Conserved Loop I of U5 Small Nuclear RNA Is Dispensable for Both Catalytic Steps of Pre-mRNA Splicing in HeLa Nuclear Extracts

VÉRONIQUE SÉGAULT,1 CINDY L. WILL,2 MARIA POLYCARPOU-SCHWARZ,3 IAIN W. MATTAJ,3 CHRISTIANE BRANLANT,1 AND REINHARD LÜHRMANN2*

UMR CNRS 7567 Maturation des ARN et Enzymologie Moléculaire Université H. Poincaré, 54506 Vandœuvre-Les-Nancy Cédex, France,1 and Institut für Molekularbiologie und Tumorforschung, Philipps Universität Marburg, 35037 Marburg,2 and EMBL, 69117 Heidelberg,2 Germany

Received 2 November 1998/Returned for modification 1 December 1998/Accepted 28 December 1998

The function of conserved regions of the metazoan U5 snRNA was investigated by reconstituting U5 small nuclear ribonucleoprotein particles (snRNPs) from purified snRNP proteins and HeLa or Xenopus U5 snRNA mutants and testing their ability to restore splicing to U5-depleted nuclear extracts. Substitution of conserved nucleotides comprising internal loop 2 or deletion of internal loop 1 had no significant effect on the ability of reconstituted U5 snRNPs to complement splicing. However, deletion of internal loop 2 abolished U5 activity in splicing and spliceosome formation. Surprisingly, substitution of the invariant loop 1 nucleotides with a GAGA tetraloop had no effect on U5 activity. Furthermore, U5 snRNPs reconstituted from an RNA formed by annealing the 5’ and 3’ halves of the U5 snRNA, which lacked all loop 1 nucleotides, complemented both steps of splicing. Thus, in contrast to yeast, loop 1 of the human U5 snRNA is dispensable for both steps of splicing in HeLa nuclear extracts. This suggests that its function can be compensated for in vitro by other spliceosomal components: for example, by proteins associated with the U5 snRNP. Consistent with this idea, immunoprecipitation studies indicated that several functionally important U5 proteins associate stably with U5 snRNPs containing a GAGA loop 1 substitution.

Nuclear pre-mRNA splicing proceeds via a two-step mechanism. In the first step, the pre-mRNA is hydrolyzed at the 5’ splice site and the 5’ end of the intron interacts concomitantly with an adenosine at the so-called branch point. The splicing intermediates thus generated include exon 1 and a lariat structure comprised of the intron and exon 2. In the second step, hydrolysis at the 3’ splice site and the concomitant ligation of exons 1 and 2 give rise to the mRNA and the excised intron in the form of a lariat. Both reactions are catalyzed by the spliceosome, a large ribonucleoprotein complex formed by the ordered interaction of numerous splicing factors and the four small nuclear ribonucleoprotein particles (snRNPs), U1, U2, U5, and U4/U6, with conserved regions of the pre-mRNA (reviewed in references 19, 27, and 34). Spliceosome assembly is initiated by the interaction of the U1 and U2 snRNPs with the 5’ splice site and branch site, respectively, thereby generating the so-called prespliceosome, or complex A. Mature spliceosomes (i.e., complexes B and C) are ultimately formed by the subsequent interaction of the U4/U6 and U5 snRNPs, in the form of a preassembled U4/U6,U5 tri-snRNP complex (reviewed in references 19 and 34).

The assembly of a catalytically active spliceosome requires the formation of a network of RNA-RNA interactions which favorably position the chemically reactive groups of the pre-mRNA for catalysis (for reviews, see references 26 and 38). The U5 snRNP has been proposed to play a central role in recognizing and aligning the 5’ and 3’ splice sites for catalysis, and its function appears to be mediated, at least in part, by base pairing interactions between the U5 small nuclear RNA (snRNA) and the pre-mRNA. In particular, at least 3 of the 9 nucleotides (nt) present in its absolutely conserved loop 1 sequence (see Fig. 1A) were shown by several methods, including cross-linking and yeast genetic studies, to interact with exon nucleotides at the 5’ and/or 3’ splice site (9, 28, 29, 30, 37, 45). The interaction of loop 1 with exon 1 is observed both prior and subsequent to the first step of splicing, whereas its interaction with exon 2 is detectable only after step 1 (30, 37). Loop 1 was thus originally proposed to play an essential role in both catalytic steps of splicing in both higher and lower eukaryotes. Recent in vitro studies with yeast have demonstrated that the first, but not the second step of splicing can occur in its absence (31). More detailed mutational analyses in vitro have also revealed that only large loop 1 deletions or insertions, as opposed to minor ones, affect the efficiency of the second step of splicing in yeast (32). Loop 1 of the U5 snRNP is currently proposed to bind and favorably position excised exon 1 for its nucleophilic attack at the 3’ splice site during the second step of splicing (31). However, since the interaction of loop 1 nucleotides with either exon is limited to 2 to 3 bp and these are often non-Watson-Crick in nature, other components of the U5 snRNP, in particular U5-specific proteins (see below), have been proposed to help stabilize U5 snRNP interactions at both the 5’ and 3’ splice site (41).

In addition to a single U5 snRNA molecule, mammalian U5 snRNPs possess eight so-called Sm or core proteins (B, B’, D1, D2, D3, E, F, and G), common to all spliceosomal snRNP species, and nine U5-specific proteins (reviewed in reference 44). Three of these U5-specific proteins, with molecular masses of 116, 200, and 220 kDa, have been shown to be evolutionarily conserved and to carry out essential functions during splicing (2, 12, 17, 23, 24). The human 220-kDa protein and its yeast homolog, Prp8p, have been shown by site-specific cross-linking experiments to interact with the 5’ and 3’ splice sites as well as...
FIG. 1. Secondary structure models of wild-type and mutant human U5 snRNAs. (A) Sequence and secondary structure model of the human U5a snRNA as originally proposed by Krol et al. (20). The conserved, single-stranded region of the Sm site is boxed. (B) The putative secondary structure of the human U5 snRNA mutants is shown schematically. All nucleotide substitutions are shown in detail.
the branch site and polypyrimidine tract (8, 25, 35, 41, 42, 45). The interaction between Prp8p and the 5′ splice site and polypyrimidine tract (8, 25, 35, 41, 42, 45). The interaction between Prp8p and the 5′ splice site and polypyrimidine tract (8, 25, 35, 41, 42, 45). The interaction between Prp8p and the 5′ splice site and polypyrimidine tract (8, 25, 35, 41, 42, 45). The interaction between Prp8p and the 5′ splice site and polypyrimidine tract (8, 25, 35, 41, 42, 45). The interaction between Prp8p and the 5′ splice site and polypyrimidine tract (8, 25, 35, 41, 42, 45). The interaction between Prp8p and the 5′ splice site and polypyrimidine tract (8, 25, 35, 41, 42, 45). The interaction between Prp8p and the 5′ splice site and polypyrimidine tract (8, 25, 35, 41, 42, 45). The interaction between Prp8p and the 5′ splice site and polypyrimidine tract (8, 25, 35, 41, 42, 45).

To this end, we have reconstituted in vitro U5 snRNPs from human or Xenopus U5 snRNA mutants and tested their ability to restore splicing to U5-depleted nuclear extracts. The data presented here demonstrate that two of the most highly conserved regions of the U5 snRNA (i.e., loop 1 and IL2) are surprisingly amenable to mutation. U5 snRNPs unexpectedly retained their ability to efficiently complement both steps of splicing even after complete deletion of loop 1. These results thus indicate that, in metazoans, the function of U5 loop 1 during its absence.

MATERIALS AND METHODS

Construction of U5 snRNA mutants. Human and Xenopus U5 snRNA deletion and substitution mutants were constructed as previously described by Jar- molowski and Mattaj (18). IL2 and sub-stem Ib were kindly provided by Albrecht Bindereif and constructed as described by Hinz et al. (16). The 5′ (nt 1 to 35) and 3′ (nt 47 to 116) halves of U5 were transcribed from PCR products containing a T7 and Sp6 promoter, respectively. Oligonucleotides used for PCR of these two U5 snRNA gene fragments were as follows: 5′ half forward primer, 5′ GGCCTAAATGCACTTAAAGATTAATCTGTGTCCT; 3′ half reverse primer, 5′ GGAAGGATATTTCCGTGGAGAGG; 3′ half reverse primer, 5′ TACGCTTGGCAGGCGAAGG. The 5′ and 3′ halves were annealed in buffer containing 20 mM HEPES–KOH (pH 7.9), 100 mM KCl, and 10 mM MgCl₂, by incubation at 70°C for 15 min and being allowed to slowly cool to room temperature.

Preparation of snRNAs, pre-mRNA, and native snRNPs. Native, RNA-free snRNP proteins (TPs) were isolated from a mixture of mG immuno-affinity-purified U1, U2, U4, and U4/U5 snRNPs by dissociation in the presence of EDTA and the anion-exchange resin DE53 (39). HeLa U5 snRNA was isolated from purified snRNPs as described previously (39). In vitro-transcribed human and Xenopus U5 snRNAs, as well as MINX pre-mRNA, were prepared as previously described (36).

U5 snRNA depletion and splicing complementation. Nuclear extracts were prepared from HeLa cells (Computer Cell Culture Center, Mons, Belgium) as described by Dignam et al. (10). U5-depleted nuclear extract was prepared by affinity selection with a 2′-O-methoxylated RNA complementary to nt 36 to 47 of the human U5 snRNA (22, 36). Mock-depleted extract was handled in an identical manner, except that oligonucleotide was omitted. Complementation with in vitro-reconstituted particles was accomplished by combining 2.6 pmol (100 ng) of authentic or in vitro-transcribed U5 snRNA, or the annealed mixture containing 100 ng each of the 5′ and 3′ halves of U5 RNA and 3.3 pmol (650 ng) of purified native snRNP proteins (TPs). RNA and TPs were incubated for 60 min at 0°C in the presence of splicing reaction mixtures lacking pre-mRNA, and splicing was initiated by the addition of the pre-mRNA. In vitro splicing and the analysis of splicing intermediates and products were performed as described previously (36). No differences in complementation efficiency were observed when reconstitution was carried out either directly in splicing extract or by additionally preincubating the U5 snRNA and TPs in the absence of extract. Splicing complex formation was analyzed by native gel electrophoresis as described by Behrens et al. (5).

Immunoprecipitation of reconstituted U5 snRNPs. 32P-labelled U5 snRNA was prepared by in vitro transcription as described above and incubated under standard reconstitution conditions. Immunoprecipitations were performed with rabbit sera directed against the U5 116-kDa protein (12), essentially as previously described (15). Briefly, protein A-Sepharose (PAS)-bound antibody was incubated for 2 h at 4°C with 12.5 μl of a splicing reaction mixture containing 10°pm (10 ng) of 32P-labelled U5 snRNA in 200 μl of IPP, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% [vol/vol] Nonidet P-40 and subsequently washed four times with IPP buffer containing 300 mM NaCl. Immunoprecipitated RNA was extracted with phenol-chloroform, precipitated with ethanol, fractionated on a 10% polyacrylamide–7 M urea gel, and visualized by autoradiography.

RESULTS

Loop 1 of the U5 snRNA is dispensable for both steps of splicing in vitro. We previously reported the establishment of an in vitro reconstitution-splicing complementation system for HeLa U5 snRNPs (36). In this system, HeLa nuclear extracts are specifically depleted of U5 snRNPs by affinity selection with a biotinylated 2′-O-alkyl RNA oligonucleotide complementary to loop 1 of the U5 snRNA. U5 snRNPs are reconstituted by incubating purified U5 snRNA and native snRNP proteins (TPs) in the presence of splicing extract. TPs, which are essentially free of any snRNA, consist predominantly of the snRNP Sm proteins B’, B, D1, D2, D3, E, F, and G (36, 39),
and the reconstitution of functional U5 snRNPs was previously shown to require their addition to the reconstitution mixture (36). The splicing activity of reconstituted U5 snRNPs is assayed directly in the reconstitution mixture by the addition of pre-mRNA. As shown in Fig. 2A, the splicing efficiency of an adenovirus major late II pre-mRNA (MINX) was significantly reduced in U5-depleted extract when compared to the mock-depleted extract (Fig. 2A, compare lanes 1 and 2). Consistent with previous results, splicing could be complemented by the addition of either authentic or in vitro-transcribed HeLa U5 snRNA plus native snRNP Sm proteins (TPs) (lanes 4 and 5). In contrast, the addition of RNA (not shown) or TPs alone (lane 3) had little or no effect on the splicing activity of U5-depleted extract.

The ability to complement splicing with in vitro-transcribed U5 snRNA allowed us to investigate the effect of U5 snRNA mutations on the activity of in vitro-reconstituted U5 snRNPs.
To this end, we constructed a number of human U5 snRNA mutants with alterations primarily in either of two conserved regions, namely loop 1 or IL2. These mutants are depicted schematically in Fig. 1B, and a more precise description of deleted and/or substituted nucleotides is presented in Table 1. As a first step, we constructed a U5 snRNA mutant in which the invariant loop 1 sequence GCCUUUUAC was substituted with a GAGA tetraloop (designated GAGA loop 1). Loop 1 was replaced by a tetraloop rather than completely deleted in order to preserve the folding of stem Ic. The structure of this RNA was verified by nuclease susceptibility assays (data not shown). The activity of U5 snRNPs reconstituted with this mutant was then assayed in our in vitro splicing complementation system. Surprisingly, the GAGA tetraloop mutant restored both steps of splicing to U5-depleted extract to a level similar to that obtained with wild-type U5 snRNA (Fig. 2A, lanes 5 and 6). (Note that the slight reduction in spliced product levels observed in wild-type extracts was observed in all experiments.) Indeed, the majority of splicing could be restored to U5-depleted extracts with a GAGA loop (sub IL2) or U5-depleted extracts by deletion of IL2 and stem Ic, as well as sub IL2 (sub-stem Ic). Interestingly, the latter U5 snRNPs were unable to restore splicing activity to U5-depleted extracts (Fig. 2C, lane 6). However, deletion of IL2 and stem Ic (ΔIL2/stem Ic) abolished the ability of U5 snRNPs to complement both steps of splicing (Fig. 2A, lane 9). To distinguish whether this loss of activity was due to deletion of IL2 or to stem Ic (which, in contrast to ΔIL2, would shorten the overall length of the major U5 5' stem-loop), reconstitutions were performed with a U5 snRNA lacking solely IL2 (ΔIL2). Interestingly, the latter U5 snRNPs were unable to restore splicing activity to U5-depleted extracts (Fig. 2C, compare lanes 5 with lanes 2 and 3). Because all of these U5 snRNA mutants exhibit similar stabilities during in vitro reconstitution and splicing, the observed losses in activity cannot be attributed to an increase in the turnover of the ΔIL2 or ΔIL2/stem Ic mutants. These results indicate that structural elements other than loop 1, namely IL2, are absolutely required for U5 snRNP function.

IL2 is dispensable for splicing in vitro. To determine whether other regions of the major U5 stem-loop are essential for splicing activity, we extended our investigation to include a Xenopus U5 deletion mutant which lacked IL1 (designated ΔI). As a negative control, the activity of a mutant lacking both IL2 and stem Ic and possessing a UUCG tetraloop substitution of loop 1 (designated ΔI) was also assayed. These mutants are shown schematically in Fig. 3A. Since only minor changes in primary sequence are observed between human and Xenopus U5 snRNAs, the latter were expected to assemble into functional hybrid U5 snRNPs in our reconstitution system. Indeed, the majority of splicing could be restored to U5-depleted extracts by the addition of U5 snRNPs reconstituted from wild-type Xenopus U5 snRNA and HeLa snRNPs (Fig. 3B, compare lanes 1 through 4). Compared to the wild type, deletion of IL1 had no significant effect on the level of splicing complementation (Fig. 3B, lane 5), demonstrating that it is dispensable for U5 snRNP function. In contrast, consistent with the results described above, deletion of IL2 and stem Ic, as well as substitution of loop 1 with a UUCG tetraloop, abolished the in vitro splicing activity of reconstituted U5 snRNPs (Fig. 3B, lane 6).

The splicing block observed upon deletion of IL2 occurs prior to or during splicing complex B formation. To determine whether U5 mutants lacking IL2 support the assembly of U4/ U6,U5 snRNPs active in spliceosome assembly, splicing complex formation was analyzed by subjecting the in vitro splicing reaction mixtures to native gel electrophoresis. For comparison, splicing reactions performed with U5 snRNPs reconstituted from wild-type or U5 snRNA, whose loop 1 sequence had been substituted by a GAGA tetraloop, were also analyzed. Consistent with the known function of U5 during spliceosome assembly, the formation of splicing complexes B and C, but not A (which contains only the U1 and U2 snRNPs), was significantly reduced in U5-depleted extract (Fig. 4, compare lanes 1 to 3 with 4 to 6). The assembly of complexes B and C could, however, be restored by the addition of U5 snRNPs

| TABLE 1. Human U5 snRNA mutants examined in this study |
|-----------------|-----------------|
| Sub 5’ IL2a     | nt 23 and 24 substituted with AU |
| Sub 5’ IL2b     | nt 20–27 substituted with AU/UAUAUA |
| Sub 3’ IL2      | nt 55–57 substituted with AAA |
| Sub IL2         | nt 20–27 substituted with AUU/AUA/AUA and 55–57 substituted with AAA |
| ΔIL2            | nt 20–27 and 55–57 deleted |
| ΔIL2/stem Ic    | nt 20–35 and 47–57 deleted |
| Sub-stem 1b     | nt 9–19 substituted with CAGAGAAGAGC |
| IL2a            | nt 36–46 deleted with CC |
| IL2b            | nt 36–46 deleted with GG |
| 5’ half         | nt 1–47 deleted with CC |
| 3’ half         | nt 36–46 deleted with GG |
| 5’ + 3’ half    | nt 5’ and 3’ halves annealed |

Il2 is required for the formation of functional U5 snRNPs. We next tested whether mutation of IL2, a second conserved region of the major stem-loop of U5 snRNA, or stem Ic, affected the splicing activity of reconstituted U5 particles. Compared to the wild type, substitution of nucleotides in either the 5’ half (sub 5’ IL2a or IL2b), 3’ half (sub 3’ IL2), or both bulged halves of IL2 (sub IL2) had no significant effect on the complementation efficiency of in vitro-reconstituted U5 snRNPs (Fig. 2A, lanes 7 and 8 and 10 [data not shown]). Thus, the precise sequence of IL2 does not appear to be relevant to U5 snRNP function. Similarly, substitution of stem Ib with a stem in which essentially the 5’ and 3’ halves of stem Ib were swapped (sub-stem Ib), resulted in only a slight reduction in the splicing activity of U5 snRNPs (Fig. 2C, lane 6). However, deletion of IL2 and stem Ic (ΔIL2/stem Ic) abolished the ability of U5 snRNPs to complement both steps of splicing (Fig. 2A, lane 9). To distinguish whether this loss of activity was due either to deletion of IL2 or to stem Ic (which, in contrast to ΔIL2, would shorten the overall length of the major U5 5’ stem-loop), reconstitutions were performed with a U5 snRNA lacking solely IL2 (ΔIL2). Interestingly, the latter U5 snRNPs were unable to restore splicing activity to U5-depleted extracts (Fig. 2C, compare lane 5 with lanes 2 and 3). Because all of these U5 snRNA mutants exhibit similar stabilities during in vitro reconstitution and splicing, the observed losses in activity cannot be attributed to an increase in the turnover of the ΔIL2 or ΔIL2/stem Ic mutants. These results indicate that structural elements other than loop 1, namely IL2, are absolutely required for U5 snRNP function.
reconstituted from in vitro-transcribed wild-type or GAGA loop 1 U5 snRNA (lanes 7 to 12). In contrast, U5 snRNPs reconstituted from the ΔII mutant were unable to support complex B and C formation (lanes 13 to 15). Similar results were obtained with the ΔI mutant (data not shown), suggesting that the deletion of IL2 inhibits either the assembly of the U4/U6.U5 tri-snRNP complex or its association with the prespliceosome (i.e., complex A).

The U5 116-kDa protein associates with U5 snRNPs lacking loop 1, but not IL2. To determine whether alterations in the U5 snRNA affected the protein composition of the U5 snRNP, immunoprecipitation studies were performed, subsequent to reconstitution with radiolabeled U5 snRNA, with antibodies reacting specifically with the 116-kDa U5-specific protein. Recent studies have demonstrated that the 116-kDa protein forms a very tight complex with the U5 220-kDa protein (1). This dimer also interacts with two other U5-specific proteins, namely of 200 and 40 kDa (1). The presence of the 116-kDa protein in a particular U5 snRNP is thus a good indication for the presence of the U5 220-kDa protein, as well as these other U5 proteins. Wild-type, GAGA loop 1, ΔI, and ΔIL2/stem Ic U5 snRNAs were quantitatively precipitated by the anti-Sm monoclonal antibody Y12, demonstrating that each supports the association of the core or common snRNP proteins (data not shown). Only minimal background precipitation of each of these RNAs, as well as ΔIL2 and sub stem Ib, was observed when immunoprecipitations were performed with nonimmune serum (Fig. 5, lanes 1, 3, 5, 7, 9, and 11). In contrast, a significant amount (compared to nonimmune serum) of wild-type, GAGA loop 1, or sub stem Ib U5 snRNA was precipitated by antibodies directed against the U5 116-kDa protein (Fig. 5, lanes 2, 4, and 12); however, in keeping with its slightly reduced splicing activity, precipitation of substem Ib was, by comparison, somewhat less efficient. Consistent with the fact that they were inactive in splicing, U5 mutants lacking IL2, either alone or in combination with other deletions, were not appreciably precipitated (Fig. 5, lanes 6, 8, and 10). Thus IL2, but not the conserved nucleotides of loop 1, is required for the stable association of the U5 116-kDa protein with the U5 snRNP. Because the U5 116-kDa protein is tightly associated with the U5 220-kDa protein, U5 snRNPs reconstituted from the GAGA loop 1 U5 snRNA most likely also contain the 220-kDa protein, which has been shown, like loop 1, to interact with both splice sites of the pre-mRNA. These results are therefore consistent with the idea that the U5 220-kDa protein and/or other U5 snRNP proteins might functionally substitute for loop 1 in its absence.

**DISCUSSION**

We have employed an in vitro reconstitution-splicing complementation system to investigate the effect of U5 snRNA
mutations on both the structure and function of the metazoan U5 snRNP. Surprisingly, substitution or deletion of the invariant U5 loop 1 sequence had no effect on the ability of reconstituted U5 snRNPs to complement either step of splicing in HeLa nuclear extracts (Fig. 2). The ability of U5 snRNPs lacking loop 1 to efficiently support the first step of splicing in HeLa nuclear extracts is consistent with results recently obtained in the yeast *S. cerevisiae*. In this instance, mutant U5 snRNAs containing substituted or deleted loop 1 nucleotides were also shown to complement the first step of splicing in U5-inactivated yeast extracts (31). Thus, although loop 1 may normally participate in the second step of splicing, other spliceosomal factors (e.g., the U5 220-kDa protein [see below]) apparently can compensate for it when it is absent. Alternatively, under normal circumstances base pairing interactions involving loop 1 nucleotides could simply play a secondary role in tethering exon 1 and aligning both splice sites for the second catalytic step of splicing. Nonetheless, the fact that U5 loop 1 nucleotides are absolutely, evolutionarily conserved, including their posttranscriptional modification (40), suggests that they contribute in some way to either the efficiency or accuracy of the splicing reaction. This function is, however, not readily apparent in our in vitro splicing system. Previous in vivo studies with HeLa cells suggested that loop 1 may contribute to 5′ splice site selection (9). The substrate used here had a single 5′ splice site, and based on the unchanged migration behavior of excised exon 1 in the presence of the GAGA tetraloop mutant (Fig. 2A) and the fact that a single nucleotide change in the length of exon 1 should be detectable under our gel electrophoresis conditions, we can exclude the possibility that the absence of loop 1 leads to aberrant 5′ splice site cleavage. Similarly, substitution or deletion of loop 1 of the yeast U5 snRNA also had no effect on the accuracy of 5′ splice site selection in yeast splicing extracts (31). However, it is conceivable that the fidelity of 3′ splice site cleavage may be altered in the absence of the invariant U5 loop 1 sequence, because small changes in the length of the mRNA or excised lariats would not be detectable under our experimental conditions. Loop 1 could also be absolutely required for some aspect of U5 snRNP or U4/U6.U5 tri-snRNP morphogenesis, such as the recycling of the tri-snRNP complex, which is thought to be dispensable in HeLa splicing extracts.
Our in vitro splicing complementation studies indicate that the functions previously attributed to loop 1 of the U5 snRNA, namely tethering of exon 1 subsequent to step 1 of splicing, as well as aligning the chemically reactive groups for the second step of splicing, can be compensated for by other spliceosomal components when loop 1 is absent. One likely candidate for this substitute is the U5 220-kDa protein. This highly conserved U5 snRNP protein (designated Prp8 in \textit{S. cerevisiae}) has been shown to be in close proximity to both splice sites, as well as to the branch site and polypyrimidine tract (8, 25, 35, 41, 42, 45). Cross-linking studies further demonstrated that its interaction with the pre-mRNA substrate persists throughout the splicing reaction (41, 42, 45). The U5 220-kDa protein has also been implicated in 3' splice site selection (42, 43). Based on these findings, the U5 220-kDa protein was proposed to assist the limited base pairing interactions between U5 loop 1 and the 5' and 3' splice sites (41). Consistent with the idea that U5 220-kDa can functionally compensate for the loss of loop 1, Prp8 has been shown to interact with the 5' and 3' splice sites even in the absence of U5 loop 1 (11).

The results of our immunoprecipitation studies are also consistent with the idea that the U5 220-kDa protein could functionally replace loop 1. In particular, the GAGA tetraloop mutant was shown to stably associate with the U5 116-kDa protein (Fig. 5), which in turn has recently been shown to form a tight protein complex with the U5 220-kDa protein (1). These results suggest that U5 snRNPs reconstituted from the GAGA loop 1 U5 snRNA also contain the U5 220-kDa protein. Indeed, immunoprecipitation studies with anti-U5 220-kDa protein antibodies suggest that this protein probably does interact with U5 snRNPs containing a GAGA loop 1 substitution (data not shown), but due to the inefficiency of immunoprecipitation, as well as high levels of background precipitation, we have not been able to demonstrate conclusively that the U5 220-kDa protein is stably associated. Consistent with our observations, recent in vivo studies employing the transient transfection of mutant U5 genes into mammalian cells detected only a 60% reduction in U5 220-kDa protein binding upon replacement of U5 loop 1 with a UUCG tetraloop (16). Furthermore, the association of Prp8 with the yeast U5 snRNA in splicing extracts was observed even in the absence of loop 1 (11). These results support the idea that the presence of loop 1 is not necessarily a prerequisite for U5 220-kDa protein association with mammalian U5 snRNPs. Of course we cannot presently rule out whether U5 proteins besides or in addition to the U5 220-kDa protein, or even non-U5 snRNP spliceosomal components (including other RNAs), could also functionally substitute for loop 1.

In addition to loop 1, other regions of the metazoan U5 snRNA were shown to be dispensable for splicing in vitro. For example, consistent with previous in vivo studies of yeast (13), deletion of IL1 had little effect on the splicing activity of U5 snRNPs (Fig. 3). On the other hand, in vitro splicing in yeast was severely inhibited by deletion or substitution of the 5' half of IL1 (11). However, this apparent difference could be attributed to differences in the IL1 mutants analyzed. Furthermore, despite its evolutionary conservation, substitutions in the sequence of either bulged half of IL2 also had no effect on U5 function, indicating it has no sequence-specific role (Fig. 2A). Similarly, substitution of stem Ib nucleotides had only a moderate effect on splicing (Fig. 2C). However, deletion of IL2 abolished U5 snRNP activity in splicing, demonstrating that this structural element is required for the formation of functional U5 snRNPs (Fig. 2C). Because the IL2 deletion, in contrast to the ΔIL2/stem Ic deletion, has little effect on the overall length of the major 5' stem-loop of the U5 snRNA, its negative phenotype is probably not simply due to the shortening of this stem-loop structure. IL2 could play an important role in determining the tertiary structure of the U5 snRNA; indeed, it has been proposed to act as a hinge which would, for example, allow folding between stems Ic and Ib (3). Consistent with our result, in yeast, IL2 has been shown in vivo to provide an essential function for the yeast U5 snRNP and to be required for efficient splicing in vitro (11, 13).

The inability of U5 snRNA mutants lacking IL2 to support splicing suggests that these deleted nucleotides play either a direct or indirect role in the splicing process. U5 snRNA mutations could directly inhibit U5 snRNP function by altering or inhibiting the association of a U5-specific protein that is involved in either catalytic step of splicing. Alternatively, splicing could be indirectly affected if the protein in question were required for the proper assembly of the U5 snRNP or its subsequent interaction with U4/U6 to form the U4/U6.U5 tri-snRNP complex. Significantly, U5 snRNPs reconstituted from ΔIL2 or ΔIL2/stem Ic U5 snRNA did not allow the formation of splicing complex B (Fig. 4 and data not shown), consistent with the idea that the assembly of the U5 snRNP and/or U4/U6.U5 tri-snRNP complex was in some way compromised in the absence of IL2. Indeed, U5 snRNPs lacking IL2 did not support the stable association of the U5 116-kDa protein, as evidenced by immunoprecipitation studies (Fig. 5). Based on the recent demonstration that the U5 220-, 116-, 200-, and 40-kDa proteins form a highly stable heteromeric complex (1), these results suggest that ΔIL2 U5 snRNPs may also lack several proteins in addition to the U5 116-kDa protein. Indeed, deletion of IL2 was shown to abolish the interaction of the U5 220-kDa protein with U5 snRNPs in vivo (16). These results are also consistent with previous nuclease and chemical protection studies which suggested that one or more U5 proteins interact with IL2 (4, 6). Whether the U5 116-kDa protein directly interacts with IL2 is presently not clear. Because protein-protein interactions appear to predominate in the U5 snRNP, immunoprecipitation studies of this kind are rather limited in their potential for drawing conclusions about RNA-protein interactions. More detailed information regarding intermolecular interactions within the U5 snRNP is clearly needed to clarify this issue.

**ACKNOWLEDGMENTS**

We thank Michael Krause for preparing biotinylated 2'-O-alkyl oligonucleotides, and Peter Kempkes and Winfried Lorenz for expert technical assistance. We are grateful to Joan Steitz for kindly providing Y12 antibodies and Albrecht Bindereif for providing the ΔIL2 and sub-stem Ib U5 snRNA mutants.

This work was supported by the Deutsche Forschungsgemeinschaft SFB 397, the French Centre National de la Recherche Scientifique, and an EC Human Capital and Mobility Network grant (EBCHRXCT 930191).

**REFERENCES**


54. Szentágothai, V., C. L. Will, B. S. Sproat, and R. Lührmann. 1995. In vitro reconstitution of mammalian U2 and U5 snRNPs active in splicing: Sm proteins are functionally interchangeable and are essential for the formation of functional U2 and U5 snRNPs. EMBO J. 14:4010–4021.


