In vivo UV cross-linking of U snRNAs that participate in trypanosome *trans*-splicing

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The maturation of mRNAs in *Trypanosoma brucei* involves a *trans*-splicing reaction whereby the 5' 39 nucleotides of a small RNA, called the spliced leader (SL) RNA, are joined with a pre-mRNA transcript. The *trans*-splicing reaction appears mechanistically similar to *cis*-splicing of nuclear pre-mRNAs, and homologs of the U2, U4, and U6 snRNAs are required for the process. In the work presented here, potential RNA–RNA interactions between the SL RNA and the U snRNAs of trypanosomes were examined by UV light induction of RNA–RNA cross-links in vivo. We detected cross-linkage between U2 and U6 RNAs and, as might be expected, between the trypanosome U4 and U6 RNAs. The latter contain extensive sequence complementarity and are thought to exist predominantly in a single RNP. We also detected an SL RNA species following in vivo UV treatment, which may represent either an intramolecular cross-link in the SL RNA or a cross-link formed between the SL RNA and an as yet unidentified small RNA. Mapping of the cross-link position between U2 and U6 RNAs is consistent with base-pairing between the 5' domain of U2 and the 3' end of U6 RNA. These results reveal the existence, in vivo, of cognate RNA–RNA interactions in the RNA homologs that participate in *trans*-splicing in trypanosomes and *cis*-splicing in other eukaryotes.

[Key Words: *Trypanosoma brucei*; UV cross-linking; *trans*-splicing; SL RNA; U2 RNA; U6 RNA]

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The removal of introns from nuclear pre-mRNAs takes place in a two-step reaction in a large ribonucleoprotein (RNP) complex called the spliceosome (Brody and Abelson 1985; Frendeway and Keller 1985; Grabowski et al. 1985; for review, see Steitz et al. 1988). In addition to pre-mRNA, the spliceosome is composed of U1, U2, U4/U6, and U5 small nuclear ribonucleoprotein particles (snRNPs), as well as several auxiliary protein factors (Konarska and Sharp 1986, 1987; Pikielny et al. 1986; Cheng and Abelson 1987; Reed et al. 1988).

Several specific RNA–RNA interactions are known to occur in the *cis*-spliceosome; these include both small nuclear RNA (snRNA)–pre-mRNA and snRNA–snRNA base-pairing interactions. U1 and U2 snRNA, for example, base-pair with the 5'-splice site and branchpoint of the pre-mRNA, respectively (Zhuang and Weiner 1986, 1989; Parker et al. 1987; Ruby and Abelson 1988; Seraphin et al. 1988; Siliciano and Guthrie 1988; Nelson and Green 1989; Wu and Manley 1989), whereas U4 and U6 snRNAs are found base-paired extensively with one another in a snRNP particle (Bringmann et al. 1984; Hashimoto and Steitz 1984; Rinke et al. 1985; Brow and Guthrie 1988) and perhaps also in the pre-spliceosome. The U4/U6 interaction may be destabilized during the

splicing reaction because U4 dissociates from the spliceosome under conditions that maintain U4/U6 interactions in the small snRNP (Pikielny et al. 1986; Cheng and Abelson 1987; Lamond et al. 1988; Blencowe et al. 1989). As we learn more about the architecture of the spliceosome, a picture of a dynamic particle undergoing multiple rearrangements during the course of the two steps of the splicing reaction is emerging. By analogy with group II self-splicing reactions, which are mechanistically identical to nuclear pre-mRNA *cis*-splicing, it has been postulated that the RNA–RNA interactions occurring within the spliceosome may form the catalytic basis of nuclear pre-mRNA splicing (see Cech 1986; Jacquier 1990). If this is the case, knowledge of the changing RNA–RNA interactions occurring within the spliceosome at each step of the reaction may be required to understand catalysis of pre-mRNA splicing.

All mRNAs in the African trypanosome *Trypanosoma brucei* contain the same 5' 39-nucleotide spliced leader (SL) sequence, and addition of the SL to pre-mRNAs is thought to occur by a mechanism similar to *cis*-splicing of nuclear pre-mRNAs (Murphy et al. 1986; Sutton and Boothroyd 1986; Laird et al. 1987; for review, see Laird 1989; Agabian 1990). The SL of trypanosomes, however, is provided to pre-mRNAs in *trans* as part of an independently transcribed ~140-nucleotide RNA termed the SL

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RNA (Campbell et al. 1984; Kooter et al. 1984; Milhausen et al. 1984). Because of the discontinuous nature of the substrate SL and pre-mRNAs, *trans*-splicing yields an intermediate in the form of a Y structure rather than the lariat produced during *cis*-splicing. Recent evidence suggests that at least some of the trypanosome U snRNA homologs (i.e., U2, U4, and U6) are required for *trans*-splicing in *T. brucei* (Tschudi and Ullu 1990). Other than by extrapolation from *cis*-splicing, little is known directly about interactions between U snRNAs and the splicing substrates in the course of the *trans*-splicing reaction. Defining the roles of the U2, U4, and U6 RNAs in *trans*-splicing is particularly important because, despite apparent similarities between *cis*- and *trans*-splicing, homologs of U1 and U5 RNA have not been found in trypanosomes. Although it is difficult to prove their absence, it is clear that if homologs of U1 and U5 exist, they are not abundant trimethylguanosine (TMG)-capped RNAs and they do not contain conserved sequence elements found in their eukaryotic counterparts (Mottram et al. 1989; K. Watkins and N. Agabian, unpubl.).

The potential for RNA–RNA interactions, which are both similar to *cis*-splicing and unique to *trans*-splicing, emerges from a comparison between the sequences of the trypanosome U snRNAs and those of their homologs in other species. Like their eukaryotic counterparts, the trypanosome U4 and U6 molecules have substantial complementarity, and their sequences conform to a phylogenetically conserved secondary structure (Brow and Guthrie 1988; Mottram et al. 1989). In contrast, the U2 RNA branchpoint recognition region (the GUAGUA box), which is perfectly conserved in all other species, is not conserved in trypanosomes (Tschudi et al. 1986, 1990; Mottram et al. 1989; Hartshorne and Agabian 1990). Instead, this and adjacent regions of the U2 RNA have the potential to base-pair with the SL RNA. This observation, together with the apparent lack of U1 RNA in trypanosomes, led to the suggestion that the U2 RNA might fulfill both U1- and U2-like functions in *trans*-splicing; namely, recognition and juxtaposition of the regions of the substrate involved in the first step of splicing (Tschudi et al. 1986; Mottram et al. 1989; Patzelt et al. 1989; Hartshorne and Agabian 1990). The U snRNAs of *T. brucei* are also distinct in that they either lack or have a poorly conserved Sm protein consensus-binding site (Tschudi and Ullu 1986; Mottram et al. 1989; Hartshorne and Agabian 1990), and the protein components of the U snRNPs are not precipitable with anti-Sm antibodies (Michaeli et al. 1990).

In the experiments described below, the potential for RNA–RNA interactions between the RNAs that are thought to participate in *trans*-splicing was investigated by using UV light-induced cross-linking in vivo. This approach has been used successfully to probe RNA secondary and tertiary interactions in vivo in *Escherichia coli* RNA (Steige et al. 1986; Mitchell et al. 1990) and, in several instances, in vitro, most notably in a plant viroid RNA and human 5S rRNA (Branch et al. 1985) and in the *Tetrahymena* ribozyme (Downs and Cech 1990). How-

ever, direct UV cross-linking has not been used previously to examine potential spliceosomal RNA interactions.

We have determined that U4 and U6 RNAs are cross-linked as a result of UV irradiation in vivo. Surprisingly, a major cross-link was also found between U6 and U2 RNAs, and an SL RNA species was detected that consists of the SL RNA cross-linked either to itself or to another RNA that is not U2, U4, or U6. Results of oligonucleotide-directed cleavage of the U2/U6 molecule with RNase H locate the region of interaction between the 3' end of U6 and the 5' end of U2 RNA. This corresponds well with results reported by Hausner et al. (1990), who used psoralen cross-linking in vitro to demonstrate an interaction between the human U6 and U2 RNAs in RNA splicing extracts. A secondary structure that may account for the observed U2/U6 cross-link is proposed.

Results

Formation of RNA–RNA cross-links following UV irradiation in vivo

The potential of UV irradiation to define specific interactions that occur in vivo between the U snRNAs involved in *trans*-splicing was investigated in *T. brucei*. Bloodstream-form trypanosomes were irradiated for various times with 254 nm light at an intensity of 10 mW/cm². The UV dosage near the center of the time course corresponds to an exposure found in other systems to produce efficient RNA–RNA or RNA–protein cross-links without substantial RNA degradation (Steige et al. 1986; Woppmann et al. 1988). Analysis of the RNA purified from irradiated cells revealed a silver-staining pattern in which several candidate cross-linked RNAs appear in the 250- to 350-nucleotide region of the gel; only minor degradation of the small RNAs was observed after 30 min of irradiation (Fig. 1). It is expected that UV-induced covalent bond formation between small RNAs would generate novel RNA species whose mobility upon gel electrophoresis would be shifted to a higher apparent molecular weight. Preliminary analysis indicates that the major new RNA species detected by silver staining (Fig. 1) consist of cross-links formed between the small fragments of the 28S rRNA (srRNAs 1, 2, 4, and 6) and an intramolecular cross-link formed in the 7S signal recognition particle RNA (see Zwieb and Schüller 1989; data not shown).

Detection of specific U snRNA and SL RNA cross-linked species

The formation of intermolecular cross-links between the *T. brucei* SL RNA or U snRNA as a result of UV treatment in vivo was assayed by Northern analysis of the RNA in Figure 1 with U2, U4, U6, and SL RNA probes. With all four probes, increasing irradiation resulted in the detection of increasing amounts of hybridizing RNAs, which migrate in the 240- to 280-nucleotide range (Fig. 2). No hybridization is apparent in this area in the unirradiated control. In some cases, the pattern of

Figure 1. Silver stain of small RNAs isolated following UV irradiation in vivo. Equal amounts of total RNA (2.5 μ g), prepared from cells after UV irradiation for the indicated times, were denatured and resolved on a 6% polyacrylamide–8 M urea gel and stained with silver. Lane M contains pBR322 digested with *Msp*I. The lane corresponding to the 1-min time point is reproduced at right to indicate more clearly the mobility and relative concentrations of the RNAs discussed in the text. The SL RNA comigrates with srRNA4 in this gel system.

hybridization of the candidate cross-linked RNAs detected with the different probes appeared to consist of similar, potentially overlapping signals. For example, hybridization with the SL RNA probe revealed a doublet, which migrates at $\sim$ 240 nucleotides in RNA from UV-irradiated cells (Fig. 2, lanes 1–6). The mobility of the doublet is comparable to that of the fastest migrating bands observed with the U2 and U6 probes. Similarly, hybridization with a U6 RNA probe produced a pattern consisting of a major species of $\sim$ 280 nucleotides and two minor bands of 260 and 240 nucleotides superimposed on a general smear of hybridization (Fig. 2, lanes 19–24). This pattern is similar to the sum of that detected with the U2 and U4 snRNA probes (Fig. 2, lanes 7–12 and 13–18, respectively). The distinct smear of hybridization at 240–280 nucleotides detected for U4 and U6 RNAs varied qualitatively from experiment to experiment. Gels run at high temperatures resulted in a more pronounced smearing of the signal, which could be resolved into more discrete bands in gels run under more standard conditions (cf. U4 hybridization in Figs. 2 and 3). This probably reflects the unusual stability of the *T. brucei* U4/U6 molecule (Mottram et al. 1989) and may be the result of resolution of alternative, partially denatured, conformers and/or multiple cross-links between the U4 and U6 RNA. In addition to the hybridizing

RNAs migrating in the 240- to 280-nucleotide range, a U4 hybridization signal of $\sim$ 50 nucleotides was also detected. Whether this is a U4 RNA fragment or whether it represents an aberrantly migrating U4 RNA with an internal cross-link is under investigation.

T. brucei contains a fourth U snRNA, designated B, that does not appear to have a homolog in other eukaryotes (Mottram et al. 1989). Hybridization of a Northern blot identical to those shown in Figure 2 with a probe complementary to the trypanosome RNA B showed only full-length RNA with no potential cross-linked species observed (data not shown).

Evidence for the formation of U4/U6, U2/U6, and SL/X cross-linked RNAs

The results shown in Figure 2 are consistent with the formation of U4/U6, U2/U6 and, perhaps, SL/U RNA cross-linked molecules as a result of in vivo UV-irradiation. To determine the composition of the candidate cross-linked RNAs, total RNA prepared from UV irradiated cells was hybridized in solution with oligonucleotides complementary to either the SL or U RNAs and digested with ribonuclease H. Alterations in the pattern of cross-linked species as a consequence of RNase H digestion was evaluated by Northern analysis of the oligonucleotide–RNase H-treated RNA. One would expect that a molecule made up of two RNAs joined by a cross-link would be altered by RNase H digestion in the presence of oligonucleotides complementary to either RNA while cross-linked RNAs, which are distinct but comigrate in the gel, would be independently affected. For example, a U4/U6 cross-linked RNA should be degraded by RNase H in the presence of either U4 or U6 complementary oligonucleotides. The resulting U4/U6 fragment would be expected to shift mobility and be detected by both U4 and U6 probes. The full-length, non-cross-linked U4 and U6 RNAs serve as internal controls to verify the specificity of the reaction.

Ribonuclease H treatment of the in vivo UV cross-linked RNA (Fig. 3) resulted in the degradation of the SL RNA doublet of $\sim$ 240 nucleotides only when the oligonucleotides complementary to the SL RNA itself were included in the reaction (Fig. 3A, lanes 4–5). The entire SL RNA sequence must be contained within the cross-linked doublet because the two oligonucleotides used, SLa and SLb, are complementary to the 3' and 5' ends of the SL RNA, respectively. From the SL RNA analysis shown in Figure 3A and the remainder of the experiment (Fig. 3B–D), which demonstrates the specificity of the reactions, it can be concluded that the SL RNA is not cross-linked to the U2, U4, U6, or RNA B molecules.

The experimental data in Figure 3 also provide evidence for UV-induced formation of U4/U6 and U2/U6 cross-links and are consistent with the suggestion made in the previous section that the U6 hybridization pattern in the 240- to 280-nucleotide range represents a summation of that detected by both U2 and U4 probes. For example, the U2 RNA and the cross-linked molecules detected with the U2 probe are specifically degraded by

Figure 2. Northern analysis of RNA isolated following UV irradiation. Aliquots of 5 μ g of each RNA sample shown in Figure 1 were resolved in four panels of two gels, transferred to a nylon membrane, and hybridized with probes complementary to the SL RNA (lanes 1–6), U2 RNA (lanes 7–12), U4 RNA (lanes 13–18), or U6 RNA (lanes 19–24). Note that on each of the two gels, one set of RNA samples was loaded in inverse order so that the mobility of any cross-linked RNAs could be compared more directly. The positions of the full-length RNAs and the approximate size of the major UV-generated RNAs are indicated. Lane M contains an end-labeled *Msp*I digest of pBR322 (sizes indicated in Fig. 1).

RNase H when the RNA is annealed with either of two U2 RNA complementary oligonucleotides (Fig. 3B, lanes 6,7). The U2a and U2b oligonucleotides do not affect the cross-linked molecules detected with the U4 probe (Fig. 3C, lanes 6,7); however, both U2a and U2b induce specific degradation of a subset of the cross-linked molecules detected with the U6 probe (Fig. 3D, lanes 6,7). In this case, a pattern of U6 hybridization remains that is similar to the pattern observed with the U4 RNA probe. This is expected because the U6 RNA probe detects both U2/U6 and U4/U6 cross-linked molecules, and the U4/U6 molecule would not be affected by RNase H treatment in the presence of U2 complementary oligonucleotides. Similarly the full-length U4 molecule, as well as molecules presumed to be U4/U6 cross-linked RNAs, is degraded by RNase H after hybridization with the U4 complementary oligonucleotide (Fig. 3C, lane 8), and a signal that we attribute to the presence of U2 cross-linked to U6 RNA remains unaltered in this sample (Fig. 3B,D, lanes 8). Finally, RNase H treatment in combination with the U6 complementary oligonucleotide results in the specific degradation of the cross-linked RNAs detected by all three U RNA probes (Fig. 3B–D, lanes 9). The major band of ~280 nucleotides, representing U2/U6 cross-linked RNA, is degraded to a fragment of the same size in these samples (Fig. 3B,D, lanes 9).

Mapping of the cross-link position of the U2 and U6 snRNAs

The hybridization patterns of the RNase H-digested U RNAs in Figure 3 allow an approximation of the site of cross-linking between U2 and U6 RNA. For example, when the U2/U6 cross-linked RNA species is digested with RNase H in the presence of either the U2a or U2b oligonucleotide, a digestion fragment is produced in the U2a-treated reaction that is smaller than that in the U2b-treated reaction (Fig. 3D, lanes 6,7; for positions of oligonucleotides, see Fig. 5, below). This suggests that the U2/U6 cross-link is positioned 5' of the U2a complementary region in the U2 RNA. Likewise, U6a oligonucleotide-directed RNase H cleavage of the U2/U6 species leaves an even larger fragment (Fig. 3B,D, lanes 9), indicating that the U2/U6 cross-link is most likely positioned 3' of the U6a complementary region in the U6 RNA. If this were not the case (i.e., if the cross-link is located, instead, in the 5' region of the U6 RNA), the small change in mobility of the U2/U6 molecule after RNase H cleavage in the presence of the U6a oligonucleotide would be surprising; U6a-mediated cleavage would be expected to leave only 27 nucleotides of U6 RNA attached to U2 RNA.

In several instances, oligonucleotide-directed RNase H

Figure 3. Oligonucleotide-directed RNase H cleavage of cross-linked RNAs. RNA (20 μ g) prepared from cells following 10 min of UV irradiation was hybridized with the oligonucleotides indicated and either mock-treated (lanes 1) or treated with RNase H (lanes 2–9). As in Fig. 2, RNA purified from each reaction was split into four aliquots, resolved in four panels of two gels, and hybridized with probes complementary to the SL, U2, U4, or U6 RNAs (A–D, respectively).

cleavage of the cross-linked molecules resulted in the formation of smaller species detected by only one of the probes. For example, U6a oligonucleotide-directed cleavage of the U4/U6 cross-linked RNA produces a molecule that migrates as a $\sim$ 190-nucleotide species (Fig. 3C, lane 9) and hybridizes faintly with the U6 probe (Fig. 3D, lane 9). This result is not surprising if one considers the proposed secondary structure of U4/U6 RNAs, the position of the U6a oligonucleotide complementary region relative to this structure, and the stringent hybridization and wash conditions used in this experiment. If the U4 and U6 RNAs are cross-linked through the helix formed with the 5' 17 nucleotides of U4 RNA (see Fig. 5, below), U6a-directed RNase H cleavage of such a molecule might leave a cross-linked species unable to form a stable hybrid with the U6 probe. A similar argument might explain our inability to detect complementary cleavage products in the U2a and U2b lanes hybridized with U2 and U6 probes (Fig. 3B,D, lanes 6,7). It is interesting that in Figure 3B (lane 7), RNase H treatment in the presence of the U2b oligonucleotide, in addition to cleaving the U2/U6 cross-linked species, completely obliterates the faint smear of hybridization seen throughout the lane. This suggests that this smear is not simply background hybridization but, instead, may represent cross-links formed between U2 RNA and molecules of heterogeneous sizes. One possibility being investigated is that this signal reflects cross-linkages between the U2 RNA and the branchpoint of pre-mRNAs.

To assign the regions of U2/U6 interaction with

greater accuracy, RNase H cleavage experiments similar to those shown in Figure 3 were carried out with gel-purified cross-linked RNA. Gel purification of the cross-linked molecules indicated that the U2/U6 cross-linked RNAs were more complex than initially predicted. The size-selected molecules, designated A–H, are displayed in Figure 4A alongside the original cross-linked sample. Although the Northern analysis shown in Figure 2 indicated the presence of a major U2/U6 cross-linked RNA of $\sim$ 280 nucleotides and two minor species of 260 and 240 nucleotides, gel purification further resolves these RNAs into at least eight distinct species.

Aliquots of the gel-purified RNA samples were annealed with either the U2a or U6b oligonucleotide and then digested with RNase H. In contrast to the experiment shown in Figure 3, in this assay, the fragments of the cross-linked molecules liberated by RNase H cleavage can be detected and compared directly with parallel reactions carried out with un-cross-linked RNA. In Figure 4, gel-purified samples A–H and un-cross-linked U2 RNA have been hybridized with a 3' U2 probe after mock RNase H treatment (Fig. 4A), incubation with the U6b oligonucleotide and RNase H (Fig. 4B), incubation with RNase H only (Fig. 4C), or treatment with the U2a oligonucleotide and RNase H (Fig. 4D). Samples treated with RNase H and the U6b oligonucleotide generate a U2/U6 fragment that is larger than the U2 RNA but substantially smaller than the fragment produced by U6a oligonucleotide-directed cleavage (Fig. 4B; cf. with Fig. 3B, lane 9). In addition, the size distribution of U2/U6

Figure 4. Oligonucleotide-directed cleavage of gel-purified U2/U6 cross-linked molecules. RNA prepared after 20 min of UV irradiation in vivo and migrating between 220 and 320 nucleotides in a denaturing 6% polyacrylamide gel was resolved into eight fractions designated A–H. Molecules migrating at 100–160 nucleotides and containing un-cross-linked U2 and U6 RNAs were also purified. The size-selected RNA was then mock-treated (A), treated with RNase H and the U6b oligonucleotide (B), treated with RNase H only (C), or incubated with RNase H and the U2a oligonucleotide (D). Reactions carried out on the cross-linked RNA are indicated by lanes A–H and on the un-cross-linked control by lane U. In A, ~1 μ g of the unpurified starting RNA was resolved in parallel (lane X). A–D were hybridized with a probe complementary to the 3' 70 nucleotides of the U2 RNA.

species A–H in Figure 4A is mirrored in the U6b digests for at least RNAs A–D, suggesting that the size differences observed for the U6 cross-linked molecules may represent different, perhaps adjacent, cross-link points. Rehybridization of the blot shown in Figure 4, A and B, with a U6 RNA probe indicated further that RNase H specifically cleaved U6 RNA only in the presence of the U6b oligonucleotide and cleaved the U6 RNA and samples A–D to yield a fragment of the same size (data not shown). Although the RNase H used in the experiment shown in Figure 4 apparently contains nonspecific ribonucleases, their presence affects the aesthetic quality but not the conclusion of the experiment. Because of the paucity of U2/U6 cross-linked material in samples E–H, we have been unable to determine whether the material in these samples consists of U2 cross-linked to U6 RNA in the same region as the major cross-link represented by samples A–D. The possibility remains that these RNAs are resolved from the major U2/U6 cross-linked species as a result of cross-linking in an additional domain (see below).

Together, these results indicate that the major cross-link between the U2 and U6 RNAs in vivo occurs 3' of the U6b oligonucleotide complementary region in the U6 RNA. The observation that U2a/RNase H treatment cleaves U2 RNA and the purified cross-linked molecules to 3' fragments of the same size (Fig. 4D) confirms that the major region of cross-linkage between U2 and U6 occurs in the 5' region of U2 RNA upstream of the U2a complementary region.

Potential secondary structure of U2/U6 snRNAs

A computer-aided search revealed two regions of complementarity between the trypanosome U6 and U2 RNAs, which are consistent with the in vivo UV cross-linking data shown above. The two areas of complementarity (Fig. 5A) correspond to adjacent blocks in the 3' end of the U6 RNA and the lower portion of the stem-loop I and adjacent nucleotides in the U2 RNA (see Hartshorne and Agabian 1990). The model shown is also the most thermodynamically stable secondary structure predicted by the RNA folding program of Zuker and Steigler (1981) for a hypothetical sequence consisting of the 3' domain of the trypanosome U6 RNA fused to the 5' end of U2 as a single molecule. A portion of this structure, italicized in Figure 5A, was suggested by Hausner et al. (1990) after finding a psoralen-induced cross-link between HeLa cell U2 and U6 RNAs in vitro. Phylogenetic comparison of U6 and U2 sequences from human, bean, and yeast (both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*) revealed the potential for the formation of similar structures that extend through this region. As an example, a potential secondary structure for the *S. pombe* U2 and U6 RNAs is shown in Figure 5B.

Discussion

Our perception of nuclear pre-mRNA splicing is based on biochemical and genetic studies in human, *Xenopus*,

Figure 5. Potential U2/U6 secondary structure. (A) The *T. brucei* U4/U6 structure is redrawn from Mottram et al. (1989), together with the putative U2/U6 interaction region discussed in the text. Only the 5' 46 nucleotides of U2 are shown. The regions of complementarity of the oligonucleotides used in Figs. 3 and 4 are indicated except for U2b, which is complementary to nucleotides 52–72. For comparison, U2a hybridizes to nucleotides 29–42 of the U2 RNA. The region of this model also proposed by Hausner et al. (1990) is italicized. (B) Hypothetical secondary structure of *S. pombe* U6 and U2 RNAs. The 5' end of U2 and the 3' end of U6 are shown; the GUAGUA branchpoint recognition region of U2 RNA is overlined.

S. cerevisiae and, more recently, *S. pombe* cells. Several components required for nuclear pre-mRNA *cis*-splicing have been purified or characterized genetically, and many of the essential regions of the U snRNAs have been defined by using *in vivo* or *in vitro* systems (for review, see Steitz et al. 1988). Progress in understanding *trans*-splicing in trypanosomes has been slowed by the inability to reproduce the reaction *in vitro* and the fact that genetic transformation systems for studying the kinetoplastidae have only recently been developed. However, evidence obtained *in vivo* indicates that the reaction mechanism of *trans*-splicing is similar to that of *cis*-splicing (Murphy et al. 1986; Sutton and Boothroyd 1986; Laird et al. 1987), and with a recently developed permeabilized cell system it has been shown that the trypanosome U2, U4, and U6 snRNAs are required for SL addition to pre-mRNAs (Tschudi and Ullu 1990). *Cis*-splicing of nuclear pre-mRNAs is accompanied by the successive formation and disruption of multiple snRNA–snRNA and snRNA–pre-mRNA base-pairing interactions. In the work presented here, potential intermolecular interactions between RNAs known to participate in *trans*-splicing were investigated by UV

irradiation *in vivo*. In addition to detecting an SL RNA cross-linked species, we also observed cross-linkages formed between U4 and U6 RNAs and a major cross-link between U6 and U2 RNAs.

What can we conclude about the SL RNA cross-linked species?

UV irradiation of trypanosomes produces a cross-linked SL RNA species that migrates as a doublet of ~240 nucleotides. RNase H cleaves the SL RNA doublet when the cross-linked RNA is hybridized to oligonucleotides complementary to either the 5' or 3' end of the ~140-nucleotide SL RNA, indicating that both intron and exon regions of the SL RNA are contained within the cross-linked species. Whether the ~240 nucleotide SL RNA produced *in vivo* following UV irradiation can be attributed to intramolecular cross-link in the ~140-nucleotide SL RNA or whether it represents an intermolecular cross-link between the SL RNA and another small *T. brucei* RNA is not known. UV irradiation of total trypanosome RNA or synthetic SL RNA preincubated over a wide range of temperatures, salt, and magnesium chlo-

ride concentrations does not yield cross-linked SL RNA any larger than ~150 nucleotides (data not shown). If the 240-nucleotide species derives from an intramolecular SL RNA cross-link, mapping of its position may give insight into the secondary or tertiary structure of the SL RNA. Attempts to map the cross-link position(s) and to determine whether only SL RNA sequences are contained within the 240-nucleotide doublet RNA have not been successful because of insufficient quantities of cross-linked RNA. Although more cross-linked RNA can be produced as in vivo irradiation time increases, in our experience RNA prepared after prolonged irradiation is refractory to secondary analysis.

If an intermolecular RNA–RNA interaction is responsible for the cross-linked SL RNA species, it is difficult to know what this RNA might be. In *cis*-splicing, two U snRNAs, U1 and U5, have been implicated in 5' exon definition (Zhuang and Weiner 1986; Seraphin et al. 1988; Siliciano and Guthrie 1988; Newman and Norman 1991), and only U1 RNA has been shown to interact directly with the pre-mRNA. However, homologs of U1 and U5 RNA have not been found in trypanosomes. Clearly, further experiments are required to determine the nature of the ~240-nucleotide SL RNA cross-linked species.

Modeling interaction of U2 and U6 RNAs

We have shown that the 5' end of the *T. brucei* U2 RNA interacts directly with the 3' end of U6 RNA in vivo. A secondary structure, derived from phylogenetic comparison of potential base-pairing interactions between U2 and U6 RNAs is proposed to account for this observation (Fig. 5). The consensus secondary structure of the 5' end of all known U2 RNAs includes a hairpin with a stem that is interrupted by one or two unpaired nucleotides (Guthrie and Patterson 1988; Ares and Igel 1989,1990; Hartshorne and Agabian 1990). We suggest that the U2/U6 interaction consists of the base-pairing of U2 sequences on either side of the hairpin with the 3' end of U6 RNA. In most cases, as illustrated by the *T. brucei* sequence in Figure 5, this interaction would disrupt the lower portion of the 5' stem of the U2 RNA. In the case of *S. pombe* (Fig. 5B), however, the complete 5' stem-loop of U2 RNA remains unaltered and the potential exists for extensive base-pairing with U6 RNA through the branchpoint recognition region of U2 RNA. Although not as striking as the *S. pombe* example, all available U2 and U6 RNA sequences have the potential to form similar structures. In some cases, the proposed U2/U6 interaction domain encompassing the branchpoint recognition region of U2 RNA is shifted 3 nucleotides in the 5' direction on the U6 RNA (not shown).

The U2/U6 RNA interactions that have been suggested here extend over a larger region of complementarity than those proposed by Hausner et al. (1990). Clearly, defining the limits of these potential interactions will require precise mapping of cross-link positions and may be aided by more extensive phylogenetic comparisons as more U2 and U6 RNA sequences become

available. The model proposed here is not meant to supersede that of Hausner et al. (1990) but, rather, to point out additional regions of potential interaction that should be investigated biochemically or genetically. Attempts to map the *T. brucei* U2/U6 RNA cross-link positions either indirectly by primer extension or directly by the splint-labeling procedure developed by Hausner et al. (1990) have not been successful. The complex pattern observed in the gel-purified samples (Fig. 4) may be a reflection of several cross-link positions or, alternatively, an RNA population consisting of a single U2/U6 cross-link with additional base modifications as a result of UV treatment. Given that UV-induced cross-links in any single RNA are relatively rare and that the U2/U6 RNA cross-links in samples A–D in Figure 4 map to the same region of the U2 and U6 molecules, the latter possibility is likely. The U2/U6 RNA cross-links in samples E–H in Figure 4, however, may represent a distinct site of cross-linkage. Preliminary primer extension results indicate that these RNAs may consist of U6 cross-linked to a region of U2 RNA 3' of the U2a oligonucleotide complementary region (data not shown). Interaction of these molecules at the U2/U6 complementary domain pointed out by McPheeters et al. (1989) may account for this observation.

Potential significance of U2/U6 RNA base-pairing

Although it is plausible that the U2/U6 base-pairing interactions described here have a role outside of the function of U2 and U6 RNAs in splicing (e.g., cotransport of snRNP particles, recycling of snRNPs, etc.), both regions of U2 and U6 RNA proposed to base-pair (Fig. 5) are required for spliceosome assembly in *cis*-splicing (Chabot and Steitz 1987; Friendewey et al. 1987; Zillmann et al. 1988; Barabino et al. 1989; Lamond et al. 1989; Bindereif et al. 1990; Vankan et al. 1990). Whereas the necessity of the 3' region of U6 RNA in *T. brucei* *trans*-splicing has not been tested, destruction of the 5' domain of U2 RNA has been shown to inhibit the reaction (Tschudi and Ullu 1990). The kinetic step in which the 5' domain of U2 RNA is required in *cis*-splicing (Barabino et al. 1989; Lamond et al. 1989) corresponds to the same stage of the reaction in which the U4/U6 RNA structure is disrupted in the *cis*-spliceosome (Pikielny et al. 1986; Cheng and Abelson 1987; Lamond et al. 1988), suggesting that perhaps the U4/U6 RNA dissociation reflects the coincident association of U6 and U2 RNA.

Although little is known about the components that participate in *trans*-splicing in trypanosomes, characterization of U snRNA homologs participating in the reaction has provided support for the widely held assumption that the reaction takes place within a particle analogous to the *cis*-spliceosome. We have shown that the majority of the SL RNA exists as a small ~10S RNP in *T. brucei* extracts (Michaeli et al. 1990). A minor fraction of the SL RNA is also found in nuclear extracts in a 40S RNP, and the Y-branched *trans*-splicing intermediate appears to be associated with a slightly larger, 50S particle (K.P. Watkins, S. Michaeli, and N. Agabian, unpubl.). The copuri-

fication of U2 and U6 RNAs with the 40S and 50S particles provides additional evidence for the existence of a *trans*-spliceosome. In the absence of an *in vitro* assay, it is difficult to prove that such RNPs represent functional *trans*-spliceosomes; however, the detection of such complexes suggests that we may be able to resolve an SL RNA-containing pre-spliceosome from a spliceosome containing splicing intermediates. In this manner it will be possible to directly characterize the RNA components required at different stages of the *trans*-splicing reaction. As demonstrated herein, the fact that U2, U4, and U6 interact in *trans*-splicing via similar base-pairing interactions as found in *cis*-splicing suggests that the molecular basis for recognition between splicing substrates is evolutionarily conserved and is dictated by similar RNA–RNA interactions.

Materials and Methods

DNA oligonucleotides, plasmids, and RNA probes

For the RNase H experiments shown in Figures 3 and 4, oligonucleotides listed in Table 1 were used.

Probes for Northern analysis of SL, U2, U4, and U6 RNAs were obtained by SP6 polymerase transcription of plasmids pSPSL2, pGU2c, pTbU4, and pTbU6, respectively. pSPSL2 was linearized with *Sal*I, while pGU2c, pTbU4, and pTbU6 were linearized with *Eco*RI. For the Northern blot shown in Figure 4, a probe complementary to the 3' end of U2 RNA downstream of nucleotide 78 was used. This was made by SP6 transcription of pGU2c linearized with *Sty*I. Each clone contains the entire coding region of the respective small RNA with variable amounts (up to ~20 nucleotides) of flanking sequences. pSPSL2, pGU2c, and transcription procedures have been described (Michaeli et al. 1990). pTbU4 and pTbU6 were cloned by polymerase chain reaction (PCR) amplification from *T. brucei* genomic DNA by using an oligonucleotide complementary to the 3' end of the respective RNAs and containing SP6 promoter sequences, and a second oligonucleotide containing the T7 promoter adjacent to a portion of the 5' end of the U4 or U6 RNAs. After *Eco*RI and *Pst*I digestion, amplification products were cloned into *Eco*RI/*Pst*I-cleaved pUC18. For each oligonucleotide shown below, the transcription start site for T7 or SP6 RNA polymerase is superscripted, U4 or U6 DNA sequences are underlined, and boldface type indicates nucleotides corresponding to *Eco*RI and *Pst*I adaptor sequences. The oligonucleotides used in the construction of pTbU4 are oU4T7+ (5'-TTTGAATTCGTAATACGACTCACTATA^GAAGCCTTGCGCAGGGAGGTGT-3') and oU4SP6– (5'-ATATCTGCAGCTATTTAGGTGACACTATA-

^GAAGCTTCCCACCCGTGGTGGGTAGCA-3'). pTbU6 was constructed by amplification with oU6T7+ (5'-TTTGAATTCGTAATACGACTCACTATA^GGAGCCCTTCGGGGACATCCA-3') and oU6SP6– (5'-ATATCTGCAGCTATTTAGGTGACACTATA^GAATTTAAAAGCTATATCTCTCGAAGATT-3').

UV light irradiation of *T. brucei* cells

RNA–RNA cross-links were induced *in vivo* with a slight modification of procedures used to investigate RNA tertiary structure in *E. coli* (Steige et al. 1986). Buffy-coat or DEAE column-purified Istat 1.1a bloodstream-form trypanosomes (~5 × 10¹⁰ cells) were suspended in 30 ml of PBSG [10 mM sodium phosphate, 145 mM sodium chloride, and 0.5% glucose (pH 7.2)] at 4°C. Aliquots of 4 ml were dispensed into 5-cm-diam. petri dishes and irradiated with 254 nm of light at an intensity of 10 mW/cm² for 1–30 min. Cells were maintained at 4°C and mixed continuously during irradiation by placing the petri dishes on a prechilled aluminum block within an ice vat and placing the vat on a rotary shaker under the light source.

RNA preparation and Northern analysis

RNA was isolated from irradiated cells by lysis with 0.5% SDS and extensive digestion with proteinase K (500 µg/ml at 45°C for 2 hr). Following phenol–chloroform extraction and ethanol precipitation the recovered RNA was dissolved in water to a concentration of ~2mg/ml and chromatographed on a Sephadex G-25 column to remove residual salts. The recovery of RNA was usually slightly lower in the irradiated samples relative to the nonirradiated control (e.g., in the experiment shown in Fig. 1, RNA recovered after 0 min of irradiation was 2.1 mg/ml, and after 30 min, 1.6 mg/ml).

Gel purification of cross-linked RNAs was carried out by electrophoresis of 500 µg of RNA prepared following 20 min of UV irradiation on a preparative denaturing polyacrylamide gel. Two fractions were eluted from the gel (relative to ³²P-labeled markers): (1) RNAs migrating between 90 and 160 nucleotides and containing un-cross-linked U2 and U6 RNA, and (2) RNAs migrating between 200 and 400 nucleotides and containing the cross-linked molecules. The latter were recovered, denatured, and loaded on a sequencing gel. After electrophoresis, eight fractions migrating between 320 and 220 nucleotides and designated A–H were cut from the gel and eluted. The double gel purification was found to be necessary to completely remove un-cross-linked RNA from the sample.

Northern analyses were carried out as described previously (Michaeli et al. 1990), with the following modifications. All gels contained 6% polyacrylamide (29:1 acrylamide/bis-acryla-

Table 1. Oligonucleotides for RNase H experiments

Oligonucleotide	Sequence (5' → 3')	Area of complementarity (nucleotides)
B-1	TTATTTCTCATTTAAGAGGTT	U'B'RNA (37–57)
SLa	GGAGGTCATCCGACCCC	SL RNA (123–139)
SLb	GATCAATATAGTACAGAACTGTTCTAATAATAGCGTTAT	SL RNA (1–39)
U2a	CAGTTTAATAACTT	U2 RNA (29–42)
U2b	GGCCCGTATCAGGAGTTACTC	U2 RNA (52–72)
U4a	AAACTTTCCTCCGAAGGAGTACCGG	U4 RNA (65–88)
U6a	TCTTCTCTGTTGAATTTCC	U6 RNA (28–46)
U6b	CAGCCTTGCGCAGGG	U6 RNA (56–70)

midel and 8 M urea. After electrotransfer of RNA to Nytran membranes, RNA was covalently attached to the wet filter by UV irradiation (2.5 mW/cm² for 65 sec) instead of in vacuo-baking at 80°C. In our hands, the sensitivity of Northern detection of small RNAs increased by ~25- to 30-fold when UV cross-linking was used instead of baking, and although not investigated thoroughly, RNA probes appear to be stripped more readily from blots prepared in this manner. *E. coli* tRNA was substituted for yeast tRNA in the hybridization buffer described by Michaeli et al. (1990), and final washes were carried out at 65°C in 0.1× SSPE/0.1% SDS.

Site-directed cleavage of RNA

Hydrolysis of specific RNAs in UV cross-linked samples was performed in 20-μl reactions containing 20 μg of total RNA and 40 pmoles of oligonucleotide in 40mM HEPES-KOH (pH 7.9), 4 mM MgCl₂, 50 mM KCl, and 1 mM dithiothreitol. Oligonucleotides were annealed to the target RNA by heating the solution to 95°C for 1 min and then cooling over 10 min to 37°C. After addition of ribonuclease H (2 units, U.S. Biochemical) and incubation at 37°C for 2 hr, RNA was extracted, ethanol-precipitated, and analyzed by Northern hybridization. For site-directed cleavage of gel-purified RNA, RNase-free *E. coli* tRNA was added as carrier.

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