Site-specific deoxynucleotide substitutions in yeast U6 snRNA block splicing of pre-mRNA *in vitro*

Chang Hee Kim, Daniel E. Ryan, Tadeusz Marciniak¹ and John Abelson²

Division of Biology 147-75, California Institute of Technology, Pasadena, CA 91125, USA

¹Present address: The Victor Chang Cardiac Research Institute, St Vincent's Medical Centre, 376 Victoria Street, Darlinghurst, NSW 2010, Australia

²Corresponding author
e-mail: abelsonj@starbase1.caltech.edu

We have identified 2'-hydroxyl groups of the U6 phosphate-ribose backbone which are required for reconstitution of splicing activity in U6-depleted yeast extract. To screen the 2'-hydroxyls of yeast U6 at nucleotides 39–88, spanning the conserved central domain, synthetic U6 RNAs were constructed with deoxyribonucleotides incorporated site specifically. Only four individual deoxynucleotide substitutions blocked splicing activity: dA51 (in the ACAGAG sequence), dA62 (next to the AGC triad), and dU70 and dC72 (both in the loop of the 3' intramolecular stem-loop). Native gel analysis revealed that these deoxy-substituted U6 RNAs were competent for assembly of spliceosomes. Interestingly, a 2'-O-methyl substituent at A51, A62, U70 or C72 did not inhibit splicing activity, indicating that the essential 2'-OH groups at these positions in U6 act as hydrogen bond acceptors or neutral coordinated ligands. The requisite 2'-hydroxyls at A62, U70 and C72 show both similarities and differences relative to the positions of essential 2'-hydroxyls of catalytic domain V of group II ribozymes. The identification of the essential 2'-hydroxyls at positions 62, 70 and 72 corroborates that the 3' intramolecular stem-loop in U6 plays an important role in pre-mRNA splicing.

Keywords: group II intron/2'-hydroxyl/spliceosome/stem-loop/U6 reconstitution

Introduction

U6 snRNA is an essential component of fully assembled spliceosomes—large (40S) ribonucleoprotein complexes that mediate the process of pre-mRNA splicing (for reviews, see Madhani and Guthrie, 1994b; Nilsen, 1994; Sharp, 1994; Ares and Weiser, 1995). The central domain of U6 (nucleotides 47–85 in yeast) is highly conserved from yeast to mammals (Brow and Guthrie, 1988; Roiha *et al.*, 1989; Shumyatsky and Reddy, 1993). Two-thirds of the conserved U6 domain is base-paired with U4 snRNA in U4–U6 snRNP complexes (Bringmann *et al.*, 1984; Hashimoto and Steitz, 1984; Rinke *et al.*, 1985; Brow and Guthrie, 1988; Vankan *et al.*, 1990); the U4–U6 snRNPs deliver U6 to a spliceosome during the ordered

process of spliceosome assembly (Pikielny *et al.*, 1986; Cheng and Abelson, 1987; Konarska and Sharp, 1987). Near completion of assembly, the U4–U6 duplex is dissociated, and U4 may leave the spliceosome (Pikielny *et al.*, 1986; Cheng and Abelson, 1987; Lamond *et al.*, 1988; Yean and Lin, 1991). Dissociation of U4–U6 allows a 5' region of the U6 conserved domain to base-pair with U2 snRNA, thus forming U2–U6 helix I in fully assembled spliceosomes (Madhani and Guthrie, 1992) (see model of the fully assembled yeast spliceosome, Figure 1). Concurrently, the 3' region of the U6 conserved domain putatively forms an intramolecular stem-loop as present in non-complexed U6 snRNPs *in vivo* (Wolff and Bindereif, 1991; Fortner *et al.*, 1994; Brow and Vidaver, 1995). The conserved ACAGAG region of U6, nucleotides 47–52 in yeast, makes physical contact with the 5' splice site of pre-mRNA, as revealed in cross-linking experiments (Sawa and Abelson, 1992; Sawa and Shimura, 1992; Wassarman and Steitz, 1992; Sontheimer and Steitz, 1993; Kim and Abelson, 1996). If U6 is associated simultaneously with U2 (at helix I) and pre-mRNA (at the 5' splice site) while U2 is base paired to the pre-mRNA at the branch site (Parker *et al.*, 1987), then the pre-mRNA could be folded to juxtapose the 5' splice site and the nucleophilic bulged adenosine of the branch site, perhaps thereby activating the first catalytic step of splicing (cf. Figure 1). Interactions between a conserved stem-loop of U5 snRNA and both the 5' and 3' splice sites are thought to position the 5' and 3' exons for ligation at the second catalytic step of splicing (Newman and Norman, 1991, 1992; Wyatt *et al.*, 1992; Sontheimer and Steitz, 1993; Frank *et al.*, 1994). The snRNA components of spliceosomes are thought to function as catalysts for the two reactive steps of pre-mRNA splicing. This catalytic RNA hypothesis is founded on the knowledge that the group II self-splicing intron, an RNA-only system (reviewed by Michel and Ferat, 1995), has intronic sequences that catalyze self-splicing via the same phosphodiester transfer reactions as occur in pre-mRNA splicing, including the formation of a 2'-5' phosphodiester-linked lariat intermediate.

The structure–function relationships of U6 in the spliceosome are not well understood but are beginning to be elucidated. Specific mutations of individual nucleotides in U6 block the first catalytic step of splicing and prevent spliceosome assembly; other mutations block the second catalytic step (Fabrizio and Abelson, 1990, 1992; Vankan *et al.*, 1990; Madhani and Guthrie, 1992). In fully assembled spliceosomes, U2–U6 helix I was identified by compensatory mutations and was demonstrated to be essential for splicing activity in yeast (Madhani and Guthrie, 1992). Two other U2–U6 helices, flanking the conserved U6 domain, were similarly identified and found to be important for splicing in mammalian cells (Hausner

Fig. 1. Model of the fully assembled yeast spliceosome. Interactions between U2, U6 and pre-mRNA are illustrated. Mutationally sensitive nucleotides, essential pro-*R* phosphate oxygen atoms, essential 2'-hydroxyl groups (this work) and U6 cross-linking interactions are highlighted.

et al., 1990; Datta and Weiner, 1991; Wu and Manley, 1991; Wolff and Bindereif, 1992; Sun and Manley, 1995), but are not important in yeast (Fabrizio *et al.*, 1989; Madhani *et al.*, 1990; Yan and Ares, 1996). A randomization-selection experiment of both U2 and U6 snRNAs provided evidence of a tertiary contact between U2 and U6 in the helix I region using a method analogous to phylogenetic co-variation analysis (Madhani and Guthrie, 1994a). Genetic suppression studies of 5' splice site mutants of pre-mRNA identified base-paired interactions between the 5' splice site and U6 that are important for 5' splice site recognition (Kandels-Lewis and Séraphin, 1993; Lesser and Guthrie, 1993). U6 structure-function relationships are key features of the fully assembled yeast spliceosome (Madhani and Guthrie, 1994b) (cf. Figure 1).

In addition to the U6 sequence and secondary structural requirements for splicing activity, other essential structural features of U6 reside on the phosphate-ribose backbone. Important phosphate oxygen atoms of U6 were identified by incorporating thiophosphate groups at selected positions in the U6 sequence (Fabrizio and Abelson, 1992; Yu *et al.*, 1995). For group II ribozymes, the catalytically important phosphate oxygens were similarly identified and suggest that domain V of group II introns has structure-function relationships analogous to the 3' stem-loop of U6 in spliceosomes (Chanfreau and Jacquier, 1994). Correlations between pre-mRNA splicing and group II self-splicing are significant because the splicing mechanisms are quite similar in both systems and because the group II system is better understood and could serve as a model for RNA structure-function relationships in spliceosomes. In addition to backbone phosphate groups, specific 2'-hydroxyl groups of ribose rings play critical roles in binding specificity and/or chemical catalysis in self-splicing RNAs and ribozymes (Bevilacqua and Turner, 1991; Pyle and Cech, 1991; Musier-Forsyth and Schimmel, 1992; Perreault and Atman, 1992; Pyle *et al.*, 1992;

Herschlag *et al.*, 1993a,b; Smith and Pace, 1993; Strobel and Cech, 1993; Pley *et al.*, 1994; Abramovitz *et al.*, 1996). These results suggest that specific 2'-hydroxyl groups of U6 may play similar roles.

In the present study, we identified which 2'-hydroxyl groups of U6 are required for the first or second step of splicing in a yeast *in vitro* splicing system (Lin *et al.*, 1985). To screen all 2'-hydroxyls of yeast U6 at nucleotides 39–88, spanning the entire conserved central domain, synthetic U6 RNAs were constructed with deoxynucleotides incorporated site-specifically at single sites or at multiple contiguous sites. Each of the 2'-deoxy U6 RNAs was assayed for reconstitution of splicing activity in U6-depleted yeast extract (Fabrizio *et al.*, 1989); thus the positions of important 2'-hydroxyl groups were revealed by assaying splicing defects for specific 2'-deoxy substituents. This study is the first to investigate deoxynucleotide substitutions in U6 snRNA.

Results

Synthesis of deoxynucleotide-substituted yeast U6 snRNAs

To construct yeast U6 RNAs substituted with a 2'-deoxy- or 2'-*O*-methyl ribonucleotide at a selected site, each full-length RNA was prepared in pieces, annealed to a complementary single strand of DNA, and ligated using T4 DNA ligase (Kleppe *et al.*, 1970; Moore and Sharp, 1992). Oligonucleotides for the central domain were chemically synthesized to incorporate a 2'-deoxy- or 2'-*O*-methyl ribonucleotide at selected sites from nucleotides 39 through 88. Initially, we prepared U6 RNA in three pieces, nucleotides 1–38, 39–59 and 60–112, such that the 5' and 3' end pieces were transcribed *in vitro* and the central piece (nucleotides 39–59) was synthesized chemically. The yield for the three-piece ligation was as high as 30%. Ligation yields are lower for incorrect 3'

and 5' ends at a ligation junction, including the lack of a 5'-monophosphate. Indeed, our attempts to transcribe nucleotides 1–58 of U6 (from *Sau3AI*-cut plasmid) primarily produced transcripts that had extra, non-coded 3'-terminal nucleotides.

Two improved strategies involved the total chemical synthesis of yeast U6 RNA in either four or five pieces. For the four-piece ligation, synthetic U6 RNA oligonucleotides 1–38, 39–59, 60–79 and 80–112 (all gel purified) were ligated to give full-length U6 in ~20% yield. For the five-piece ligation, U6 RNA oligonucleotides 1–38, 39–59, 60–76, 77–94 and 95–112 were ligated in ~12% yield. The four-piece ligation was used to incorporate a deoxynucleotide at each individual site from 60 through 79, and the five-piece ligation was used for each site from 80 through 88. We found that large quantities of RNA were best prepared via chemical synthesis of the entire RNA in segments for ligation.

For the all-RNA pieces of U6, gel purification removed nearly all of the synthetic byproducts. To expedite preparation of the 2'-deoxy substituted RNA pieces (29 total), an improved method for automated RNA synthesis was followed (see Materials and methods). Synthetic 5'-*O*-trityl-protected oligonucleotides were purified on Nensorb columns to partially remove the aborted by-products of the chemical synthesis, and the eluted full-length 5'-OH oligonucleotides were phosphorylated and used for ligation. For a 0.2 μ mol scale automated synthesis, the quantities of Nensorb-purified 18mer and 20mer products ranged from 1 to 30 nmol. For the five-piece ligations, we started with 40 pmol each of the all-RNA oligonucleotides and 60 pmol of the Nensorb-purified, deoxy-substituted oligonucleotide and obtained 2–3 pmol of gel-purified, full-length U6 RNA. In the four-piece ligations, the same amounts of the individual starting materials yielded 6–9 pmol of gel-purified full-length U6 RNAs. As less than 50 fmol of U6 RNA are needed per splicing assay in U6-depleted yeast extract, ample quantities were obtained to conduct the experiments described.

Splicing activity of U6 RNAs substituted with multiple deoxynucleotides in the 5' portion of the central domain

Splicing assays were conducted in yeast extract using [α - ^{32} P]uridine-labeled actin pre-mRNA as the substrate. In order to assay U6 RNAs substituted with deoxynucleotides, the endogenous U6 snRNA in the yeast extract was depleted by oligo-directed RNase H digestion using a deoxyoligonucleotide (d1) complementary in sequence to nucleotides 28–54 of yeast U6 RNA (Fabrizio and Abelson, 1990). Over the period of incubation, oligonucleotide d1 is itself destroyed by endogenous DNases in the extract. In U6-depleted extract, each of the 2'-deoxy U6 RNAs was assayed for reconstitution of splicing activity (Fabrizio *et al.*, 1989). As a control, wild-type U6 RNA was ligated from three pieces (see above), and the gel-purified, ligated U6 restored splicing activity in U6-depleted extract as efficiently as fully transcribed U6 (Figure 2, lanes 3 and 4). To screen the 2'-hydroxyl groups of U6 RNA at nucleotides 39–59 rapidly, we constructed three U6 RNAs substituted with seven contiguous deoxynucleotides at specific sites (deoxy box 39–45, 46–52 and 53–59) and assayed each for reconstitution of splicing

Fig. 2. Splicing activity of U6-depleted yeast extract reconstituted with U6 RNAs substituted with seven deoxynucleotides at specific contiguous sites. Samples were incubated with [α - ^{32} P]uridine-labeled actin pre-mRNA at 23°C for 30 min, then total RNA was isolated and separated on a denaturing polyacrylamide gel to assay splicing activity: mock-treated extract (lane 1); U6-depleted extract, i.e. extract treated with oligonucleotide d1 to digest endogenous U6 snRNA (lane 2); reconstitution of splicing activity with *in vitro* transcribed wild-type U6 snRNA (lane 3), wild-type U6 RNA ligated from three pieces (lane 4) and ligated U6 RNAs substituted with deoxynucleotides at nucleotides 39–45, 46–52 and 53–59 (lanes 5–7, respectively). Splicing substrate, reaction intermediates and products are labeled as follows: lariat intron–exon 2 (IVS-E2); lariat intron (IVS); pre-mRNA substrate (pre-mRNA); spliced mRNA product (mRNA).

activity (Figure 2, lanes 5–7). Only U6 RNA with deoxy box 39–45 (Figure 2, lane 5) reconstituted splicing activity, although not as efficiently as wild-type U6 RNA (Figure 2, lanes 3 and 4). Since deoxy box 39–45 was competent for splicing, we did not subsequently make 2'-deoxy substitutions at single sites for nucleotides 39–45. For deoxy boxes 46–52 and 53–59, the inability to reconstitute splicing activity (Figure 2, lanes 6 and 7) suggested that at least one 2'-OH group in this region is crucial for splicing or that multiple deoxynucleotide substitutions are inhibitory. Subsequently, we prepared U6 RNAs substituted with a single deoxynucleotide at each site from 46 through 59, and the singly substituted U6 RNAs were screened for reconstitution of splicing activity.

Splicing activity of U6 RNAs substituted with a single deoxynucleotide at each site from 46 through 88

Figure 3 shows results of splicing assays for U6 RNAs substituted with a single deoxynucleotide at each site from 46 to 59. For dA51-substituted U6, splicing activity was completely blocked (Figure 3, lane 12). Interestingly, splicing activity was not inhibited when A51 was replaced by a 2'-*O*-methyladenosine (Figure 3, lane 13). The 2'-*O*-methyl group cannot act as a hydrogen bond donor, nucleophile or coordinated anion (in contrast to 2'-OH groups), therefore 2'-*O*-methyl A51 does not function as such in spliceosomes. Nucleotide A51 is part of the functionally important ACAGAG sequence (Fabrizio and Abelson, 1990; Kandels-Lewis and Séraphin, 1993; Lesser and Guthrie, 1993).

Surprisingly, single deoxynucleotide substitutions at

Fig. 3. Splicing activity of U6-depleted yeast extract reconstituted with U6 RNAs substituted site-specifically with a single deoxynucleotide at each site from 46 through 59 (or with a 2'-O-methyl nucleotide at position 51). Samples were incubated with [α - ^{32} P]uridine-labeled actin pre-mRNA at 23°C for 30 min, then total RNA was isolated and separated on a denaturing polyacrylamide gel to assay splicing activity: mock-treated extract (lanes 1 and 8); U6-depleted extract (lanes 2 and 9); reconstitution of splicing activity with wild-type U6 RNA ligated from three pieces (lanes 3 and 10) and ligated U6 RNAs substituted at nucleotides 46–59 with a single deoxynucleotide (lanes 4–7 and 11–21, respectively).

Fig. 4. Splicing activity of U6-depleted yeast extract reconstituted with U6 RNAs substituted site-specifically with a single deoxynucleotide at each site from 60 through 73. Samples were incubated with [α - ^{32} P]uridine-labeled actin pre-mRNA at 23°C for 30 min, then total RNA was isolated and separated on a denaturing polyacrylamide gel to assay splicing activity: mock-treated extract (lane 1); U6-depleted extract (lane 2); reconstitution of splicing activity with wild-type U6 RNA ligated from four pieces (lane 3) and ligated U6 RNAs substituted at nucleotides 60–73 with a single deoxynucleotide (lanes 4–17, respectively).

positions 53–59 had no effect on splicing (Figure 3, lanes 15–21), despite the result that U6 with deoxy box 53–59 blocked splicing activity (cf. Figure 2).

Figures 4 and 5 show results of splicing assays for U6 RNAs substituted with a single deoxynucleotide at each site from 60 to 88. In this region, one deoxynucleotide substitution, dA62 (Figure 4, lane 6), completely blocked splicing, and two others showed only traces of spliced products, dU70 (lane 14) and dC72 (lane 16, Figure 4). Nucleotide A62 is next to the functionally important CAGC sequence, part of U2–U6 helix I (Fabrizio and Abelson, 1990; Madhani and Guthrie, 1992), and nucleotides U70 and C72 are both part of the loop of the U6 intramolecular 3' stem-loop (Fortner *et al.*, 1994) (cf. Figure 1). As observed for a 2'-O-methyl substituent at nucleotide A51 (see above), a single 2'-O-methyl substituent at A62, U70 or C72 did not inhibit splicing activity (Figure 6), in contrast to the inhibitory effects of deoxynucleotide substitutions at these sites.

Using a set of synthetic oligonucleotide stocks, we were able to reproduce our results. To verify that dA51- and dA62-substituted U6 completely blocked splicing activity and that dU70- and dC72-substituted U6 severely inhibited splicing, we prepared duplicate stocks of the deoxy-substituted oligonucleotides by resynthesizing and purifying them. Using the duplicate stocks, replication of the assay results for dA51-, dA62-, dU70- and dC72-substituted U6 RNAs (data not shown) confirmed that these individual deoxynucleotide substituents caused the splicing defects observed (cf. Figures 3 and 4).

Native gel analysis of spliceosome assembly in yeast extracts reconstituted with deoxynucleotide-substituted U6 RNAs

For the 2'-deoxy U6 RNAs that blocked splicing activity, it was important to distinguish whether a spliceosome

Fig. 5. Splicing activity of U6-depleted yeast extract reconstituted with U6 RNAs substituted site-specifically with a single deoxynucleotide at each site from 74 through 88. Samples were incubated with [α - ^{32} P]uridine-labeled actin pre-mRNA at 23°C for 30 min, then total RNA was isolated and separated on a denaturing polyacrylamide gel to assay splicing activity: actin pre-mRNA substrate (lane 1); mock-treated extract (lane 2); U6-depleted extract (lanes 3 and 4); reconstitution of splicing activity with *in vitro* transcribed wild-type U6 snRNA (lane 5) and ligated U6 RNAs (from five pieces) substituted at nucleotides 74–88 with a single deoxynucleotide (lanes 6–20, respectively).

assembly step was blocked or whether spliceosomes were fully assembled and the block occurred at the first catalytic step. Hence, U6 reconstitution experiments which blocked or inhibited splicing were repeated exactly as for the splicing assays except that product mixtures were split in order to assay both splicing activity (on a denaturing gel) and spliceosome assembly (on a native gel). A number of spliceosome assembly intermediates have been separated on native gels and characterized biochemically (Cheng and Abelson, 1987; Moore *et al.*, 1993). In yeast, the biochemically characterized assembly intermediates are the ‘commitment complex’ containing pre-mRNA and U1 snRNP, the B complex (U2 snRNP addition), the A2-1 complex (U4–U5–U6 tri-snRNP addition) and the A1 complex (fully assembled spliceosomes). Complexes that contain first-step splicing products (A2-2) and second-step products (A2-3) were identified as well, but these are usually overlapped with A2-1 complexes on native gels. In the standard native gel assay for yeast spliceosome assembly, radiolabeled actin pre-mRNA is the assembly substrate, and partially and fully assembled spliceosomes migrate according to size with the fully assembled A1 complex at the top of the gel. The complexes are identified by inspection of the native gel with reference to a wild-type control lane. Because the A complexes are U6 dependent, they are readily identified via comparison with a U6-depleted control lane. To determine whether the samples generated A2-2 and A2-3 complexes (defined by the presence of spliced intermediates and products), the split portion of each sample was assayed for splicing activity on a denaturing gel.

Figure 7 shows the spliceosome assembly assays for U6 RNAs substituted with a single deoxynucleotide at position 51, 61, 62, 68, 70, 71 or 72 (or with a 2'-O-

Fig. 6. Splicing activity of U6-depleted yeast extract reconstituted with U6 RNAs substituted site-specifically with a single 2'-O-methyl nucleotide at position 62, 70 or 72. Samples were incubated with [α - ^{32} P]uridine-labeled actin pre-mRNA at 23°C for 30 min, then total RNA was isolated and separated on a denaturing polyacrylamide gel to assay splicing activity: mock-treated extract (lane 1); U6-depleted extract (lane 2); reconstitution of splicing activity with wild-type U6 RNA ligated from four pieces (lane 3) and ligated U6 RNAs substituted at positions 62, 70 and 72 with a single deoxynucleotide (lanes 4–6) or a single 2'-O-methyl nucleotide (lanes 7–9).

Fig. 7. Spliceosome assembly of U6-depleted yeast extract reconstituted with U6 RNAs substituted site-specifically with a single deoxynucleotide at position 51, 61, 62, 68, 70, 71 or 72 (or with a 2'-O-methyl nucleotide at position 51). Samples were incubated with [α - ^{32} P]uridine-labeled actin pre-mRNA at 23°C for 30 min, treated with heparin, and separated on a native polyacrylamide gel: mock-treated extract (lane 1); U6-depleted extract (lane 2); reconstitution of spliceosome assembly with wild-type U6 RNA ligated from four pieces (lane 3) and ligated U6 RNAs substituted at the positions noted with a single deoxynucleotide (lanes 4–11).

methyl nucleotide at position 51). Split samples were assayed concurrently for reconstitution of splicing activity (data not shown). The mock-treated yeast extract assembled A and B complexes (Figure 7, lane 1), and the corresponding splicing assay showed the formation of lariat intermediate and mRNA product, indicating that the A2-2 and A2-3 complexes had formed in this sample. When endogenous U6 was depleted by oligo-directed RNase H digestion, no splicing occurred and no A

complexes were assembled (Figure 7, lane 2), as expected for complexes that require U6 snRNP for assembly. When wild-type, ligated U6 RNA was added to the same stock of U6-depleted extract as a control, splicing activity and assembly of A complexes were restored (Figure 7, lane 3). Likewise in the same U6-depleted extract, the deoxynucleotide-substituted U6 RNAs which blocked splicing activity (i.e. dA51-, dA62-, dU70- and dC72-substituted U6), were all competent for assembly of A complexes (Figure 7, lanes 4–11). Although assembly of A complexes for dA62-substituted U6 was relatively diminished in the experiment shown (Figure 7, lane 7), replicate experiments (not shown) revealed that dA62 U6 assembled A complexes at relatively high levels. For dA51- and dA62-substituted U6, the absence of spliced intermediates and products on the corresponding denaturing gel (not shown) indicated that the A2-2 and A2-3 complexes were not produced in these samples.

Concentration effects of deoxynucleotide-substituted U6 RNAs in U6-reconstituted yeast extract

We tested various concentrations of U6 RNA in U6-reconstituted samples to determine whether the four inactive deoxy-substituted U6 RNAs (see above) were capable of reconstituting splicing activity at unusually high or low concentrations. Fabrizio *et al.* (1989) had titrated the concentration of wild-type U6 RNA for reconstitution of splicing activity in U6-depleted extract. As suggested by their titration data, the concentrations of added U6 RNA in our reconstituted samples were in the range of 15–20 nM. In addition to the four inhibitory deoxy-substituted U6 RNAs reported here, we tested the A51U mutant U6 RNA which blocks the second step of splicing and causes an accumulation of A2-2 complexes (Fabrizio and Abelson, 1990). As controls for normal assembly and splicing activity, we tested wild-type and dC66-substituted U6 RNAs. Reconstituted samples were split and assayed concurrently for splicing activity and spliceosome assembly (data not shown). For all reconstituted samples, the lowest concentration of added U6 (0.2 nM) produced the lowest levels of reconstituted activity, as expected from the published titration data. As the concentration of added U6 was increased in 10-fold increments (2, 20 and 200 nM), the levels of splicing activity increased for wild-type U6, dC66-substituted U6 and A51U U6 RNAs (this mutant blocked the second step of splicing as expected); however, no splicing activity was observed for dA51-, dA62-, dU70- and dC72-substituted U6 RNAs over the 10³-fold range of concentrations. All of the reconstituted samples showed assembly of complete spliceosomes (A complexes) at increasing levels as the concentration of added U6 was increased, except at the highest level of added U6 (200 nM) which caused a shift from A and B complexes to poorly understood C complexes (Cheng and Abelson, 1987). This shift occurred for all U6 RNAs tested, and suggests that the 200 nM level of added U6 was high enough to assess the upper limits of U6 concentration effects. Therefore, the 15–20 nM concentration range for U6 reconstitutions was appropriate, and no unusual concentration effects were found for the four deoxy-substituted U6 RNAs that blocked or severely inhibited splicing activity.

Discussion

In this study of the essential functional groups of the U6 phosphate-ribose backbone, the 2'-OH groups of U6 ribose rings were substituted site-specifically with 2'-H groups to determine which 2'-hydroxyls of U6 are required for splicing in yeast extract. The 2'-hydroxyl group of RNA can contribute binding energy to a bonded interaction or can stabilize a transition state to catalyze a reaction (Bevilacqua and Turner, 1991; Pyle and Cech, 1991; Musier-Forsyth and Schimmel, 1992; Perreault and Atman, 1992; Pyle *et al.*, 1992; Herschlag *et al.*, 1993a,b; Smith and Pace, 1993; Strobel and Cech, 1993; Pley *et al.*, 1994; Abramovitz *et al.*, 1996). In either case, it can act as a hydrogen bond donor or acceptor. In binding interactions, the 2'-hydroxyl group can be important for recognition of a ribonucleotide or a structural domain. In catalysis, it can act as an active site nucleophile, especially when deprotonated (e.g. by a metal hydroxide; Cech, 1987), and it can coordinate to active site metal ions that catalyze a reaction (Pley *et al.*, 1994). Also, 2'-hydroxyls may help to organize water molecules for catalysis in an active site. In contrast, a 2'-H group cannot participate in hydrogen bonding or coordinate to metal ions. A deoxyribose ring in DNA conforms to a C2' endo ring pucker, whereas a ribose ring in RNA conforms to a C3' endo ring pucker. The single deoxyribonucleotide substitutions made in our experiments are not expected to alter the C3' endo conformations of RNA structures; however, the multiple contiguous substitutions may or may not do so (Egli *et al.*, 1993; Ban *et al.*, 1994).

In our initial studies of the 2'-hydroxyls of yeast U6 RNA, we prepared three U6 RNAs substituted with seven contiguous deoxynucleotides (deoxy box 39–45, 46–52 and 53–59). Deoxy box 39–45 reconstituted splicing activity, whereas deoxy boxes 46–52 and 53–59 blocked splicing activity. Surprisingly, single deoxynucleotide substitutions at each site from 53 through 59 did not inhibit splicing. Therefore, deoxy box 53–59 was defective in splicing because it lacked two or more functionally essential 2'-hydroxyl groups. Recently, in the X-ray structure determination of a group I intron domain (P4–P6), a new binding motif was observed between adjacent phosphate-ribose backbones of the folded RNA which involved pairs of 2'-hydroxyls on contiguous nucleotides (T.R.Cech, personal communication). Such motifs are not necessarily sensitive to a single deoxynucleotide substitution but may be destabilized by multiple substitutions. In our subsequent studies, the 2'-hydroxyl groups of U6 at nucleotides 46–88 were screened individually.

Screens of the fifty 2'-hydroxyls of yeast U6 RNA at nucleotides 39–88, spanning the entire conserved central domain, showed that only two 2'-hydroxyl groups are required for splicing activity: A51 (in the conserved ACAGAG sequence) and A62 (next to the conserved AGC triad; Figure 1). A deoxynucleotide substitution at either position blocked the first catalytic step of splicing *in vitro*. Two other 2'-hydroxyl groups are required for efficient splicing: U70 and C72 (both in the loop of the 3' intramolecular stem-loop; Figure 1). A deoxynucleotide substitution at either of these positions severely inhibited splicing, and only traces of spliced products were generated. Native gel analysis revealed that dA51-, dA62-,

dU70- and dC72-substituted U6 RNAs were all competent for complete (or nearly complete) assembly of spliceosomes. The splicing defects for dA51-, dA62-, dU70- and dC72-substituted U6 RNAs were verified by repeating the chemical syntheses, ligations, purifications and splicing assays. The concentrations of added U6 RNA in U6-reconstituted samples were in the range of 15–20 nM, as suggested by the titration data for U6 reconstitutions (Fabrizio *et al.*, 1989). We tested a 10^3 -fold range of concentrations, spanning the titration data of Fabrizio *et al.*, and found no unusual concentration effects for the four inhibitory deoxy-substituted U6 RNAs.

In our experiments to define the chemical roles of the essential 2'-hydroxyl groups of U6, a 2'-*O*-methyl substituent at nucleotide A51, A62, U70 or C72 did not inhibit splicing activity, indicating that the 2'-OH groups at these positions in U6 do not act as hydrogen bond donors, nucleophiles or coordinated anions but rather as hydrogen bond acceptors or a neutral coordinated ligands. (In each case, the presence of the 2'-*O*-methyl group was confirmed by mass spectrometry.)

In previous studies to identify the functional elements of U6, mutagenesis experiments showed that three sequences in U6 RNA, all in the conserved central domain, are essential for splicing: the ACAGAG sequence (nucleotides 47–52 in yeast), the CAGC sequence (nucleotides 58–61) and the region of the 3' stem-loop bulge (nucleotides 80 and 81) (Fabrizio and Abelson, 1990, 1992; Vankan *et al.*, 1990; Madhani and Guthrie, 1992) (cf. Figure 1). Particular mutations of any one of these nucleotides will inhibit splicing as observed by *in vitro* and *in vivo* splicing assays. Some mutations block splicing activity whereas others specifically block the second step of splicing. In yeast, mutations of A51, G52 (both in the ACAGAG sequence), C58 or C59 (both in the CAGC sequence) are second-step blocks, whereas mutations of the other nucleotides block the first step of splicing. All of the first-step effects were found to block spliceosome assembly at the stage of U4–U5–U6 tri-snRNP addition to the pre-spliceosomal complex of pre-mRNA–U1–U2 (B complex); therefore, the tri-snRNP-containing A complexes were not formed. Mutational analysis of yeast U6 snRNA revealed that nucleotides 54–59 are base paired to U2 snRNA in U2–U6 helix I, an essential interaction for splicing activity *in vivo* (Madhani and Guthrie, 1992).

Cooperative with the splicing functions of U6 nucleotide bases are the functions of particular phosphate and ribose groups of the U6 backbone. Backbone phosphate groups of ribozymes and other RNAs provide catalytic metal-binding sites as well as binding sites for proteins and other RNA components (Cech, 1987). In U6, phosphate oxygen atoms that are required for splicing activity were identified at each of the three essential sequences of U6 (discussed above) by introducing specific phosphorothioate substitutions (Fabrizio and Abelson, 1992; Yu *et al.*, 1995). Transcription of phosphorothioate-substituted nucleotides introduces thiophosphate linkages, each with a non-bridging, (*R*)-configured sulfur atom. In yeast U6, a thiophosphate linkage between A49 and G50 or between A79 and U80 blocked splicing activity, just as observed for base mutations of the adjoining nucleotides A49, G50 and U80 (Figure 1). A thiophosphate linkage between C58 and A59 blocked the second catalytic step of splicing,

as observed for base mutations of the adjoining nucleotides C58 and A59 (Figure 1). The second step was strongly inhibited by a thiophosphate linkage between A51 and G52, nucleotides that specifically block the second step when mutated. The functions of the required phosphate oxygen atoms are correlated strikingly to the functions of the adjoining, essential nucleotide bases.

Comparing our 2'-deoxy substitution results with the results for base mutations and phosphorothioate substitutions in U6 RNA, we note the following. (i) A dA51 substitution in yeast U6 promoted spliceosome assembly but blocked the first step of splicing; therefore, the 2'-hydroxyl group at A51 is essential for splicing catalysis but not for spliceosome assembly. In contrast, base mutations of A51 blocked the second step of splicing. In spliceosomes, nucleotide A51 is held in close proximity to the 5' splice site of pre-mRNA (and to the same site in the lariat intermediate) as demonstrated by cross-linking experiments (Sontheimer and Steitz, 1993; Kim and Abelson, 1996). (ii) Similar to dA51-substituted U6 RNA, a dA62 substitution in the 3' stem-loop of yeast U6 promoted spliceosome assembly but blocked the first step of splicing; therefore the 2'-hydroxyl group at A62 is essential for splicing catalysis but not for spliceosome assembly. (iii) Interestingly, the splicing defects observed for dA51- and dA62-substituted U6 RNA appear similar to those observed previously for phosphorothioate-substituted U6 RNAs produced via transcription using α -S GTP or α -S UTP (Fabrizio and Abelson, 1992). All of these U6 analogs have modifications in the phosphate-ribose backbone that allow complete (or nearly complete) assembly of spliceosomes but block first-step catalysis. In contrast to the first-step effects of these backbone substituents, all nucleotide base mutations of yeast U6 which blocked the first catalytic step were found to block spliceosome assembly at the stage of U4–U5–U6 tri-snRNP addition (Fabrizio and Abelson, 1990). (iv) Yeast U6 with a dU70 or dC72 substitution in the 3' stem-loop promoted spliceosome assembly but severely inhibited the first catalytic step of splicing. Therefore, the 2'-hydroxyls of U70 and C72 are not absolutely required for splicing activity but are essential for splicing efficiency. The 2'-hydroxyl groups of U70 and C72 are the first functional elements identified in the loop region of the U6 3' stem-loop. (v) In contrast to the results for base mutations and phosphorothioate substitutions in U6, no deoxynucleotide substitutions specifically blocked the second catalytic step of splicing. Second-step effects might have been expected for dA51-, dG52-, dC58- or dA59-substituted U6 (cf. Figure 1). Of these, only dA51-substituted U6 blocked splicing activity and did so prior to the first catalytic step.

Three of the four 2'-hydroxyl groups identified as functionally essential in yeast U6 are part of the 3' stem-loop (Figures 1 and 8). Previously, the only known functional elements of the 3' stem-loop were the nucleotide bases at positions 80 and 81 (at the bulge) and the phosphate group of the adjacent A79–U80 linkage (discussed above). Our results corroborate that the 3' stem-loop plays an important role in splicing catalysis.

Recent results from investigations of phosphorothioate substitutions in U6 RNA and in domain V of group II self-splicing introns suggest that the intramolecular 3' stem-loop of U6 and the domain V stem-loop may be

Fig. 8. A comparison of secondary structure models for the 3' intramolecular stem-loop of yeast U6 (Fortner *et al.*, 1994) and for the domain V stem-loop of group II self-splicing introns (reviewed by Michel and Ferat, 1995). The essential 2'-hydroxyl groups identified for yeast U6 (this work) and for the domain V stem-loop (Abramovitz *et al.*, 1996) are illustrated.

related structurally and/or functionally. The three sites of phosphorothioate inhibition found in yeast U6 (at linkages A49–G50, C58–A59 and A79–U80) are equivalent to those found recently in U6 of the nematode *A. lumbricoides* (Fabrizio and Abelson, 1992; Yu *et al.*, 1995), and the effects on splicing were the same except that the thiophosphate linkage equivalent to A49–G50 in yeast had a weaker effect in *A. lumbricoides*. In the nematode system, an additional phosphate group was found to participate in splicing—a thiophosphate linkage equivalent to A59–G60 in yeast completely blocked splicing activity. It was known that mutations of the G60 nucleotide in yeast U6 also blocked splicing activity (discussed above). The additional phosphate group identified in spliceosomes. Unfortunately, the A59–G60 phosphate linkage of yeast U6 could not be screened via phosphorothioate substitution in the yeast experiments (Fabrizio and Abelson, 1992). The phosphate groups of nematode U6 equivalent to linkages A59–C60 and A79–U80 in yeast U6 correlate structurally with two phosphate groups found to be essential for the catalytic activity of domain V of group II self-splicing introns (Chanfreau and Jacquier, 1994) (Figure 8). These intriguing similarities between the U6 and domain V stem-loops support the hypothesis that both pre-mRNA splicing and group II self-splicing evolved from a common ancestor. Furthermore, the 3' stem-loop of U6 may have

structure–function relationships which are analogous to those of the domain V stem-loop (cf. Figure 8).

The noted similarities between the stem-loops of U6 and domain V allow us to compare the essential 2'-hydroxyl groups of the U6 stem-loop with those recently identified in the domain V stem-loop (Abramovitz *et al.*, 1996). In both yeast U6 and domain V stem-loops, the nucleotide linked to the 3' end of the conserved AGC triad, namely A62 in yeast U6 or C8 in domain V, possesses a 2'-hydroxyl group that is essential for splicing activity (Figure 8). The well-defined kinetic system of the group II ribozyme allowed for the assignment of a binding function to the C8 2'-hydroxyl group. Unfortunately, our splicing assay does not allow us to clearly distinguish between a binding function and a catalytic role. The two other 2'-hydroxyl groups of the U6 stem-loop that severely hindered splicing, at U70 and C72, are located in the loop (Figure 8). The 2'-hydroxyl groups at these two positions are essential for splicing efficiency and may be important for binding interactions in the spliceosome rather than for catalysis. In domain V, two 2'-hydroxyls in the GNRA-type tetraloop were identified as important for binding (Figure 8). Although the specific binding interactions of a GNRA tetraloop (Costa and Michel, 1995) are expected to differ from those of the pentaloop in the U6 stem-loop, the loop regions in both systems may be important binding domains.

The positions of essential 2'-hydroxyl groups in the yeast U6 stem-loop (i.e. A62 adjoining the AGC triad and loop nucleotides U70 and C72) correlate generally to positions of essential 2'-hydroxyls in domain V; however, several other 2'-OH groups of domain V were found to be important for splicing activity, especially on the 3' side of the stem-loop (Figure 8). In fact, U6 RNA appears to lack any counterparts to the domain V hydroxyls involved in catalysis. In the domain V studies, deoxynucleotide substitution of a catalytic nucleotide resulted in a 10- to 20-fold decrease in k_{cat}/K_m , reflecting inhibition of the rate-limiting step. Pre-mRNA splicing may have different rate-limited steps that are not affected strongly by analogous deoxynucleotide substitutions. The correlations between the functional phosphate groups of U6 and domain V (discussed above) certainly suggest that the U6 and domain V stem-loops are comparable structures that may be functionally equivalent. Comparing the essential 2'-hydroxyls of both stem-loops, we note that the few similarities found may point to key features in common for these two systems. Additional information about the tertiary structures and functional groups of U6 and domain V is required before one can make meaningful comparisons. The unknown role of the U6 3' stem-loop is intriguing, and studies are underway to incorporate photoreactive cross-linking nucleotides in this stem-loop to trace its interactions in yeast spliceosomes.

Materials and methods

Synthesis of U6 RNA fragments

Synthetic RNA oligonucleotides were chemically synthesized on an Applied Biosystems synthesizer model 380A. Nucleotide phosphoramidites were commercially obtained from Glen Research. Oligonucleotides were prepared for wild-type and deoxynucleotide-substituted sequences of U6 RNA: nucleotides 39–59 for the three-piece ligation, nucleotides 1–38, 39–59, 60–79 and 80–112 for the four-piece ligation

and nucleotides 1–38, 39–59, 60–76, 77–94 and 95–112 for the five-piece ligation strategy. For ligation of the RNA pieces of U6 using T4 DNA ligase (see below), complementary DNA oligonucleotides were synthesized to anneal to U6 nucleotides 20–100 (three- and four-piece ligations) and nucleotides 20–112 (five-piece ligation). For the all-RNA pieces of U6, oligonucleotides were purified via denaturing polyacrylamide gel electrophoresis. RNA was visualized by UV shadowing, eluted and ethanol precipitated. For deoxynucleotide substitutions in RNA oligonucleotides 60–79 and 77–94, a new procedure for RNA synthesis (Wincott *et al.*, 1995) was followed using *S*-ethyltetrazole (24 mol equiv; Aldrich) to activate phosphoramidites and using a new TBDMS-deprotection reagent, a mixture of *N*-methyl-pyrrolidinone (2.25 ml, anhydrous), triethylamine (1 ml) and triethylamine:3 HF (1.5 ml) (Aldrich), combined just prior to use (in the order listed) in a plastic tube kept at 50–60°C. A 250 µl aliquot of this reagent was added to each tube of base-deprotected oligonucleotide solid. After 1.5 h at 65°C, the fractions of each oligonucleotide were recombined and diluted with 4 ml of 0.1 M TEAA, pH 7, for purification on Nensorb Prep columns (DuPont) according to the manufacturer's instructions. After lyophilization, the partially purified oligonucleotides were dissolved in 10 mM Tris-HCl, 1 mM EDTA (or TE) buffer pH 7.5.

Pieces A(1–38) and C(60–112) of U6 RNA for the three-piece ligation were transcribed *in vitro* from a synthetic DNA template with T7 RNA polymerase (cf. Fabrizio and Abelson, 1990). Each transcription mixture (3 ml total) contained 40 mM Tris-HCl pH 8.1, 22 mM MgCl₂, 5 mM dithiothreitol (DTT), 1 mM spermidine, 0.01% Triton X-100, 8% polyethylene glycol (PEG) 8000, 4 mM each NTP, 5 mM DNA template, 660 U/ml RNasin (Promega), 10 U/ml PPase and 5000 U/ml T7 RNA polymerase (NEB) and was incubated at 37°C for 4 h. The C(60–112) transcription mixture was supplemented additionally with 16 mM GMP (to cap the 5' terminus as a 5'-monophosphate for ligation). For full-length U6 RNA, the template for transcription was *Bam*HI-cleaved pUC18 with the U6 gene (plus an extra coded G at the initiation site) under a T7 promoter. For radiolabeled U6 RNA with low specific activity, a 20 µl reaction mixture contained 1.2 mCi/ml [α -³²P]UTP (USB-Amersham) and 3 mM cold UTP. High specific-activity [α -³²P]uridine-labeled actin pre-mRNA was transcribed *in vitro* using SP6 polymerase (Boehringer Mannheim) as described by Lin *et al.* (1985) except that the reaction mixture was prepared with 3.4 mCi/ml [α -³²P]UTP and 50 µM cold UTP. All transcripts were purified via denaturing polyacrylamide gel electrophoresis. RNA was visualized by UV shadowing, eluted, extracted with phenol/chloroform pH 5.2 and ethanol precipitated.

Analysis of the deoxynucleotide-substituted U6 RNA fragments

After synthesis and partial purification on Nensorb columns, deoxy-substituted U6 oligonucleotides (nucleotides 39–59, 60–79 or 77–94) were analyzed by mass spectrometry to identify the full-length oligonucleotide and by partial alkaline hydrolysis to verify the position of the deoxynucleotide substituent. Aliquots of the U6 RNA fragments to be analyzed by mass spectrometry were precipitated by adding a 1/4 volume of 3 M ammonium acetate and 3 volumes of ethanol followed by cooling at –20°C overnight. The RNA pellet was washed with 75% ethanol, redissolved in 200 µl of sterile double-distilled water (ddH₂O) and precipitated again as before. The pellet was redissolved in sterile ddH₂O, and 1 µg of each sodium-free oligonucleotide was diluted to 10 µl (ddH₂O) and submitted for analysis by mass spectrometry. To verify the position of a deoxynucleotide substituent, 40–60 pmol of each deoxy-substituted U6 oligonucleotide (39–59, 60–79 or 77–94) was radiolabeled at the 5' end using [γ -³²P]ATP (~15 µCi), T4 polynucleotide kinase (NEB; 1 U/pmol RNA), 100 µM ATP, 70 mM Tris-HCl pH 7.5, 10 mM MgCl₂ and 5 mM DTT at 37°C for 1 h. The radiolabeled oligonucleotides were purified via denaturing polyacrylamide gel electrophoresis. After elution from the gel, each sample was treated with 6 µg of carrier tRNA and precipitated. Pellets were dissolved in 0.2 M NaHCO₃ buffer pH 9.2 to give ~0.25 µM solutions. Aliquots (20 µl) were heated at 90–95°C for 30 min, then put on ice. A 5 µl portion of each sample was separated on a 20% denaturing polyacrylamide gel to produce a partial hydrolysis ladder with a missing band indicative of the position of the deoxyribonucleotide substituent. Xylene cyanole and bromophenol blue dyes were restricted to lanes without sample.

Ligation of the U6 RNA fragments

Synthetic RNA oligonucleotides require a 5'-terminal monophosphate group for ligation. For Figures 3, 4 and 6, each oligonucleotide piece (except the 5' piece) of U6 RNA (100 pmol each piece) was

phosphorylated by incubating with 1 U/pmol of T4 polynucleotide kinase (NEB) and 5 µCi of [γ -³²P]ATP (USB-Amersham) in the supplied buffer (1× 70 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 5 mM DTT) at 37°C for 30 min, then ATP was added (72 µM) and incubation was continued for an additional 30 min. The specific activity of each phosphorylated oligonucleotide was determined by Cerenkov counting a 1 µl aliquot of the reaction mix (5 µM RNA) spotted on a Whatman DE-81 filter and washed three times in 0.3 M phosphate buffer to remove unincorporated [γ -³²P]ATP (Sambrook *et al.*, 1989). For Figure 5, the oligonucleotide pieces (except the 5' piece) of each U6 RNA were phosphorylated as a group (40–60 pmol each piece) using 1 U/pmol of T4 polynucleotide kinase, 0.1 mM ATP and 0.25 µCi of [γ -³²P]ATP/pmol of RNA in the supplied buffer at 37°C for 1 h.

For the three- and four-piece ligations, the oligonucleotide pieces of U6 were annealed to a DNA oligonucleotide complementary to U6 nucleotides 20–100 and then ligated by T4 DNA ligase as described by Moore and Sharp (1992), except that RNA oligonucleotides were heated at 90°C for 2 min for annealing and each ligation mixture (20 µl) contained 66 mM Tris-HCl pH 7.6, 6.6 mM MgCl₂, 10 mM DTT, 0.66 mM ATP, 3 µM each RNA piece and cDNA (0.9 mol equiv), and 1 U/µl of T4 DNA ligase (USB-Amersham, high concentration). The product mixtures were extracted with phenol/chloroform pH 5.2 and ethanol precipitated. Full-length ligated U6 RNAs were purified by denaturing polyacrylamide gel electrophoresis. For the five-piece ligations, U6 oligonucleotides in 20 mM Tris-HCl pH 8, 1 mM EDTA solution were annealed to a DNA oligonucleotide complementary to U6 nucleotides 20–112 and then ligated as described by Moore and Sharp (1992), except that the ligation mixture was ~0.5 µM for each RNA piece and cDNA (0.9 mol equiv).

U6 reconstitution of splicing activity in yeast extract

Yeast whole-cell extract was prepared according to Lin *et al.* (1985). To prepare U6-depleted extract, yeast extract was incubated with oligonucleotide d1 (complementary to yeast U6 nucleotides 28–54) to digest endogenous U6 snRNA via endogenous RNase H activity. For U6 depletion, each sample contained 40% yeast extract, 60 mM KPO₄ pH 7, 3 mM MgCl₂, 2 mM ATP, 1 mM spermidine, 3% PEG-8000 and 0.3 µM oligonucleotide d1 and was incubated for 30 min at 37°C, or 40 min at 34°C. As a control, extract without d1 was heated under parallel conditions. The U6-depleted extract was divided into 5 µl aliquots for the addition of a deoxynucleotide-substituted or wild-type U6 RNA to reconstitute splicing activity as described by Fabrizio *et al.* (1989). U6 RNA (1 µl of 0.1 µM U6, or ddH₂O for controls) was added to each 5 µl sample, and the reconstituted extracts were incubated at 23°C for 10 min to assemble U6 snRNPs. Then [α -³²P]uridine-labeled actin pre-mRNA (0.5 µl of 2 nM actin in 3× buffer) was added to each sample and incubated at 23°C for 30 min. The reactions were terminated by digestion and extraction of proteins as described by Lin *et al.* (1985). The radiolabeled actin pre-mRNA products were separated on a 7 or 8% denaturing polyacrylamide gel (29:1 acrylamide/bisacrylamide) to assay for splicing intermediates and products.

In vitro complex formation

Native polyacrylamide gel electrophoresis was performed as described by Cheng and Abelson (1987) for half of each splicing assay mixture (see above) in order to assay assembly of spliceosomes. Heparin (40 µg per 2 µl of yeast extract) was added to samples reconstituted with deoxy-substituted or wild-type U6 RNA (see above), then samples were loaded directly onto the native gel. Electrophoresis at 200 V was conducted at 4°C for 5 h.

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