Evidence for base-pairing between mammalian U2 and U6 small nuclear ribonucleoprotein particles

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Intramolecular and intermolecular snRNA cross-links were generated by irradiating HeLa nuclear extract with 365 nm light in the presence of the psoralen derivative AMT. After deproteinization, cross-linked RNAs were resolved by gel electrophoresis and identified as anomalously migrating species by Northern blotting. In addition to the U4/U6 snRNA cross-link, we detected an intermolecular U2/U6 cross-link, as well as several apparently intramolecular U1, U2, and U5 cross-links. Photoreversal of the U2/U6 cross-link with 254 nm irradiation released stoichiometric amounts of U2 and U6 snRNA. To localize the U2/U6 cross-link, the 3' end of U2 in the purified U2/U6 complex was labeled selectively using a novel oligonucleotide "splint" technique. The labeled U2/U6 complex was then subjected to rapid enzymatic RNA sequencing or to targeted digestion of the U2 and U6 components of the complex by RNase H and a panel of complementary oligonucleotides. The U2/U6 cross-link is located upstream of nucleotide 15 in U2 and downstream of nucleotide 85 in U6, suggesting that the phylogenetically conserved base-pairing between these regions (6 consecutive base pairs in human, *Drosophila melanogaster*, and *Caenorhabditis elegans*, 7 in *Schizosaccharomyces pombe* and *Trypanosoma brucei*, 8 in *Pisum sativum*, 11 in *Saccharomyces cerevisiae*) is significant.

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In eukaryotes, introns are removed from nuclear mRNA precursors by a multicomponent ribonucleoprotein complex called the spliceosome (for review, see Steitz et al. 1988). The fully assembled spliceosome consists of four separate small ribonucleoprotein particles (the U1, U2, U5, and U4/U6 snRNPs) and numerous auxiliary protein factors. The U1, U2, and U5 snRNPs each contain a single small nuclear RNA (snRNA), whereas the U4/U6 snRNP consists of two snRNAs base-paired to each other (Bringmann et al. 1984; Hashimoto and Steitz 1984; Rinke et al. 1985; Brow and Guthrie 1988). All four spliceosomal snRNPs share a core of six common proteins; other proteins are unique to a single kind of snRNP (Lührmann 1988).

During spliceosome assembly, the U1 snRNP base-pairs with the 5'-splice site (Zhuang and Weiner 1986; Legrain et al. 1988; Ruby and Abelson 1988; Seraphin et al. 1988; Siliciano and Guthrie 1988; Seraphin and Rosbash 1989) and the U2 snRNP interacts with the 3' region of the intron (Black et al. 1985; Chabot et al. 1985; Ruskin et al. 1988) initially through the protein factor U2AF (Ruskin et al. 1988) and later by base-pairing with

the branch site (Parker et al. 1987; Nelson and Green 1989; Wu and Manley 1989; Zhuang and Weiner 1989; Zhuang et al. 1989). In contrast, only protein factors are required for initial recognition of the 3'-splice site (Gerke and Steitz 1986; Tazi et al. 1986; Ruskin et al. 1988; Swanson and Dreyfuss 1988) and the polypyrimidine tract (Ruskin et al. 1988; Garcia-Blanco et al. 1989). The U4/U6 and U5 snRNPs are known to associate with each other before assembling into the spliceosome (Cheng and Abelson 1987; Konarska and Sharp 1987; Lossky et al. 1987; Lamond et al. 1988), but the role of these three snRNAs in mRNA splicing remains elusive.

The spliceosome undergoes dynamic changes in conformation and composition during mRNA splicing. The strongest evidence is that extensive base-pairing between U4 and U6 snRNA in the U4/U6 snRNP is labilized or lost when the first phosphoester bond transfer occurs (Pikielny et al. 1986; Cheng and Abelson 1987; Lamond et al. 1988; Blencowe et al. 1989). Thus, it is possible that base pairs between spliceosomal snRNAs may be formed or broken in the course of assembly, catalysis, and disassembly of the spliceosome. In an effort to detect such base-pairing interactions, we have induced intermolecular and intramolecular RNA cross-links in HeLa nuclear extract by 365 nm irradiation in the presence of the psoralen derivative (4'-amino-methyl-4,5',8-trimethylpsoralen (AMT; for review, see

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Cimino et al. 1985). Psoralen cross-linking appears to be specific for base-paired regions of RNA and DNA and has been used previously to demonstrate base-pairing between U1 and U2 snRNA and nuclear RNA (Calvet and Pederson 1981; Calvet et al. 1982), between U4 and U6 snRNA (Rinke et al. 1985), within U1 snRNA (Rinke et al. 1985; Calvet and Myers 1987), and probably between U3 and rRNA precursor sequences (Maser and Calvet 1989; Stroke and Weiner 1989). Here we report the identification and characterization of a novel, phylogenetically conserved base-pairing interaction between the 5' end of U2 and the 3' end of U6 snRNA.

Results

Initial characterization of the covalent U2/U6 cross-link

We generated intermolecular and intramolecular RNA cross-links in HeLa nuclear extract by 365 nm irradiation in the presence of the psoralen derivative (AMT). After cross-linking, the extract was deproteinized and the snRNAs were identified by Northern blotting. Cross-linking in the presence of AMT generated two slowly migrating bands of comparable abundance, labeled U4/U6 and U2/U6, which were not observed after irradiation in the absence of AMT (Fig. 1A, lanes 1 and 2). We attempted to increase the yield of U2/U6 (estimated to be <5% of total U2) by incubating a human β -globin mRNA precursor in nuclear extract pretreated with EDTA to block cleavage at the 5'-splice site and cause accumulation of spliceosomes (Abmayr et al.

1988); however, the yield of U2/U6 was unaffected (Fig. 1A, cf. lanes 3 and 4 with lane 2).

We tentatively identified the two major slowly migrating bands as cross-linked U4/U6 and U2/U6 complexes by probing identical Northern blots of cross-linked RNA for each spliceosomal snRNA separately (Fig. 1B). The slowest migrating band that reacts with the U4 and U6 probes almost certainly corresponds to the U4/U6 complex (Bringmann et al. 1984; Hashimoto and Steitz 1984; Brow and Guthrie 1988), which is known to be susceptible to psoralen cross-linking (Rinke et al. 1985). The faster migrating band reacted with U2 and U6 probes. We also detected a band, migrating slightly above U1, which probably corresponds to the intramolecular U1 cross-link described previously (Rinke et al. 1985; Calvet and Myers 1987). Separation of the cross-linked RNAs on higher percentage polyacrylamide gels revealed additional cross-linked products that probably represent intramolecular U1, U2, and U5 cross-links (data not shown). (In Fig. 1B, the U4 probe was slightly contaminated with the U2 probe.)

That the U2/U6 cross-link is as abundant as the previously characterized U4/U6 cross-link (Rinke et al. 1985) can be seen when the blots are probed for all five snRNAs simultaneously (Fig. 1A, lanes 2–4). Although a third slowly migrating band can be seen between U4/U6 and U2/U6 (Fig. 1B, lane U6), this species is at least 10-fold less abundant than U2/U6 and would not be detected in our subsequent analysis of the U2/U6 cross-link. The third band is seen only when a blot is probed with oligonucleotide U6_{90–105} and overexposed to compensate for the inefficiency of this probe, which reacts well with uncross-linked U6 but very poorly with U2/U6 (Fig. 1B, lane U6) because the oligonucleotide lies close to the U2/U6 cross-link (see below).

We then used targeted digestion of U2 to demonstrate that the U2/U6 band is an intermolecular cross-link between U2 and U6, not comigrating intramolecularly cross-linked U2 and U6 RNAs (Fig. 2). Cross-linked RNA was digested with RNase H and oligonucleotide U2_{154–172} (complementary to U2 nucleotides 154–172), and a Northern blot of the digestion products was probed for U2 or U6 RNA. As expected for a covalent intermolecular U2/U6 cross-link, RNase H cleavage of U2 increased the mobility of the U2/U6 complex to the same extent regardless of whether the Northern blot was probed for U2 (Fig. 2, cf. lanes 1 and 2) or U6 (cf. lanes 3 and 4). Targeted digestion of U2 did not affect the mobility of the U4/U6 complex. Similar results were obtained when U6 was digested with RNase H and a complementary oligonucleotide (data not shown).

To determine the stoichiometry of U2 and U6 in the U2/U6 complex, we analyzed the cross-linked complex from cells that had been metabolically labeled with [³²P]phosphate for a generation (Fig. 3). Nuclear extract from labeled cells was cross-linked, and spliceosomal snRNPs were immunoprecipitated with anti-Sm antibodies. The U2/U6 and U4/U6 complexes are precipitated efficiently under these conditions (data not shown). The immunoprecipitated RNAs were separated

Figure 1. Northern blot of cross-linked snRNAs generated by psoralen cross-linking of nuclear extract. (A) Irradiation of nuclear extract in the absence (lane 1) or presence (lane 2) of AMT psoralen; irradiation in the presence of AMT psoralen after pretreatment of nuclear extract with 4 mM EDTA for 30 min at 0°C followed by incubation under splicing conditions without (lane 3) or with (lane 4) 10 ng H β 6 mRNA precursor (Abmayr et al. 1988). This blot was probed for U1, U2, U4, U5, and U6 simultaneously. (B) Irradiation in the presence of AMT psoralen but without mRNA precursor. Identical lanes were probed separately for U1, U2, U4, U5, or U6 snRNA to identify cross-linked species. A labeled *Hpa*II digest of pBR322 was used to facilitate alignment.

Figure 2. RNase H digestion of the apparent U2/U6 complex confirms that U2 and U6 are covalently cross-linked. Cross-linked RNAs were annealed with a DNA oligonucleotide complementary to U2_{154–172} and incubated with (lanes 2 and 4) or without (lanes 1 and 3) RNase H. RNAs were identified by Northern blotting with a U2_{79–98} probe (lanes 1 and 2) or a U6_{43–60} probe (lanes 3 and 4).

by two-dimensional denaturing polyacrylamide gel electrophoresis (5% first dimension, 9% second dimension) with or without photoreversal of the cross-links between dimensions by 254 nm light (Rabin and Crothers 1979). Two pairs of discrete spots can be seen (U4 and U6, U2 and U6) that comigrate in the first dimension but separate in the second dimension of the photoreversed gel (Fig. 3, left); without photoreversal, the U4/U6 and U2/U6 complexes are obscured by background labeling (Fig. 3, right). The snRNAs were cut out of the gel, and Cerenkov radiation was measured with a scintillation counter. When the counts per minute were corrected for background (using a gel fragment adjacent to each measured spot) and for the size of each snRNA, the ratio of U2 to U6 was 1.1 and the ratio of U4 to U6 was 1.0. We conclude that the U2/U6 complex contains equimolar amounts of U2 and U6.

Although we have not strictly excluded the existence of an additional undetected RNA component in the U2/U6 complex, this seems improbable because (1) the U2/U6 complex migrates faster than U4/U6 in all gel systems tested, (2) inefficient cross-linking is unlikely to produce a ternary cross-link, and (3) no evidence for a third RNA was found in experiments designed to localize the U2/U6 cross-link (see Figs. 5–7). We note, however, that the U2/U6 band was occasionally diffuse (cf. Fig. 2 with Fig. 1), perhaps reflecting minor variations in the cross-linking protocol, gel electrophoresis, or the precise site of U2/U6 cross-linking.

Oligonucleotide splint technique for selective 3'-end labeling of RNA

Initially, we had hoped to identify the exact site of cross-linking between U2 and U6 by the rapid RNA sequencing strategy of Calvet and Myers (1987). However, we were unable to label the 3' end of either RNA in the

U2/U6 complex using RNA ligase and ³²P-labeled pCp, perhaps because psoralen monoadducts or the conformation of the U2/U6 complex rendered both 3' ends inaccessible. Nor were we able to identify the site of cross-linking by primer extension with reverse transcriptase, perhaps because psoralen monoadducts blocked efficient extension. We therefore developed a new technique for selectively labeling the 3' end of either U2 or U6 RNA in total deproteinized nuclear extract. An snRNA/DNA hybrid with a recessed RNA 3' end is created by annealing an appropriate oligonucleotide to the 3' end of the snRNA (see diagram in Materials and methods). Extension of the RNA 3' end with T7 DNA polymerase and a labeled deoxynucleoside triphosphate generates a full-length RNA bearing a unique, labeled, covalent DNA tail (Fig. 4A). The labeled products (~10⁵ cpm/band) were eluted from the gel shown in Figure 4A and identified as U2 and U6 by rapid enzymatic sequencing (Donis-Keller et al. 1977; data not shown, but see Fig. 5, U2, lanes 1–4). Using the same labeling protocol we were able to label U2 but not U6 in the U2/U6 complex (Fig. 4B); however, U6 was labeled in the U4/U6 complex, albeit inefficiently. The identity of the tailed cross-linked complexes in Figure 4B was subsequently confirmed by Northern blotting (data not shown). (Trace labeling of U2 in Fig. 4B, lane 3, is due to slight contamination of the U6 oligonucleotide with the U2 oligonucleotide purified on the same gel.)

Localization of the U2/U6 cross-link by rapid enzymatic RNA sequencing

Labeled U2/U6 complex was eluted from the gel shown in Figure 4B and subjected to rapid enzymatic se-

Figure 3. Photoreversal releases stoichiometric amounts of intact U2 and U6 from the U2/U6 complex. Nuclear extract from HeLa cells metabolically labeled with [³²P]phosphate was cross-linked, immunoprecipitated with Y12 antibodies, and separated by two-dimensional gel electrophoresis with (left) or without (right) photoreversal between dimensions. Autoradiograms were quantified as described in the text.

Figure 4. Oligonucleotide splint technique for selective labeling of snRNAs in total deproteinized nuclear extract. (A) U6 or U2 was labeled separately in 2 μ l deproteinized nuclear extract. (B) U2 (lane 2) or U6 (lane 3) was labeled separately in 15 μ l deproteinized cross-linked extract. As a control, U2 and U6 were labeled simultaneously in 2 μ l deproteinized extract (lane 1). Labeled products were fractionated on a 7% denaturing polyacrylamide gel and autoradiographed for 15 min with Kodak X-Omat AR film. Markers (lane M) are a *Hpa*II digest of pBR322.

quencing with RNase T1 (Fig. 5, lanes 9 and 10). As controls, labeled uncross-linked U2 snRNA (lanes 1–4) and labeled cross-linked U2 containing psoralen monoadducts (lanes 5–8) were sequenced in parallel. Any cross-link involving the labeled RNA (whether intramolecular or intermolecular) would be expected to generate a gap in the sequencing ladder (Calvet and Myers 1983). Because we were able to identify a continuous T1 ladder from nucleotide 183 to 31 for U2 in the U2/U6 complex (Fig. 5, cf. lane 10 with lanes 2–4), the cross-link must lie 5' to G₃₁ in U2. The T1 ladders of the U2/U6 complex (lane 10) and cross-linked U2 (lanes 6–8) are less distinct than that of uncross-linked U2 (lanes 2–4), presumably because random psoralen monoadducts cause band broadening.

Mapping the U2/U6 cross-link by targeted RNase H digestion

Knowing the the U2/U6 cross-link lies upstream of U2 nucleotide 31, we used the strategy shown in Figure 6A to localize the cross-link more precisely on U2. The 3' end of U2 in the U2/U6 complex was labeled by the oligonucleotide splint technique, and the labeled U2/U6 complex was digested with RNase H in the presence of one of three complementary oligonucleotides spanning U2 nucleotides 1–42 (see Fig. 8, below). As controls, uncross-linked U2 and U6 were labeled and digested in parallel. We conclude that the cross-link must be located near the 5' end of U2 within oligonucleotide A (nucleotides 1–15) because RNase H digestion with oligonucleotides B (nucleotides 16–30) and C (nucleotides 28–42) yields the same size U2 fragment with both the cross-linked U2/U6 complex and the uncross-linked U2 control (Fig. 6B) but does not digest the uncross-linked

U6 control (data not shown). Note that poly(U) must be added to the RNase H digestion to prevent self-cleavage caused by annealing of the poly(dA) tail with internal U-rich RNA sequences (Fig. 6B, cf. first three lanes).

We used the same experimental strategy to localize the U2/U6 cross-link on U6 (Fig. 7A). The 3' end of U2 snRNA in the U2/U6 complex was labeled as in Figure 6, and the labeled complex was digested with RNase H in the presence of one of eight complementary oligonucleotides spanning the entire length of U6 (see Fig. 8). As controls, uncross-linked U2 and U6 were labeled by the oligonucleotide splint technique and digested in parallel; all eight oligonucleotides digested tailed U6 RNA (Fig. 7B,C, left) but not tailed U2 RNA (data not shown). We deduce that the cross-link must be located near the 3' end of U6, downstream of nucleotide 85, because (1) digestion with oligonucleotides A, B, C, D, and E yields progressively faster migrating species, all of which migrate more slowly than intact tailed U2 snRNA (Fig. 7B,C); (2) the smallest major U6 digestion product ob-

Figure 5. Localization of the U2/U6 cross-link by rapid enzymatic RNA sequencing. The 3' end of U2 in the U2/U6 complex was labeled selectively by the oligonucleotide splint technique. After purification by gel electrophoresis, the labeled complex was sequenced enzymatically with T1 ribonuclease (lanes 9 and 10). As controls, uncross-linked unirradiated U2 (lanes 1–4) and uncross-linked irradiated U2 containing psoralen monoadducts (lanes 5–8) were labeled and sequenced in parallel. No T1 ribonuclease (lanes 1, 5, and 9); 0.1 μ g (lanes 2 and 6), 0.05 μ g (lanes 3, 7, and 10), or 0.02 μ g (lanes 4 and 8) T1 ribonuclease. Numbers (left) indicate positions of guanosines in human U2 sequence [for references, see Reddy 1986].

Having localized the cross-link between nucleotides 1 and 15 at the 5' end of U2 and nucleotides 85 and 106 at the 3' end of U6, we are left with the paradox that U2 oligonucleotide A (directed against one side of the cross-link) cleaves both U2 and U2/U6, while U6 oligonucleotide G (directed against the other side of the cross-link) cleaves U6 but not U2/U6. Apparently, RNA structure (which is altered by the cross-link) affects the ability of a complementary oligonucleotide to form a cleavable duplex, and even a short RNA/DNA duplex is sufficient to assure cleavage by RNase H.

We have demonstrated that irradiation of HeLa nuclear extract with 365 nm light in the presence of the psoralen derivative AMT induces a covalent cross-link between the 5' end of U2 (nucleotides 1–15) and the 3' end of U6 (nucleotides 85–106). Psoralen cross-linking is thought to be specific for adjacent pyrimidines on opposite strands, either within or at the end of a base-paired duplex (Calvet and Pederson 1981; Calvet et al. 1982; Rinke et al. 1985; Calvet and Myers 1987; Stroke and Weiner 1989; Teare and Wollenzien 1990; for review, see Cimino et al. 1985). We therefore looked for potential base-pairing interactions in the cross-linked region

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Figure 7. Mapping the U2/U6 cross-link by targeted digestion of U6 with RNase H and complementary oligonucleotides. (A) Deletion mapping strategy (for explanation, see Fig. 6A). (B) (Lanes U2/U6) The 3' end of U2 in the U2/U6 complex was labeled selectively, and the complex was digested by RNase H in the presence of oligonucleotides A (U6₁₋₁₇), B (U6₁₈₋₂₉), C (U6₃₀₋₄₂), D (U6₄₃₋₆₀), E (U6₆₁₋₇₄), F (U6₇₅₋₈₉), and G (U6₉₀₋₁₀₅). (Lane U2) The 3' end of uncross-linked U2 was labeled selectively to determine the mobility of intact U2 snRNA. (Lanes U6) Labeled uncross-linked U6 snRNA was digested with RNase H and the same panel of oligonucleotides as a control. All RNase H digestions contained 1 μ g poly(U). (C) The experiment in B was repeated with oligonucleotides D, E, F, and an additional oligonucleotide, E' (U6₆₅₋₈₄). In both B and C, oligonucleotide E is slightly contaminated with F. Markers (lane M) are as in Fig. 4.

lational initiation codon, and the work of Been and Cech (1987) suggests that conformational flexibility may actually be characteristic of RNA-catalyzed reactions.

Our physical evidence for a U2/U6 cross-link, combined with phylogenetic conservation of the potential for base-pairing in the region of the cross-link, strongly suggests that U2 and U6 form base pairs at some step in mRNA splicing. What might this step be? In principle, base-pairing between U2 and U6 could occur during spliceosome assembly, sequential phosphoester bond transfer, spliceosome disassembly, or recycling of spliceosomal snRNPs. Distinguishing between these possibilities may be difficult. We found that the U2/U6 cross-link could be generated in nuclear extract prepared according to Heintz and Roeder (1984) but not otherwise specially treated; however, the yield of cross-link was quite variable (data not shown) and was not affected by the addition of all four nucleoside triphosphates or by treatment of the extract with alkaline phosphatase to

deplete endogenous nucleoside triphosphates (data not shown). Nor was the yield of cross-link affected by incubating an mRNA precursor under splicing conditions with untreated extract or with extract pretreated with EDTA (Abmayr et al. 1988) to block cleavage at the 5'-splice site (Fig. 1 and data not shown). Although cross-linking between U2 and U6 might thus appear to be independent of mRNA splicing, no conclusions can be drawn because snRNPs in the extract are present in vast excess over the added mRNA precursor, and endogenous U2/U6 complexes generated in vivo might outnumber those generated during active mRNA splicing in vitro.

The spliceosome is dynamic

Our evidence for base-pairing between U2 and U6 is consistent with the idea that the spliceosome is a dynamic structure in which RNA folds and refolds in the course of the reaction. For example, the proposed U2/U6 base-

Figure 8. Location of cross-link on secondary structure models of U2 and U4/U6 snRNAs. Oligonucleotides used in the targeted RNase H digestion of U2 and U6 snRNAs (Figs. 6 and 7) are indicated. The cross-linked regions, as defined by experiments shown in Figs. 6 and 7, are boxed and include the proposed U2/U6 base pairs (Table 1). U2 is drawn according to Ares and Igel (this issue), and U4/U6, according to Brow and Guthrie (1988). For clarity, the maximally base-paired U4/U6 structure is shown, although the ability of U6 to base-pair with U4 in the U4/U6 complex and with U2 in the U2/U6 complex could be mutually exclusive (see text).

pairing would disrupt much of phylogenetically conserved U2 stem I (particularly in *S. cerevisiae*; Guthrie and Patterson 1988). This suggests that intramolecular U2 base-pairing and intermolecular U2/U6 pairing are mutually exclusive and occur at different steps in the splicing reaction. A related issue concerns U4 and U6, which are extensively base-paired in the U4/U6 snRNP prior to spliceosome assembly, as documented by melting studies (Bringmann et al. 1984; Hashimoto and Steitz 1984; Brow and Guthrie 1988), by psoralen cross-linking (Rinke et al. 1985), and by phylogenetic comparisons (Brow and Guthrie 1988). Yet U4/U6 base-pairing is either lost (Pikielny et al. 1986; Cheng and Abelson 1987; Lamond et al. 1988) or partially unwound after lariat formation (Blencowe et al. 1989), possibly by the action of an RNA helicase (Burgess et al. 1990). Can U6 form base pairs simultaneously with U4 and U2, or must the U4/U6 interaction be partially or completely disrupted before the U2/U6 interaction can take place? We are currently addressing this question experimentally. Finally, given the many compelling arguments that mRNA splicing is mechanistically (and perhaps ancestrally) related to group II self-splicing (Sharp 1985; Cech 1986; Bruzik and Steitz 1990), it is tempting to

speculate that the proposed U2/U6 base-pairing plays a role in catalysis itself; however, the absence of a consensus sequence for the proposed U2/U6 interaction suggests that this role is more likely to be structural than enzymatic.

Published data regarding the function of the 5' end of U2 and the 3' end of U6 are difficult to interpret unambiguously. Consistent with our proposed U2/U6 interaction, mammalian splicing is inhibited by RNase H digestion of the 5' end of U2 RNA (Black et al. 1985; Krainer and Maniatis 1985; Frendewey et al. 1987; Zillmann et al. 1988), by antisense oligonucleotides directed against the 3' end of U6 (Blencowe et al. 1989), and by mutations in U2 nucleotides 3–6 (J. Wu and J.L. Manley, pers. comm.). On the other hand, deletion of most of U2 stem I may have little effect on splicing in a *Xenopus* oocyte assay system (Hamm et al. 1989, but see Pan and Prives 1989), deletion or mutation of the 3' end of yeast U6 does not invariably abolish splicing (Frabizio et al. 1989), a hybrid human/yeast U2 functions in *S. cerevisiae* although the proposed U2/U6 base-pairing is severely affected (Shuster and Guthrie 1990; Ares and Igel, this issue), and a triple mutation within the proposed base-pairing region of U2 in *S. cerevisiae* has no apparent

Table 1. Phylogenetically conserved base-pairing between U2 and U6 snRNAs

Human	U2 5'	1 10 AUCGCUUCUCG : : : : : : : : U6 3' GCGA <u>A</u> GUGC - 10 - AGUAGGAA 95 87
<i>Drosophila melanogaster</i>	U2 5'	1 10 AUCGCUUCUCGG : : : : : : : : U6 3' GCGA <u>A</u> GUGC - 9 - AGUAGGAA 95 86
<i>Schizosaccharomyces pombe</i>	U2 5'	1 8 AUUCUCUC : : : : : : : : U6 3' AAGAGAG - 12 - AGUAGGAA 88 82
<i>Saccharomyces cerevisiae</i>	U2 5'	1 10 ACGAAUCUCUUUG : : : : : : : : U6 3' UUU <u>A</u> GAGAAAC - 9 - AGUAGGAA 102 92
<i>Caenorhabditis elegans</i>	U2 5'	1 8 AUCGCUUC : : : : : : : : U6 3' GCGA <u>A</u> G - 13 - AGUAGGAA 90 85
<i>Trypanosoma brucei</i>	U2 5'	1 7 AUAUCUU : : : : : : : : U6 3' UAU <u>A</u> GAG - 11 - AGUCGGAA 93 87
<i>Pisum sativum</i>	U2 5'	1 10 AUACCUUUCUCGG : : : : : : : : U6 3' AA <u>A</u> GAGCU - 10 - AGUAGGAA 86 79

For tabulation of all sequences except *C. elegans* U2 (Thomas et al. 1990) and *P. sativum* (B.A. Hanley and M.A. Schuler, in prep.), see Reddy (1986, 1990). Three additional base pairs can be drawn for *P. sativum* by bulging U₉₀ in U6. The distance between the proposed U2/U6 base-pairing and the conserved U6 sequence 5'-AAGGAUGA-3' spanning the end of stem II in the U4/U6 interaction domain (Brow and Guthrie 1988) is given in nucleotides. Two other conserved U6 sequences, 5'-GNGA-3' and 5'-CAAA-3', are underlined and doubly underlined, respectively.

phenotype (D.J. Field, D.M. McMaster, and J.D. Friesen, pers. comm.). How might these apparent contradictions and paradoxes be resolved? Although fundamentally similar, splicing in yeast and mammals differs significantly; the branch site, for example, is essential in one system (Parker et al. 1987) but almost dispensable in the other except under special circumstances (Wu and Manley 1989; Zhuang and Weiner 1989; Zhuang et al. 1989). Thus, evidence against the proposed U2/U6 base-pairing in yeast systems may simply indicate that this U2/U6 interaction is not rate limiting or is structurally redundant under the conditions tested; the proposed U2/U6 interaction might even be unnecessary for splicing but might rather function, for example, in efficient recycling of snRNPs that remain bound to the intron lariat after exon ligation. In any case, many highly conserved sequence elements, including the entire

stem-loop I of U1 (Liao et al. 1990) and much of the base-pairing between U4 and U6 (Fabrizio and Abelson 1990) appear to be dispensable in yeast, genetically or biochemically. The implication is that evolution provides a far more sensitive test of function than laboratory assays and that spliceosomal snRNA structures and sequences are both redundant and robust.

Clearly, additional experiments will be required to test the significance of the proposed U2/U6 base-pairing, but taken together, the biochemical and phylogenetic data build a strong case for the importance of this interaction between the two most highly conserved spliceosomal snRNAs. Compensatory base changes in U1 snRNA can suppress 5'-splice site mutations both in mammals (Zhuang and Weiner 1986) and in yeast (Seraphin et al. 1988; Siliciano and Guthrie 1988; Seraphin and Rosbash 1989), and compensatory base changes in

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the addition of oyster glycogen carrier (1 μ g), the RNA was deproteinized, ethanol-precipitated, purified by denaturing gel electrophoresis, and eluted into 400 μ l of elution buffer [10 mM Tris-HCl (pH 7.8), 500 mM sodium acetate, 1 mM EDTA, 1% (wt/vol) SDS], and 400 μ l phenol/chloroform/isoamyl alcohol (50 : 49 : 1) by gentle overnight agitation at room temperature on a Nutator (Adams). Carrier tRNA was added, and the eluted RNA was ethanol-precipitated and subjected to rapid enzymatic RNA sequencing with T1 ribonuclease as described by Donis-Keller et al. (1977).

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