonucleotide-directed RNase H cleavage experiments. The fact that the shortened form of the endogenous U6 snRNA, lacking ~31 nucleotides of the 3'-terminal domain (approximately at nucleotides 82–112), is capable of mediating splicing of actin pre-mRNA with the same effects in the level of mRNA and intron lariet makes the preceding results stronger. The 3'-terminal domain is highly divergent in sequence, although almost constant in length (Brow and Guthrie 1988). Our data suggest that the phylogenetic conservation of the size of this region of U6 may have biological importance. Interestingly, fidelity and/or efficiency of restoration of splicing are not only perturbed by shorter RNA species (95 nucleotides)

but also longer species (121 nucleotides) than the reference U6 molecule (113 nucleotides).

Mutational analysis of the 3'-terminal domain of U6 snRNA confirms that conserved nucleotides of this region contribute to the efficiency of reconstitution of splicing activity but are not absolutely required. Taken together, these results argue for a minor importance of the sequence of the 3'-terminal domain of U6 and suggest that its length may have a role.

Substitution of the invariant G81 with C in synthetic U6 RNA abolishes its ability to restore splicing activity. Although base-pairing with U4 in this U6 RNA variant is still very efficient, its ability to participate in spliceosome formation is lost. Thus, the U4/U6 snRNP forms correctly, but mature spliceosomes do not assemble (Table 1).

The failure of human U6 to function in yeast extracts

In striking contrast to other yeast spliceosomal RNAs, U6 is very similar in size, sequence, and structure to its mammalian homolog. Indeed, there is 60% identity over

Table 1. U6 RNA sequence requirements for splicing and reconstitution

U6 RNA runoff and mutants	Length (nucleotides)	Splicing efficiency (%)		Interaction with U4	Spliceosome assembly
		mRNA	IVS		
<i>DraI</i>	113	80	80	yes	yes
<i>BamHI</i>	121	62	58	NT ^a	NT ^a
<i>FokI</i>	95	34	100–130	yes	yes
<i>EcoNI</i>	74	0	0	no	no
<i>BclI</i>	59	0	0	no	no
M2C/U6	113	0	0	yes	no
M2G/U6	113	30	30	yes	yes
M3/U6	113	45	45	yes	yes

U6 RNAs of various lengths and U6 RNA mutants of the 3'-terminal domain listed were tested for their ability to reconstitute splicing activity in vitro. The interaction with the endogenous U4 was analyzed by immunoprecipitation with anti-m₃G antibodies. Spliceosome assembly was examined on nondenaturing polyacrylamide gel (Cheng and Abelson 1987). Splicing efficiency was quantified by Cerenkov emission of isolated bands corresponding to the spliced message and the excised intron, respectively, of the 10 nm T7–U6 reconstitution. The value of the control extract was taken as 100%.

^a(NT) Not tested.

the full length of yeast and mammalian U6, increasing to >80% identity in the middle third of the RNAs. Microinjection experiments in *Xenopus laevis* oocytes have shown that snRNP-like particles can be assembled with snRNAs from a wide variety of organisms, even from *S. cerevisiae* (Riedel et al. 1987; Tollervey and Mattaj 1987), whose snRNAs are the most divergent in size and sequence from those of higher eukaryotes (Kretzner et al. 1987). In contrast, our data demonstrate that the human SP6/U6 RNA does not restore splicing activity nor does it interact with endogenous yeast U4 in yeast extracts and, as a result, it is not incorporated into the yeast spliceosome (data not shown). Why is the human U6 transcript inactive in yeast extract? One explanation is that the 5'-terminal region is different in length and sequence between yeast and mammals. Alternatively, in mammalian U6, the central domain is predicted to base-pair with a region in the 3'-terminal domain to form a stem that cannot be formed in yeast U6 (Brow and Guthrie 1988). These differences may prevent the human U6 RNA from assembling correctly in yeast extracts. Why does the human U6 transcript not interact with the endogenous yeast U4? To understand the reason for the lack of base-pairing, we analyzed the U4/U6 interaction domain of both human and yeast U6 RNAs. It has been proposed (Brow and Guthrie 1988) that two main regions of U4 and U6 are base-paired and are separated by a U4 stem–loop structure. Within stem I, HeLa and yeast U6 RNAs differ in sequence at one position (Fig. 9C, underlined nucleotides), but the difference does not affect the base-pairing with the yeast U4 snRNA. Within stem II, they differ at four positions, three of which could interfere with the base-pairing. Actually, the interaction of human U6 with human U4 is not better; three nucleotides are still unpaired. Perhaps, the sequence complementarity is not the only element important for the association between U4 and U6, the contribution of species-specific RNA-binding proteins, which stabilize the base-pairing, may be important. The last hypothesis may be especially true in mammals, as it was impossible to reassociate the deproteinized snRNAs

U4 and U6 once the complex had been temperature-dissociated (Hashimoto and Steitz 1984; Rinke et al. 1985).

Conclusions

We assembled synthetic U6 RNA in vitro into a functional snRNP that is physically identical to the endogenous U6 snRNP, is immunoprecipitated by the same antibodies, and participates in spliceosome formation. There are many examples of reconstitution of snRNPs using *X. laevis* egg extracts or HeLa cell S100 extracts (Hamm et al. 1987, 1988; Patton et al. 1987; Patton and Pederson 1988; Kleinschmidt et al. 1989; Pikielny et al. 1989) and examples of functional assembly of human snRNA with amphibian or HeLa proteins (Krainer 1988; Pan and Prives 1988). This work and that of McPheeters et al. (1989) are the first examples of synthetic U-RNAs that are able to restore splicing in vitro. An important outcome of this experimental approach is that it constitutes a powerful tool for the identification of the sequences in snRNAs that are required for particle formation, for pre-mRNA splicing, and for spliceosome assembly. This is especially important in the case of U6, as it has been speculated that the extreme conservation of size, sequence, and structure reflects a fundamental role, perhaps one at the heart of *trans*-mediated catalysis of splicing (Guthrie and Patterson 1988).

Materials and methods

Subcloning of the yeast U6 gene under the T7 promoter and construction of mutants

Because of the paucity of convenient restriction sites of the U6 snRNA gene (Brow and Guthrie 1988), the linkage to the bacteriophage T7 promoter was done by self-assembly of six synthetic oligodeoxynucleotides (Reyes and Abelson 1987), as shown in Figure 1A. The six synthetic oligomers were purified by electrophoresis on denaturing 15% polyacrylamide gels and phosphorylated in a separate reaction mixture with T4 polynucleotide kinase. Stoichiometric amounts of each oligomer were combined and annealed by evaporation to dryness. This construct was then cloned into the *EcoRI*/*BamHI* sites of

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M13mp18. The identity of the insert was proved by the dideoxy sequencing method (Sanger et al. 1977) with Sequenase (U.S. Biochemicals). An extra G was added at the 5' end of the U6 gene to improve the T7 promoter strength (Milligan et al. 1987). Transcription by T7 polymerase starts at the extra G added at the 5' end of the U6 gene and terminates at the *DraI* site at the 3' end to give a 113-nucleotide RNA molecule with a sequence identical to an unmodified yeast U6 RNA. The T7 promoter/U6 gene construct was subcloned in the *EcoRI/PstI* site of the vector pUC18 to give a recombinant plasmid designed pT7U6 to obtain larger quantities of DNA.

U6 RNA variants of the 3'-terminal domain were constructed by total gene synthesis. Both oligonucleotides e and f (Fig. 1A) were changed to obtain the mutations listed in Figure 8A. In all cases, the sequence of the mutant gene insert was determined by the dideoxy sequencing method, as above.

T7 RNA synthesis

For the unlabeled RNA synthesis, 10 μ g of pT7U6 DNA was cut with the appropriate restriction endonucleases and incubated in a volume of 100 μ l containing 40 mM Tris-HCl (pH 8.0), 106 mM MgCl₂, 2 mM spermidine, 10 mM DTT, 2.5 mM ATP, UTP, GTP, and CTP, 300 units of T7 polymerase (or SP6 polymerase, in the case of the human U6), and 160 units of RNasin, at 37°C for 2 hr. Transcription reaction was terminated by adding formamide loading buffer. RNAs were visualized by UV shadowing and eluted from the gel in 0.5 M sodium acetate (pH 5.0), 1.0 mM EDTA (pH 8.0), and 2.5% phenol/chloroform at 4°C for 13–15 hr and then phenol/chloroform extracted and ethanol precipitated. U6 RNA (5–10 μ g) was synthesized by this method, as determined by absorption at 260 nm. ³²P-labeled transcripts were synthesized as described above, except that the reaction (20.0 μ l) contained 1 mM ATP, GTP, and CTP and 0.2 mM UTP, 6 mM MgCl₂, and 50 μ Ci of [α -³²P]UTP (3000 Ci/mmol).

Oligonucleotide-directed RNase H cleavage and in vitro functional assay

For the cleavage of the endogenous U6 snRNA, splicing extracts were incubated for 30 min at 30°C with the appropriate oligonucleotide as indicated at the top of Figure 2A and B. Note that oligonucleotide b contains 18 nucleotides of T7 promoter (at the 5' end) and oligonucleotide f contains 8 nucleotides of the restriction sites (at the 3' end) that are not complementary to the U6 snRNA. Typical cleavage reactions were performed for 2 μ l of splicing extract in 4- μ l reaction mixtures containing 2.5 mM MgCl₂, 3% PEG-8000, 60 mM potassium phosphate (pH 7.0), 2 mM spermidine, and 0.3 or 3.0 μ M oligonucleotides in the presence or absence of 2 mM ATP. No exogenous RNase H was added because yeast splicing extract contains endogenous RNase H activity. For in vitro functional assay, after treatment of the extract with 0.3 μ M oligonucleotide d1 in the presence of 2 mM ATP, 0.5 μ l of in vitro-synthesized U6 RNA (resuspended in water) was added and allowed to assemble for 5–10 min at 23°C. Then, 1 fmole of the ³²P-labeled actin precursor was added in a volume of 0.5 μ l of the same buffer as above, except that the concentration of the reagents was 2 $\times$. The samples were incubated for 30 min at 23°C. The reactions were terminated as described by Lin et al. (1985).

Preparation of the splicing extract and substrates

Yeast whole-cell extracts and actin pre-mRNA substrate were prepared according to Lin et al. (1985). Unlabeled actin transcripts were synthesized as described above.

Stability of oligonucleotides in yeast extract

Oligonucleotide d (30 pmole) was 3'-terminal-labeled with 5' [³²P]pCp and T4 RNA ligase, as described by Ruby et al. (1989). This gives the penultimate 3' phosphate a radioactive label. Labeled oligonucleotides were gel-purified and incubated into whole-cell yeast extract for 30 min at 30°C, as described for the in vitro functional assay. After phenol extraction and ethanol precipitation, they were analyzed on a denaturing 15% acrylamide gel.

Immunoprecipitation

As described by Cheng and Abelson (1986), 1.5–2.5 μ l serum, 2.5 mg of protein A–Sepharose (Pharmacia), 0.5 ml of NET-2 buffer [50 mM Tris-HCl (pH 7.5), 0.05% Nonidet P-40, and 150 mM NaCl] were mixed for 1 hr at 4°C, and the beads washed three times with 0.5 ml of the same buffer. After the reconstitution, the mixtures were immunoprecipitated by adding the antibody-bound protein A–Sepharose to the in vitro assembly mix in a total volume of 0.5 ml, and the mixture was rolled for 2 hr at 4°C. The beads were then washed three times with 1 ml of NET buffer, and the precipitated RNAs released by digestion with proteinase K for 15 min at 37°C. RNAs were extracted with phenol/chloroform, precipitated, fractionated on a 5% or 8% acrylamide–8 M urea gel, and electroblotted as described below.

In vitro complex formation

Nondenaturing polyacrylamide gel electrophoresis was performed according to Cheng and Abelson (1987). Electrophoresis for splicing complex analysis was at 300 V for 30 min, followed by electrophoresis at 230 V for 5 hr at 4°C. For RNPs, analysis electrophoresis was at 300 V for 1 hr at 4°C in a buffer containing 20 mM Tris-HCl (pH 8.0) and 1 mM EDTA.

Northern blot analysis

RNAs were electroblotted onto GeneScreen membranes (NEN) at 4°C in 25 mM sodium phosphate buffer (pH 6.5), for 20 hr at 25 V for denaturing gels and for 2–4 hr at 40 V for nondenaturing gels. The blots were UV-irradiated for 10 min, as described by Cheng and Abelson (1987). Prehybridization and hybridization were carried out at 42°C in a solution containing 50% formamide, 25 mM sodium phosphate (pH 6.5), 6 $\times$ SSC, 5 $\times$ Denhardt's solution, 0.5% SDS, and 0.1 mg/ml heat-inactive calf thymus DNA. After hybridization, membranes were washed twice for 5 min in 2 $\times$ SSC and 0.5% SDS at room temperature, twice for 5 min in 2 $\times$ SSC and 0.1% SDS at room temperature and, finally, once for 30 min in 2 $\times$ SSC and 0.1% SDS at 50°C. The U6 probe was a mixture of oligonucleotides b, d, and f, listed in Figure 1A, whereas the U4 probe was a mixture of oligonucleotides complementary to the full-length U4 snRNA (Siliciano et al. 1987). Oligonucleotides were ³²P-labeled at the 5' end by T4 polynucleotide kinase and included in the hybridization buffer at 5 $\times$ 10⁶ to 10 $\times$ 10⁶ cpm in each 25 ml of the hybridization solution.

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