The EMBO Journal vol.14 no.16 pp.4010–4021, 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

Véronique Ségault, Cindy L.Will, Brian S.Sproat[1] and Reinhard Lührmann[2]

Institut für Molekularbiologie und Tumorforschung, Philipps Universität Marburg, 35037 Marburg and [1]Ribonetics GmbH, D-37079 Göttingen, Germany

[2]Corresponding author

Communicated by R.Lührmann

An *in vitro* reconstitution/splicing complementation system has been developed which has allowed the investigation of the role of mammalian U2 and U5 snRNP components in splicing. U2 or U5 snRNP cores are first reconstituted from purified native snRNP core proteins and snRNA in the absence of cellular extract and are subsequently added to splicing extracts depleted of either U2 or U5 snRNP. When snRNPs reconstituted with HeLa U2 or U5 snRNA were added to U2- or U5-depleted nuclear extract, splicing was complemented. Addition of naked snRNA, on the other hand, did not restore splicing, demonstrating that the core proteins are essential for both U2 and U5 snRNP functions in splicing. Hybrid U2 or U5 snRNPs, reconstituted with core proteins isolated from U1 or U2 snRNPs, were equally active in splicing complementation, indicating that the snRNP core proteins are functionally interchangeable. U5 snRNPs reconstituted from *in vitro* transcribed U5 snRNA restored splicing to a level identical to that observed with particles reconstituted from authentic HeLa U5 snRNA. In contrast, splicing could not be restored to U2-depleted extract by the addition of snRNPs reconstituted from synthetic U2 snRNA, suggesting that U2 snRNA base modifications are essential for U2 snRNP function.

Keywords: *in vitro* reconstitution/pre-mRNA splicing/Sm proteins/snRNPs/snRNA modification

Introduction

Pre-mRNA splicing occurs via a two-step transesterification reaction that is catalysed by a dynamic ribonucleoprotein complex termed the spliceosome. Spliceosome formation involves the association of the small nuclear ribonucleoproteins (snRNPs) U1, U2, U4/U6 and U5, and numerous non-snRNP protein factors, with the pre-mRNA substrate (for reviews, see Green, 1991; Moore *et al.*, 1993). U1 snRNP binds to the pre-mRNA first, followed by U2 and, ultimately, by U4/U6 and U5 which are pre-assembled into a tri-snRNP complex before their incorporation into the spliceosome.

SnRNP particles consist of one or two small nuclear RNAs (snRNAs) which possess a modified 5′ cap structure. U1, U2, U4 and U5 snRNAs contain a hypermethylated trimethylguanosine (m_3G) cap (Reddy and Busch, 1988), whereas U6 possesses a γ-methylphosphate cap (Singh and Reddy, 1989). The m_3G cap, in conjunction with core snRNP proteins (see below), forms the karyophilic signal required for the import of snRNPs into the nucleus subsequent to their morphogenesis in the cytoplasm (Fischer and Lührmann, 1990; Hamm *et al.*, 1990a). The spliceosomal snRNAs, especially U1, U2, U5 and U6, are also modified at numerous positions internally; these modifications include base methylations and the conversion of uridine to pseudouridine (5-β-D-ribofuranosyluracil) (Reddy and Busch, 1988). Interestingly, these snRNA modifications are found, for the most part, in functionally important regions. For example, the evolutionarily conserved nucleotides of U5 snRNA loop I, which are involved in essential interactions at both the 5′ and 3′ splice sites (Newman and Norman, 1991, 1992; Wassarman and Steitz, 1992), are modified at four out of nine positions; these modifications are also evolutionarily conserved (Szkukalek *et al.*, 1995). A significant number of modified nucleotides are also present in those regions of U2 snRNA which interact with the pre-mRNA branch site or with U6 snRNA (Reddy and Busch, 1988). However, at present nothing is known about the role of modified nucleotides in splicing.

The spliceosomal snRNAs are complexed with two classes of polypeptide. The first class, the so-called Sm or core proteins (B, B′, D1, D2, D3, E, F and G), are common to U1, U2, U5 and U4/U6 snRNPs (Lührmann *et al.*, 1990). The snRNP core proteins interact with the Sm site, an evolutionarily conserved structural element present on U1, U2, U4 and U5 snRNAs, which consists of a single-stranded, uridylic acid-rich region that is typically flanked by two hairpin loops (Branlant *et al.*, 1982). The snRNP Sm proteins appear to be identical in all of the spliceosomal snRNPs. Differences in the Sm protein composition of U1, U2, U5 and U4/U6 snRNPs have not been detected; proteins isolated from individual particles exhibit the same electrophoretic and immunological properties (Lührmann, 1988). Further, the morphology of the core RNP structures formed upon interaction of the Sm proteins with the various snRNAs appears identical under the electron microscope (Kastner *et al.*, 1990). However, the observation that certain regions of the Sm site are not interchangeable between U1 and U5 snRNA has led to speculation that subtle differences exist between those Sm proteins binding to U1 snRNA and those binding to U5 snRNA (Jarmolowski and Mattaj, 1993). The existence of bona fide Sm protein variants is unlikely given the recent experimental evidence suggesting that each of the snRNP core proteins arises from a single gene (Lehmeier *et al.*, 1994; Hermann *et al.*, 1995; Séraphin, 1995). It is conceivable, however, that Sm

proteins possessing, for example, differences in post-translational modification, are specifically associated with a given snRNP species.

The second class of snRNP proteins consists of the particle-specific proteins which are found associated with only one type of snRNP. Mammalian U1 snRNPs possess three specific proteins (i.e. 70K, A and C) (Hinterberger *et al.*, 1983; Bringmann and Lührmann, 1986), whereas 17S U2 snRNPs (Behrens *et al.*, 1993b) and 20S U5 snRNPs (Bach *et al.*, 1989) contain twelve and nine specific proteins, respectively. Interestingly, some proteins specifically associated with the mammalian 17S U2 snRNP particle have recently been shown to be identical to those comprising the essential splicing factor SF3a (Behrens *et al.*, 1993a; Brosi *et al.*, 1993). Additional particle-specific proteins include two polypeptides which associate with 12S U4/U6 snRNPs (Gozani *et al.*, 1994; J.Lauber and R.Lührmann, manuscript in preparation) and five which are specifically present in 25S (U4/U6.U5) tri-snRNP particles (Behrens and Lührmann, 1991). In addition to these functionally active forms, snRNP particles lacking defined subsets of particle-specific proteins can be isolated biochemically (Lührmann *et al.*, 1990). For example, 12S U2 snRNPs containing, in addition to the Sm proteins, only two U2-specific proteins (A′ and B′′) have been isolated. Further, U1, U2, U5 and U4/U6 snRNPs consisting solely of the Sm proteins (so-called core snRNP particles) have also been described (Bach *et al.*, 1990).

Whereas the biochemical characterization of mammalian snRNP constituents has steadily progressed, functional characterization, particularly of the snRNP proteins, has remained elusive. It is clear that the Sm proteins play an important role in the biogenesis of snRNPs; they have been shown to be required for snRNA cap hypermethylation (Mattaj, 1986; Plessel *et al.*, 1994), the formation of an snRNP nuclear localization signal (Mattaj and De Robertis, 1985; Fischer *et al.*, 1993), and, lastly, for the association of U1-specific proteins (Nelissen *et al.*, 1994). Their role, if any, in metazoan splicing is currently ill-defined; *in vitro* studies by Wersig and Bindereif (1992) have suggested that the Sm proteins, at least those associated with U4 snRNA, are dispensable for splicing. On the other hand, genetic studies in yeast have demonstrated that at least one of the Sm proteins, namely D1, is essential for splicing *in vivo* (Rymond *et al.*, 1993). A small subset of particle-specific proteins have also been shown to be essential splicing factors both in metazoans and yeast (Beggs, 1993). It is generally thought that most of the particle-specific proteins are responsible for fulfilling a given snRNP's specific function(s) during splicing (Will *et al.*, 1993).

Nonetheless, functional characterization of both the RNA and proteins comprising the mammalian spliceosomal snRNPs has been hampered by the difficulty of reconstituting splicing active snRNPs *in vitro*. Functional reconstitution of *Xenopus* snRNPs or hybrid human–*Xenopus* snRNPs has been accomplished in *Xenopus* oocytes by microinjecting either purified, authentic U1 or U2 snRNAs, or the genes that encode them, subsequent to the depletion of endogenous U1 or U2 snRNAs by oligonucleotide-directed RNase H cleavage (Pan and Prives, 1988, 1989; Hamm *et al.*, 1989, 1990b). The microinjection of various snRNA mutants led to the identification of functionally important domains of U1 and U2 snRNA. A similar system has been established in yeast splicing extracts; subsequent to oligonucleotide-directed degradation of endogenous U2 or U6 snRNA, splicing activity could be restored by the addition of *in vitro* transcribed U2 or U6 snRNA, respectively (Fabrizio *et al.*, 1989; McPheeters *et al.*, 1989). The reconstitution of splicing active snRNPs upon addition of snRNA to splicing extracts prepared from mammalian cells, however, has only been achieved with the U4 and U6 snRNAs. Pikielny *et al.* (1989) initially demonstrated that U4/U6 snRNPs reconstituted in nuclear or S100 extract with *in vitro* transcribed snRNA could be chased into spliceosomal complexes. Wolff and Bindereif (1992) subsequently established an *in vitro* reconstitution/splicing complementation system through which *in vitro* reconstituted U4/U6 snRNPs were shown to be active, not only in spliceosome formation, but also in splicing. In this system, splicing extracts are first depleted of U4 or U6 RNPs by streptavidin–agarose affinity selection with biotinylated 2′-*O*-Me RNA oligonucleotides, and *in vitro* transcribed U6 or U4 snRNA, which reassemble into an snRNP particle in the extract, are subsequently added (Wersig and Bindereif, 1992; Wolff and Bindereif, 1992). This system has allowed the investigation of structurally and functionally important regions of the mammalian U4 and U6 snRNAs (Wersig and Bindereif, 1990; Wolff and Bindereif, 1993). However, attempts to establish a similar system for mammalian U1, U2 and U5 snRNPs have, until now, been unsuccessful.

Here, we describe the development of an *in vitro* reconstitution/splicing complementation system which allows the analysis of the functional contributions of U2 and U5 snRNP components. In the absence of cellular extract, U2 or U5 snRNPs are first reconstituted from purified native snRNP proteins and snRNA that has either been isolated from snRNP particles or transcribed *in vitro*. The *in vitro* reconstituted particles are then added to splicing extracts depleted of U2 or U5 snRNPs and, before splicing, particle-specific proteins which are present in the extracts are allowed to assemble onto the 10S U2 or U5 snRNPs and form 17 and 20S particles, respectively. SnRNPs reconstituted in this way are capable of restoring splicing to extracts specifically depleted of a given snRNP species. Using this system we demonstrate that the snRNP Sm proteins are essential for U2 and U5 snRNP function in splicing and are also functionally interchangeable. In addition, we provide evidence that neither the presence of an m_3G cap nor the formation of pseudouridines is a prerequisite for U5 snRNP splicing activity, since U5 snRNPs reconstituted from *in vitro* transcribed U5 snRNA lacking a hypermethylated cap complement U5-depleted extract. In contrast, U2 snRNPs reconstituted from synthetic U2 snRNA do not restore splicing to U2-depleted extract, suggesting that post-transcriptional base modifications are important for U2 snRNP structure and/or function. The ability to complement splicing with synthetic U5 snRNA will allow future detailed investigations of structurally and functionally important regions of the mammalian U5 snRNA.

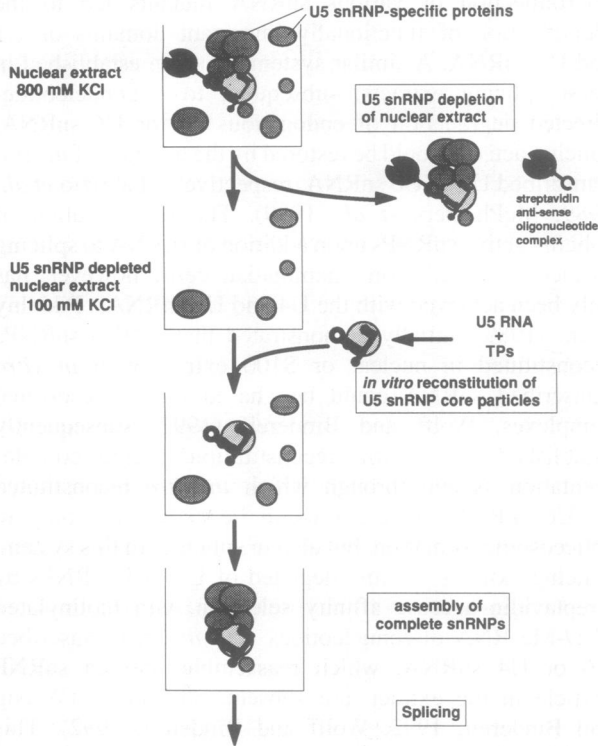

Fig. 1. Scheme of the *in vitro* reconstitution/splicing complementation assay. The U5 snRNP depletion of nuclear extract through a biotinylated 2′-*O*-alkyloligoribonucleotide and streptavidin–agarose, and the reconstitution of U5 snRNPs with U5 snRNA and native snRNP proteins (TPs) are presented schematically.

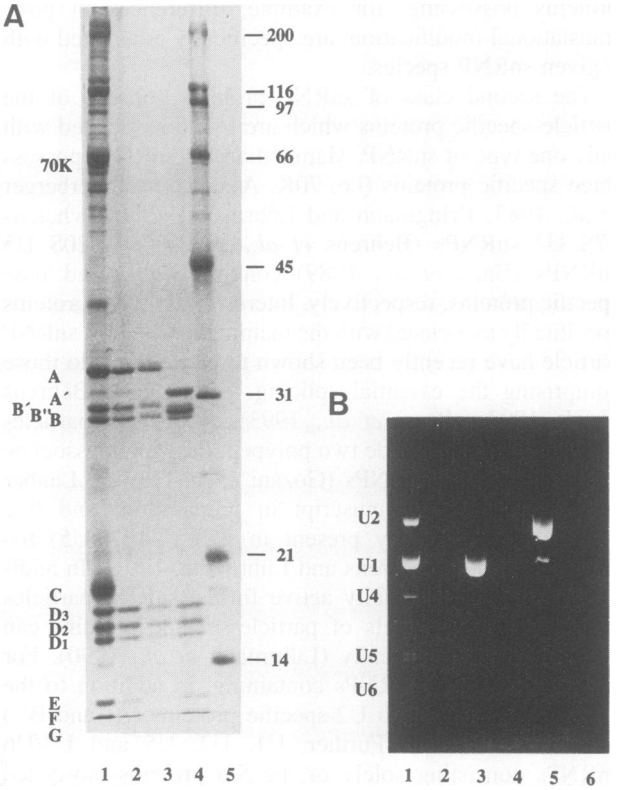

Fig. 2. Protein and RNA composition of native snRNP protein preparations. (**A**) SnRNP proteins used for U2/U5 snRNP reconstitution were prepared under non-denaturing conditions as described in Materials and methods and fractionated on a 12% SDS–polyacrylamide, high TEMED gel (Lehmeier *et al.*, 1990). Native proteins were isolated from 5 μg anti-m_3G purified U1-U6 snRNPs (lane 2), 5 μg Mono Q purified U1 snRNP (lane 3), and 5 μg Mono Q purified U2 snRNP (lane 4). SnRNP proteins extracted under denaturing conditions from U1–U6 snRNPs (lane 1) and molecular weight standards (Biorad) (lane 5) are shown for comparison. Molecular weights are shown at the right and a subset of the snRNP proteins are indicated at the left. Although the G protein is no longer visible on this particular gel due to an abnormally long electrophoresis time, it is consistently present in TP preparations (Sumpter *et al.*, 1992). (**B**) RNA composition of purified snRNPs and native snRNP protein preparations. RNA was isolated by phenol/chloroform extraction of 5 μg (protein) of U1–U6 snRNPs (lane 1), U1 snRNPs (lane 3), and U2 snRNPs (lane 5) or from 5 μg of native snRNP proteins prepared from U1–U6 snRNPs (lane 2), U1 snRNPs (lane 4), or U2 snRNPs (lane 6). SnRNAs were fractionated on a 10% polyacrylamide–7 M urea gel and visualized by staining with ethidium bromide (Bringmann *et al.*, 1986). The various snRNA species are indicated on the left.

Results

In vitro reconstitution of U2 and U5 snRNPs

Reconstitution of U2 and U5 snRNPs was achieved by a two-step procedure which includes an initial, extract-free incubation step (Figure 1). In this first step, purified snRNA, that has either been isolated from snRNP particles or transcribed *in vitro*, is incubated with native, RNA-free, snRNP proteins (denoted total core proteins or TPs). TPs were prepared from a mixture of U1, U2, U5 and U4/U6 snRNPs or from purified U1 or U2 snRNPs by dissociation in the presence of EDTA and the ion exchange resin, DE53 (Sumpter *et al.*, 1992). The composition of these native snRNP protein preparations is shown in Figure 2. All three preparations contain predominantly the snRNP core proteins, B, B′, D1, D2, D3, E, F and G (Figure 2A). Only a very limited number of snRNP-specific proteins, such as the U1-A protein (Figure 2A, lanes 2 and 3) or the U2-A′ and U2-B″ proteins (lanes 2 and 4) are present to a significant extent. Not surprisingly, snRNPs reconstituted from TPs have been shown to be comprised almost exclusively of the snRNP core proteins (Sumpter *et al.*, 1992). As shown in Figure 2B, snRNA is quantitatively removed during the TP preparation such that the native snRNP protein preparations used for snRNP reconstitution are essentially RNA-free (compare lanes 1, 3 and 5 with 2, 4 and 6, respectively).

Since TPs lack the majority of 17S U2 snRNP-specific proteins, as well as 20S U5 and 25S (U4/U6.U5) tri-snRNP specific proteins, the reconstitution of splicing active forms of U2 and U5 required an additional incubation step with nuclear extract (Figure 1). As the *in vitro* reconstituted particles were ultimately to be tested for splicing activity, extracts specifically depleted of U2 or U5 snRNPs were prepared by affinity selection with biotinylated 2′-*O*-methyl or 2′-*O*-alkyl RNA oligonucleotides complementary to U2 or U5 snRNA, respectively (Barabino *et al.*, 1990; Lamm *et al.*, 1991), and the second reconstitution step was performed directly in these depleted extracts. Depletion was carried out at an ionic strength sufficient to dissociate all 17S U2- (Behrens *et al.*, 1993b) and 25S tri-snRNP-specific proteins, as well as the majority of 20S U5-specific proteins (Behrens and Lührmann, 1991; Blencowe *et al.*, 1993); the optimal salt concentration for depletion, as determined by titration experiments, was 0.8 M KCl. Analysis of the RNA composition of the depleted

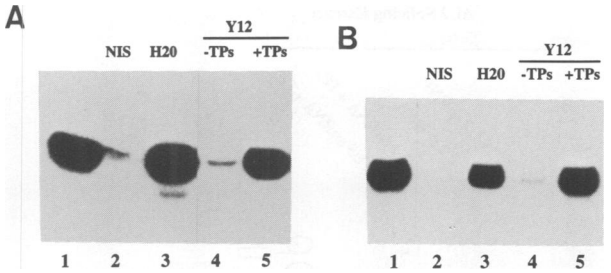

Fig. 3. Immunoprecipitation of reconstituted U2 and U5 snRNPs. (**A**) Particles were reconstituted with ^{32}P-pCp-labelled HeLa U2 snRNA in the presence (lanes 2, 3 and 5) or absence (lane 4) of native snRNP proteins, incubated with U2-depleted extract and then immunoprecipitated as described in Materials and methods with the following antibodies: rabbit non-immune serum (NIS) (lane 2), anti-m_3G H20 (lane 3), and anti-Sm Y12 (lanes 4 and 5). Input radiolabelled U2 snRNA is shown in lane 1. (**B**) Particles were reconstituted with ^{32}P-pCp-labelled HeLa U5 snRNA in the presence (lanes 2, 3 and 5) or absence (lane 4) of native snRNP proteins, incubated with U5-depleted extract, and then immunoprecipitated as described in the Materials and methods with the following antibodies: rabbit non-immune serum (NIS) (lane 2), anti-m_3G H20 (lane 3), and anti-Sm Y12 (lanes 4 and 5). Input radiolabelled U5 snRNA is shown in lane 1. In both panels, RNA was isolated from the immunoprecipitates, fractionated on a 10% polyacrylamide–7 M urea gel, and detected by autoradiography. Similar results were also obtained with synthetic U2 and U5 snRNA.

extracts demonstrated the selective removal of U2 or U5 snRNA from U2- or U5-depleted extract, respectively (data not shown). Thus, U2-depleted extracts, while quantitatively lacking U2 snRNA and, presumably, U2 snRNP core proteins, should contain sufficient amounts of U2-specific proteins to support the formation of 17S U2 snRNPs after initial reconstitution of 10S U2 snRNPs with TPs. Similarly, U5-depleted extracts were expected to support the formation of 20S U5 snRNPs after the addition of *in vitro* reconstituted 10S U5 snRNPs.

To test for the formation during our two-step reconstitution procedure of core U2 and U5 snRNPs, reconstitution was carried out with radiolabelled U2 or U5 snRNA and RNP formation was then assayed by immunoprecipitation. As shown in Figure 3A, significant amounts of U2 snRNA were precipitated by the anti-Sm monoclonal antibody, Y12, (compare lane 5 with the background control in lane 2), demonstrating the association of core snRNP proteins with U2 during the two-step reconstitution procedure. The efficiency with which Y12-precipitable particles are formed appears to be rather high (assuming equal precipitation efficiencies), since a similar amount of U2 is precipitated by the monoclonal antibody, H20 (compare lanes 3 and 5); the latter recognizes the m_3G cap of U snRNAs (Bochnig *et al.*, 1987) and, thus, both naked U2 snRNA and reconstituted U2 snRNPs are precipitated. Significantly, Y12-precipitable particles were not formed to a significant extent when naked U2 snRNA was added directly to the depleted extract (i.e. without prior incubation with TPs) (Figure 3A, lane 4). Thus, one or more core snRNP protein, which is required for the formation of 10S U2 snRNPs, is limiting in the U2-depleted extracts. Similar results were also obtained with *in vitro* reconstituted U5 snRNPs. As shown in Figure 3B (lanes 3 and 5), significant amounts of U5 snRNA were precipitated by H20 and Y12 under standard reconstitution conditions. However, precipitation with Y12 was not observed if U5 snRNA was added directly to the U5-depleted extract (in the absence of TPs) (lane 4). Thus, both U2- and U5-depleted extracts appear to contain only limiting amounts of snRNP core proteins, thus necessitating the addition of TPs to the reconstitution mixture.

In vitro reconstituted U2 and U5 snRNPs are active in splicing

We next tested whether the *in vitro* reconstituted U2 and U5 snRNPs were active in splicing. Since the second reconstitution step is carried out in splicing buffer and in the presence of U2- or U5-depleted extract, the formation of splicing active snRNPs after reconstitution could be assayed directly by adding a radiolabelled pre-mRNA to the mixture and incubating at 30°C. U5- or U2-depleted extracts were first assayed for their ability to splice a radiolabelled adenovirus pre-mRNA. As shown in Figure 4, splicing activity was significantly reduced in both the U5- and U2-depleted extracts, although the pre-mRNA substrate was efficiently spliced in mock-depleted extract (ME) (compare lanes 1 and 2 in both Figure 4A and B). Consistent with the known functions of U5 and U2 during spliceosome formation, native gel electrophoresis demonstrated that A complex formation was significantly reduced in the U2-depleted extract, whereas B complex formation was inhibited in the U5-depleted extract (data not shown). Splicing activity could, however, be restored to the U5-depleted extract by the addition of Mono Q purified 10S U5 snRNPs which contain solely the core snRNP proteins (Figure 4A, lane 3), demonstrating that the block to splicing was specifically due to the absence of U5 snRNPs. Similarly, complementation of the U2-depleted extract was achieved by the addition of 12S U2 snRNPs which contain the snRNP core proteins and two of the more tightly-associated U2-specific proteins (i.e. A′ and B″) (Figure 4B, lane 3). Significantly, the addition of TPs alone (not shown in Figure 4, but see Figures 5 and 6), or HeLa U5 or U2 snRNA to their respective depleted extract did not restore splicing (Figure 4A and B, lane 4). The exogenously added snRNAs were not significantly degraded in either splicing extract (data not shown), demonstrating that the absence of splicing complementation was not due to decreased U5 or U2 snRNA stability. However, U5 and U2 snRNA incubated with TPs and depleted extract before splicing (i.e. under standard reconstitution conditions) restored splicing to a level similar to that observed with the native 10S U5 or 12S U2 snRNPs (Figure 4A and B, lane 5). The specificity of splicing complementation with snRNA plus TPs was confirmed by adding U5 snRNA and TPs to the U2-depleted extract, and by adding U2 snRNA and TPs to the U5-depleted extract; in both instances no complementation was observed (Figure 4A and B, lane 6). Since splicing complementation is observed only upon addition of both snRNA and the core protein-enriched TP preparation, the results presented in Figure 4 indicate that snRNP core proteins are absolutely required for the formation of splicing active U5 and U2 snRNPs. Although TP preparations also typically contain a limited number of particle-specific proteins (see Figure 2A), subsequent hybrid snRNP reconstitution experiments demonstrate that these additional proteins are not responsible for the splicing

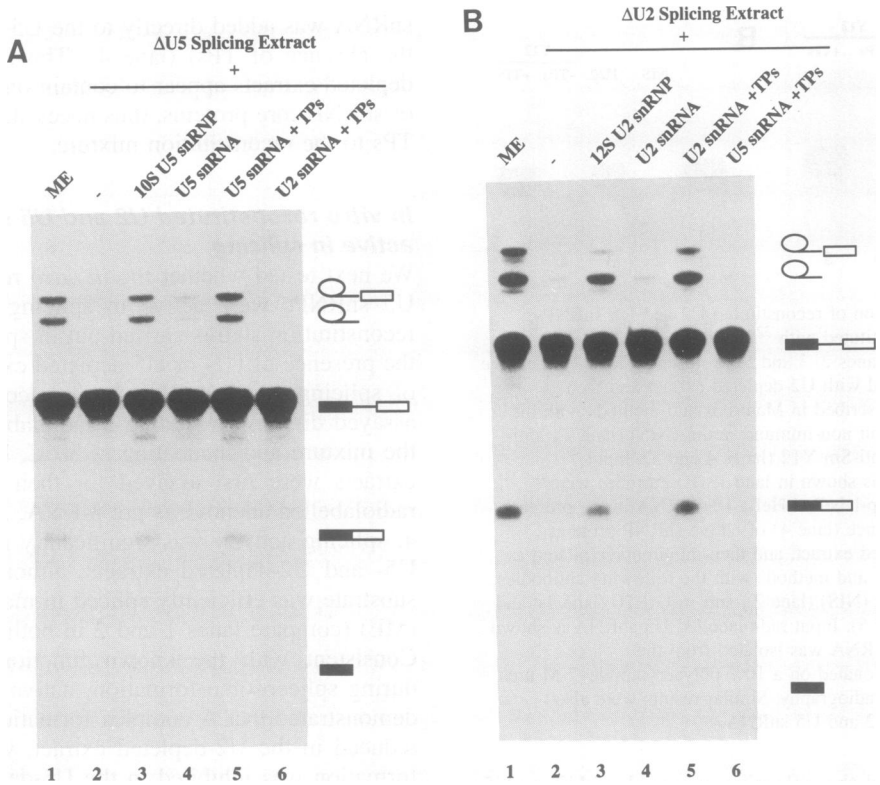

Fig. 4. Splicing complementation of U5- or U2-depleted nuclear extract. (**A**) *In vitro* reconstitution/splicing reactions were performed with mock depleted extract (ME) (lane 1), U5-depleted extract (lane 2) or U5-depleted extract plus the following: 100 ng of Mono Q purified 10S U5 snRNP (lane 3), 100 ng HeLa U5 snRNA (lane 4), 100 ng HeLa U5 snRNA and 650 ng purified snRNP protein (TPs) (lane 5) or 100 ng HeLa U2 snRNA and 650 ng TPs (lane 6). (**B**) *In vitro* reconstitution/splicing reactions were performed with mock depleted extract (lane 1), U2-depleted extract (lane 2) or U2-depleted extract plus the following: 100 ng Mono Q purified 12S U2 snRNP (lane 3), 100 ng HeLa U2 snRNA (lane 4), 100 ng HeLa U2 snRNA and 650 ng purified snRNP protein (TPs) (lane 5) or 100 ng HeLa U5 snRNA and 650 ng TPs (lane 6). The position of the pre-mRNA, and the splicing intermediates and products are indicated at the right of both panels. The amount of exogenously added U2 or U5 snRNA (100 ng) is approximately equivalent (in the case of U2) or 2-fold greater (in the case of U5) than that present in the mock-depleted splicing reactions.

complementation observed with reconstituted U2 or U5 particles (see below).

SnRNP core proteins are functionally interchangeable

Although biochemically indistinguishable, at least in terms of their gel migration behaviour (Lehmeier *et al.*, 1990), the snRNP proteins common to all spliceosomal snRNPs could conceivably exhibit small differences (e.g. differences in post-translational modification) which are specific for a given snRNP species. To test whether the core snRNP proteins associating with one snRNP species can functionally replace those which associate with another, native snRNP proteins were isolated from highly purified 12S U1 or U2 snRNPs, and hybrid U5 and U2 particles were reconstituted and functionally assayed as described above. As shown in Figure 5A, U5-depleted extracts were complemented to a similar extent by U5 particles reconstituted with core proteins prepared either from a mixture of snRNP particles (TPs) (lane 7) or from highly purified U1 snRNPs (lane 8). U5 snRNA, TPs or U1 proteins alone did not complement splicing to a measurable extent (lanes 4–6). The U1 snRNPs from which the U1 proteins were prepared do not contain detectable amounts of U5 (Figure 2B) and, assuming that U5 core proteins in the depleted extract do not associate during the second step of reconstitution (significant amounts of these proteins do not appear to be present in the U5-depleted extract), these results demonstrate that U5 snRNPs containing U1 core proteins are active in splicing. Similar results were obtained with native snRNP proteins isolated from Mono Q purified U2 snRNPs. Whereas TPs or U2 proteins alone had no significant effect on splicing efficiency (Figure 5B, lanes 4 and 5), U5 snRNPs reconstituted from TPs or U2 core proteins restored splicing to a U5-depleted extract to a similar extent (Figure 5B, lanes 6 and 7).

The ability of hybrid snRNP particles to complement splicing was not limited to U5 snRNPs. Efficient complementation of U2-depleted extracts was observed not only when reconstitution was performed with proteins isolated from a mixture of snRNPs (TPs), but also with U2 snRNPs reconstituted from U1 proteins (Figure 5C, lanes 4 and 5). TPs or U1 proteins alone had no measurable effect on splicing efficiency (data not shown). The results presented in Figure 5 demonstrate that the core snRNP proteins associating with one snRNP species are functionally interchangeable with those from another. Since TPs prepared from U1 or U2 snRNPs contain no U5-specific proteins, these results also demonstrate that the formation of splicing active U5 snRNPs upon pre-incubation with TPs is not due to the association of a U5-specific protein which is present in the TPs but missing in the U5-depleted extract. Similarly, the splicing complementation observed when U2 snRNA is pre-incubated with TPs cannot be attributed merely to the presence of one or more U2-specific protein in the TP preparation.

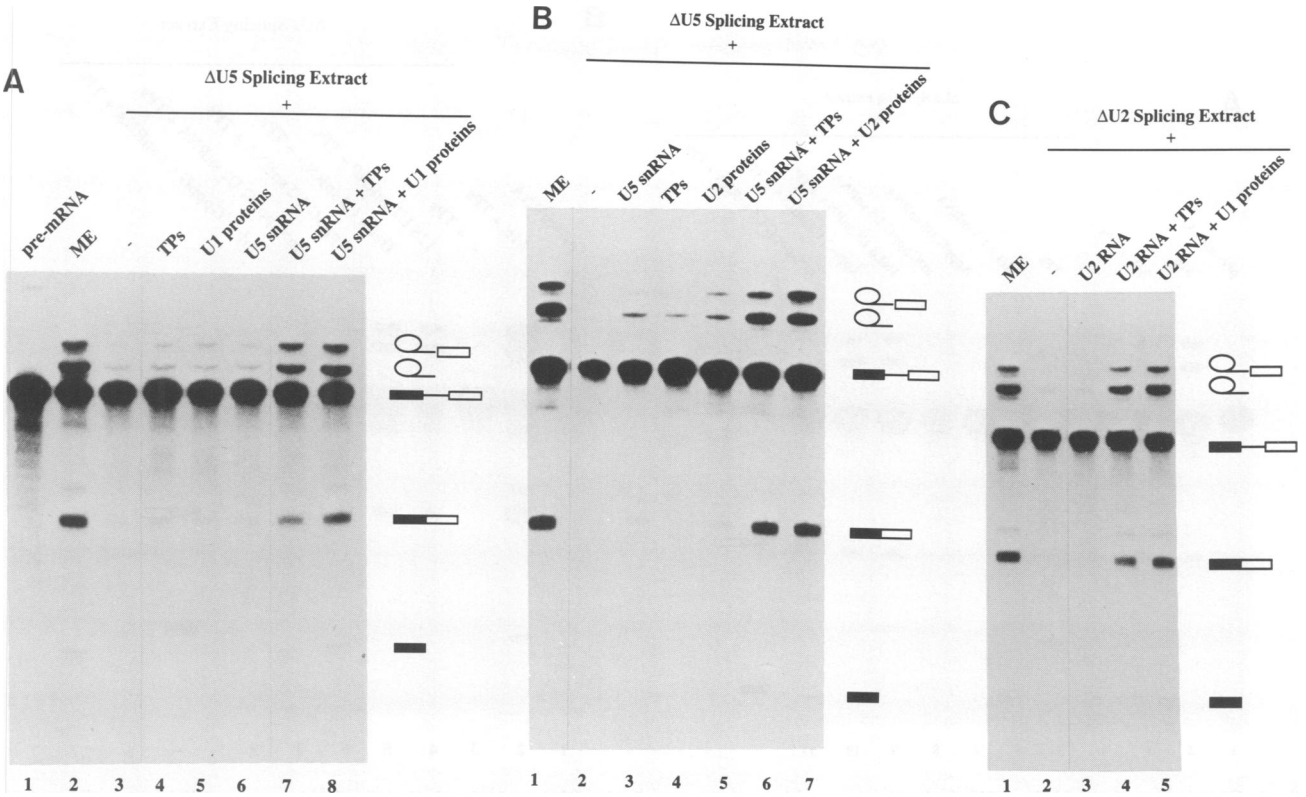

Fig. 5. Complementation of U5- or U2-depleted extract with hybrid reconstituted particles. (**A**) U5-depleted splicing extracts are complemented by U5 snRNPs reconstituted from U1 proteins. Reconstitution and splicing reactions were performed with mock depleted extract (lane 2), U5-depleted extract (lane 3), or U5-depleted extract plus the following: 650 ng TPs (lane 4), 650 ng U1 snRNP protein (lane 5), 100 ng HeLa U5 snRNA (lane 6), and 100 ng HeLa U5 snRNA plus 650 ng TPs (lane 7) or 650 ng U1 proteins (lane 8). The positions of the pre-mRNA (lane 1) and splicing intermediates and products are indicated schematically. (**B**) U5-depleted extracts are complemented by U5 snRNPs reconstituted from U2 proteins. Reconstitution and splicing reactions were performed with mock depleted extract (lane 1), U5-depleted extract (lane 2), or U5-depleted extract plus the following: 100 ng HeLa U5 snRNA (lane 3), 650 ng TPs (lane 4), 350 ng U2 snRNP protein (lane 5), and 100 ng HeLa U5 snRNA plus 650 ng TPs (lane 6) or 350 ng U2 proteins (lane 7). (**C**) U2-depleted extracts are complemented by U2 snRNPs reconstituted from U1 proteins. Reconstitution and splicing reactions were performed with mock depleted extract (lane 1), U2-depleted extract (lane 2), or with U2-depleted extract plus the following: 100 ng HeLa U2 snRNA (lane 3), 100 ng HeLa U2 snRNA plus 650 ng TPs (lane 4) or 650 ng U1 proteins (lane 5). The addition of TPs or U1 proteins alone did not restore splicing to a significant extent (not shown).

U5 but not U2 snRNPs reconstituted from in vitro transcribed snRNA can complement splicing

As a prerequisite to study the role of individual snRNA nucleotides in snRNP function, we first tested whether *in vitro* transcribed U5 snRNA was able to restore splicing to a U5-depleted extract. To test additionally whether the 5′-trimethylguanosine cap is essential for U5 snRNP function, we prepared *in vitro* transcribed U5 snRNAs with either an ApppG or m^7GpppG cap structure. Since the enzyme responsible for cap hypermethylation does not appear to be present in splicing extracts (cap hypermethylation is a cytoplasmic event) (Mattaj, 1986), it is unlikely that the m^7G cap is converted to the normally present m_3G form. The addition of HeLa or synthetic U5 snRNA alone to U5-depleted extract had no effect on splicing (Figure 6A, lanes 6–8). However, when pre-incubated with purified snRNP proteins, synthetic U5 snRNAs restored splicing to a level identical to that obtained with HeLa U5 snRNA, regardless of the cap structure (Figure 6A, compare lanes 9 to 11). Thus, a trimethylguanosine cap structure appears to be dispensable for U5 snRNA function in splicing.

Similar splicing complementation studies were also carried out with *in vitro* transcribed U2 snRNA. As previously shown, efficient complementation of a U2-depleted extract was obtained with purified 12S U2 snRNPs and U2 snRNPs reconstituted from HeLa U2 snRNA and TPs (Figure 6B, lanes 4 and 5). However, in contrast to U5, U2 snRNPs reconstituted from *in vitro* transcribed U2 snRNA and TPs did not stimulate splicing to a significant extent (Figure 6B, lanes 6–8). The level of product formation observed in lanes 6–8 is only slightly higher than the background level arising from the residual splicing activity present in U2-depleted extracts and represents an ~2-fold increase, as compared with the 20-fold increase observed with HeLa U2 snRNA. Significant levels of complementation were not observed with U2 snRNPs reconstituted from synthetic U2 snRNA even when the snRNA possessed a normal trimethylguanosine 5′ cap (lane 8). Further, an increase in splicing activity could not be achieved by adding higher amounts of synthetic U2 snRNA (up to 500 ng) or by denaturing and subsequently renaturing the snRNA before reconstitution.

U5 and U2 snRNPs reconstituted from synthetic snRNAs lack pseudouridine modifications

In contrast to authentic HeLa U5 snRNA which contains methylated nucleotides at positions 1, 2, 37, 41 and 45, and pseudouridine (Ψ) at positions 43, 46 and 53 (in the case of U5a RNA) (Reddy and Busch, 1988), *in vitro*

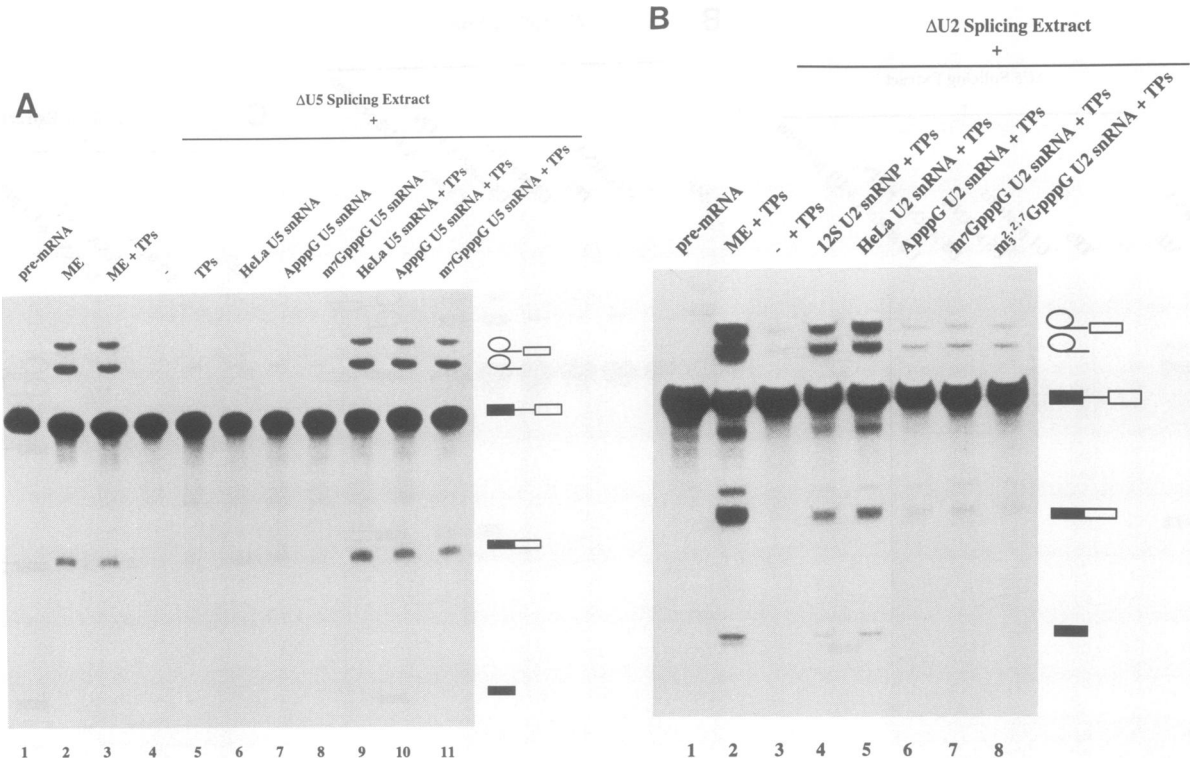

Fig. 6. Complementation of U5- or U2-depleted extract with *in vitro* transcribed snRNAs. (**A**) Synthetic U5 snRNA restores splicing regardless of 5′ cap structure. Reconstitution and splicing complementation were performed with mock depleted extract in the absence (lane 2) or presence of purified snRNP proteins (lane 3), with U5-depleted nuclear extract (lane 4) or with U5-depleted extract plus the following: 650 ng TPs (lane 5), 100 ng HeLa U5 snRNA alone (lane 6) or with 650 ng TPs (lane 9), 100 ng ApppG capped U5 snRNA alone (lane 7) or with 650 ng TPs (lane 10) and m^7GpppG capped U5 snRNA alone (lane 8) or with 650 ng TPs (lane 11). (**B**) Synthetic U2 snRNA does not restore splicing to U2-depleted extracts. Reconstitution and splicing complementation were performed with mock depleted extract (lane 2), U2-depleted extract (lane 3) or U2-depleted extract plus the following: 100 ng Mono Q purified 12S U2 snRNP (lane 4), 100 ng HeLa U2 snRNA (lane 5), 100 ng ApppG capped U2 snRNA (lane 6), 100 ng m^7GpppG capped U2 snRNA (lane 7), or 100 ng m_3GpppG capped U2 snRNA (lane 8); 650 ng TPs were added to all of the reconstitution/splicing assays. The position of the pre-mRNA and the splicing intermediates and products are indicated schematically at the right.

transcribed U5 snRNA lacks all internal modifications. Thus, the ability of the latter to support *in vitro* splicing in U5-depleted extracts suggested that internal U5 snRNA modifications do not play an essential role in splicing. In contrast, in light of the failure of synthetic U2 snRNA to complement splicing, U2 snRNP function could require base methylations or the formation of pseudouridine. In the case of U2, a large percentage of its uridines (13 out of 56) is normally converted to Ψ (Reddy and Busch, 1988). To determine whether U2 or U5 snRNA was modified subsequent to *in vitro* transcription, we assayed whether Ψ formation occurred during the *in vitro* reconstitution and *in vitro* splicing reactions. Consistent with previous studies (Patton, 1991, 1994; Patton *et al.*, 1994), Ψ formation could be detected when *in vitro* transcribed, radiolabelled U5 or U2 snRNA was incubated with both S100 and nuclear extract (Figure 7A and B, lane 1). However, when radiolabelled, synthetic U5 or U2 snRNA was subjected to standard reconstitution conditions, no Ψ formation could be detected (lane 2). Subsequent incubation at 30°C for up to 90 min (as occurs during the *in vitro* splicing reaction) did not lead to the formation of detectable levels of Ψ (lanes 3–5). These results suggest that at least one form of base modification does not occur to a significant extent during *in vitro* reconstitution and splicing and thus the majority of particles reconstituted from synthetic snRNA appear to lack pseudouridine.

Discussion

We have established a two-step *in vitro* reconstitution system which generates U2 and U5 snRNPs that are active in an *in vitro* splicing complementation assay. The reconstitution/splicing complementation system described here should facilitate future investigation of both structural and functional aspects of the U2 and U5 snRNPs. The reconstitution of functionally active U2 and U5 snRNPs was accomplished by initially incubating purified snRNA with native, RNA-free, snRNP core proteins (TPs), and subsequently incubating with splicing extract that had been specifically depleted of U2 or U5 snRNPs by streptavidin–agarose affinity selection with biotinylated 2′-*O*-methyl or 2′-*O*-alkyl RNA oligonucleotides, respectively. The formation of 10S U2 and U5 snRNPs during this two-step reconstitution procedure was demonstrated by immunoprecipitation assays with the anti-Sm monoclonal antibody Y12 (Figure 3). Further, they demonstrated that core U2 or U5 snRNP formation did not occur if U2 or U5 snRNA was added directly to the U2- or U5-depleted extract respectively, suggesting that the depleted extracts do not contain significant amounts of free Sm proteins. Splicing was restored to a U2- or U5-depleted extract after addition of both authentic HeLa snRNA and TPs, but not after the addition of only one of these components (Figure 4A and B). Since a subset of

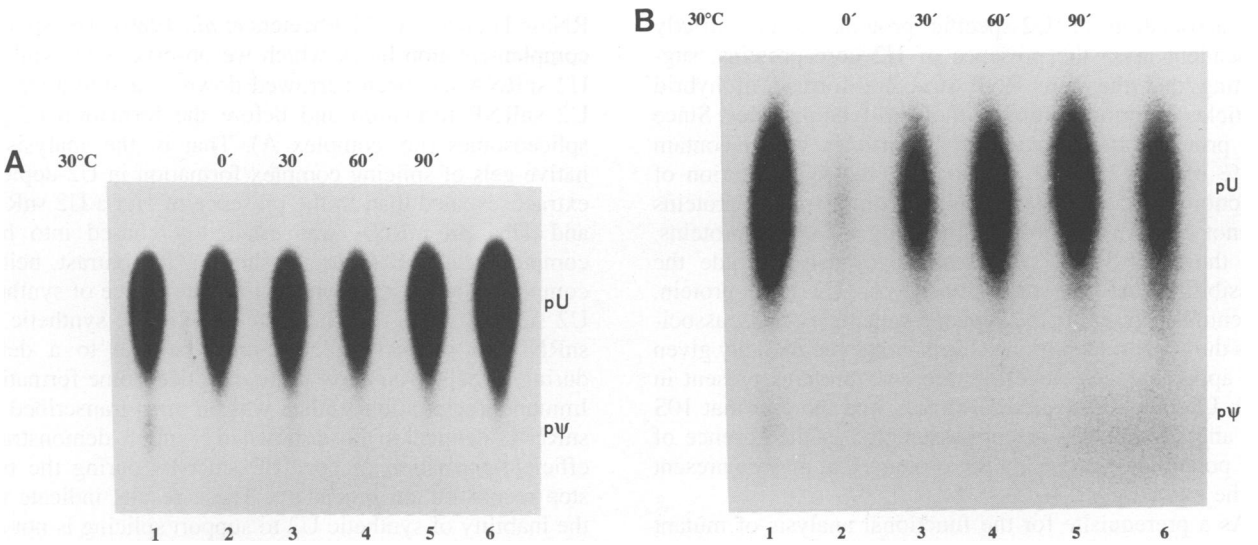

Fig. 7. Pseudouridine modification of *in vitro* transcribed U2 and U5 snRNA. Pseudouridine is not detected in synthetic U5 (**A**) or U2 snRNA (**B**) after *in vitro* reconstitution and splicing. ^{32}P-labelled, *in vitro* transcribed U5 or U2 snRNA was incubated in the presence of S100 and nuclear extract for 60 min (lane 1) or, after two-step *in vitro* reconstitution (as described in Materials and methods), for 0 (lane 2), 30 (lane 3), 60 (lane 4) or 90 min (lane 5) at 30°C. Reconstituted particles were immunoprecipitated with Y12 antibody and the precipitated RNA was isolated, hydrolysed with nuclease P1 and subjected to one-dimensional chromatography on TLC plates as described in Materials and methods. As a negative control, hydrolysed U5 or U2 snRNA that had not been incubated with extract is shown in lane 6. Radiolabelled pU and pΨ were detected by autoradiography and their positions are indicated at the right of each panel. The reduction in the amount of radioactivity observed in lane 2 of Figure 7B is due to the loss of the sample during its preparation. However, Ψ formation is not observed at the zero time point if an amount equivalent to that present at later time points is analysed.

U2 snRNP-specific proteins (Behrens *et al.*, 1993a; Brosi *et al.*, 1993), as well as the formation of an intact (U4/U6.U5) tri-snRNP complex (Lamm *et al.*, 1991; Blencowe *et al.*, 1993), have been shown to be essential for splicing, the ability of *in vitro* reconstituted U2 and U5 snRNPs to complement splicing would suggest that both 17S U2 and 20S U5, as well as 25S (U4/U6.U5) tri-snRNP complexes, are formed at some point in the splicing extract.

The observation that naked U2 or U5 snRNA alone is not able to restore splicing indicates that one or more component essential for the reconstitution of functional U2 or U5 snRNPs is not present in the depleted extracts. Since the combination of snRNA and TPs restored splicing, proteins present in the TP mixture must ultimately be required for splicing complementation. Hybrid reconstitution experiments demonstrated that particle-specific proteins present in TPs are not required and, thus, snRNP core proteins must play an essential role. The requirement for Sm proteins does not appear to be at the level of snRNA stabilization. Significant degradation of exogenously added U2 or U5 snRNA, whether authentic or transcribed *in vitro*, was not observed in depleted extracts in the absence of TPs. Based on immunoprecipitation studies, the essential role of the Sm proteins may be at the level of RNP formation. Namely, immunoprecipitation studies with the anti-Sm protein antibody, Y12, confirmed that Sm proteins do not associate with naked U2 or U5 snRNA to a measurable extent after incubation with depleted extract (Figure 3); rather, Sm protein association and thus the formation of 10S U2 or U5 particles was dependent upon the presence of TPs. Recent RNP assembly studies with the U1 snRNP have demonstrated that the association of the U1-specific proteins, 70K and C, is partially mediated by Sm proteins (Nelissen *et al.*, 1994). The requirement for Sm proteins in our splicing complementation assay could indicate that the subsequent association of U2- or U5-specific proteins during 17S U2 and 20S U5 snRNP formation is absolutely dependent upon the prior association of the snRNP core proteins. Thus, the essential role which the Sm proteins play in our reconstitution/complementation system may simply be a structural one. While it is conceivable that Sm proteins play a direct role in spliceosome formation or splicing proper, the data presented here do not allow any conclusions in this regard.

The absolute requirement of Sm proteins for splicing complementation allowed us to investigate whether Sm proteins associated with one snRNP species could functionally replace those of another. In light of the observation that the Sm site of U5 is not functionally interchangeable with that of U1, at least when the transport activity of such hybrid snRNAs is assayed in *Xenopus* oocytes (Jarmolowski and Mattaj, 1993), the question as to whether structural, and as a consequence, functional heterogeneities exist between U1 and U5 Sm proteins is not a trivial one. As shown in Figure 5A and B, hybrid U5 snRNPs reconstituted from proteins isolated either from a mixture of spliceosomal snRNPs or highly purified U1 or U2 snRNPs complemented a U5-depleted extract to a similar extent. Since U5 snRNPs are significantly less abundant than U1 or U2 snRNPs, it was not feasible to prepare TPs from purified U5 snRNPs. The low percentage of U5 in the TPs prepared from a mixture of snRNPs suggests that even in the case of TPs, hybrid U5 snRNPs are probably formed. Similarly, hybrid U2 snRNPs reconstituted from proteins isolated from purified U1 snRNPs are active in our reconstitution/complementation assay (Figure 5C). These results demonstrate that U1 core proteins support the formation of functional U5 snRNPs and thus the association of U5-specific proteins during 20S U5 snRNP formation does not require U5 core proteins. Similarly,

the association of U2-specific proteins is not strictly dependent upon the presence of U2 core proteins, suggesting that the core RNP structure formed in hybrid particles is similar to that of the wild-type particles. Since U1 proteins are prepared from particles which contain <1% of both U2 and U5 snRNPs, the reconstitution of functional U2 and U5 snRNPs with U1 core proteins cannot be attributed to contaminating U2 or U5 proteins. On the other hand, we cannot rigorously exclude the possibility that one or more U5 or U2 core protein, potentially present in the depleted splicing extracts, associates during reconstitution. This is, however, unlikely given the apparently low level of free core proteins present in both U2- and U5-depleted extracts, and the fact that 10S U2 and U5 snRNPs are pre-assembled in the absence of any potentially competing Sm proteins that may be present in the extract.

As a prerequisite for the functional analysis of mutant U2 and U5 snRNAs, we tested whether *in vitro* transcribed snRNAs are assembled into splicing active snRNPs in our reconstitution/complementation system. Synthetic U5 snRNA, possessing either an m^7GpppG or ApppG cap, was as active in splicing as its endogenous counterpart demonstrating that a trimethylguanosine cap structure is dispensable for U5 function (Figure 6A). Interestingly, no pseudouridine formation was detected with U5 snRNPs reconstituted from synthetic U5 snRNA, even after prolonged incubation at 30°C in the U5-depleted extract (Figure 7A). Although previous studies examining Ψ formation in reconstituted U5 and U2 snRNPs suggested that nuclear extracts contain pseudouridine synthase activity (the formation of sub-stoichiometric amounts of pseudouridine were observed upon incubation in nuclear extract) (Patton, 1991, 1994; Patton *et al.*, 1994), the ratio of nuclear extract to U5 snRNA was 150-fold higher than that employed in our reconstitution/complementation assay. Our results provide the first evidence that U5 snRNA base modifications are not essential to U5 snRNP function. In particular, although we cannot at present rule out an effect of internal base methylation, the conversion of uridine at positions 43, 46 and 53 to Ψ apparently has no effect on the splicing activity of U5 snRNA. These results are consistent with the previous observation that Ψ formation is not required for U5 snRNP assembly (Patton, 1991) and would further suggest that it has no influence upon (U4/U6.U5) tri-snRNP assembly, since formation of the latter is a prerequisite for splicing. It should be noted, however, that due to our inability to detect low levels of pseudouridine (only 8% of the uridine residues present in U5a snRNA are Ψ), we cannot rigorously exclude the possibility that a small percentage of the U5 snRNAs, in particular those which may take part in the splicing reaction, do contain pseudouridine.

Interestingly, in contrast to U5, significant splicing complementation was not observed with U2 snRNPs reconstituted from synthetic U2 snRNA (Figure 6B). Previous attempts to restore splicing to U2-inactivated oocytes upon microinjection of *in vitro* transcribed HeLa U2 snRNA were also unsuccessful (Pan and Prives, 1989). On the other hand, low levels of splicing complementation were observed when *in vitro* transcribed HeLa U2 was added to yeast splicing extracts whose endogenous U2 snRNA had been degraded by oligonucleotide-directed RNase H cleavage (McPheeters *et al.*, 1989). The splicing complementation block which we observe with synthetic U2 snRNA has been narrowed down to a step after 10S U2 snRNP formation and before the formation of prespliceosomes (i.e. complex A). That is, the analysis on native gels of splicing complex formation in U2-depleted extract revealed that, in the presence of HeLa U2 snRNA and TPs, pre-mRNA was efficiently chased into both complex A and B (data not shown). In contrast, neither complex A nor B was formed in the presence of synthetic U2 snRNA and TPs. Thus, the inability of synthetic U2 snRNA to support splicing must be due to a defect during or before an early stage of spliceosome formation. Immunoprecipitation studies with *in vitro* transcribed U2 snRNA, identical to those shown in Figure 3, demonstrated efficient formation of core U2 snRNPs during the two-step reconstitution procedure. These results indicate that the inability of synthetic U2 to support splicing is not due to a decrease in its stability relative to that of HeLa U2 snRNA since similar amounts of core U2 snRNPs are reconstituted in both cases (data not shown). Since TPs contain a relatively high amount of A′ and B″, it does not seem likely that reconstituted U2 snRNPs lack these U2-specific proteins. However, conclusive evidence for the formation of 17S U2 snRNPs is currently lacking. To date, we have been unable to detect 17S U2 snRNP formation by glycerol gradient centrifugation with both HeLa and synthetic U2 snRNA, apparently due to the insufficient sensitivity of our assay. That is, since particles reconstituted with HeLa U2 snRNA restore splicing to U2-depleted extracts, it follows that, despite our inability to detect them, 17S U2 snRNPs are formed during the two-step reconstitution with HeLa U2 snRNA. We are currently attempting to refine our 17S U2 snRNP detection methods, and thereby establish whether the inability of synthetic U2 snRNA to support splicing is due to a defect at the level of particle assembly or, alternatively, at an early stage of spliceosome formation.

The inactivity of the synthetic U2 snRNA could be due to a number of factors. A trivial explanation could be the presence of three additional nucleotides at its 5′ end. These extra nucleotides could conceivably alter the conformation of the U2 snRNA and/or hinder the binding of a functionally important U2 protein. However, in the aforementioned oocyte complementation studies (Pan and Prives, 1989), removal of two of these extra nucleotides, such that only one additional nucleotide was present, had no effect on the splicing activity of the synthetic U2 snRNA. A second possible explanation for the inactivity of the synthetic U2 snRNA could be the lack of post-transcriptional modification. Significant levels of pseudouridine formation were not detected in the synthetic U2 snRNA after reconstitution of U2 snRNPs, even after prolonged incubation at 30°C (Figure 7B). Since 22% of the U2 uridine residues are normally converted to pseudouridine, and we estimate that <1% of the total counts are present as pseudouridine, the results presented in Figure 7B indicate that only very low amounts of pseudouridine (at most 5%) have potentially been formed. Although we, at present, have no information regarding the presence or absence of other types of internal modification (namely base methylations), and also cannot rule out the presence of low levels of pseudouridine, these initial

results are consistent with the hypothesis that modified nucleotides are required for U2 snRNA splicing activity. Interestingly, even though the function of post-transcriptional snRNA modifications is not known, they are usually present in RNA domains that are highly conserved throughout evolution and that are often involved in RNA–RNA interactions. In U2 snRNA, modified nucleotides are localized, for the most part, in the 5′-domain which is involved in base-pairing interactions with both the branch site of the pre-mRNA (Parker *et al.*, 1987; Wu and Manley, 1989; Zhuang and Weiner, 1989) and with U6 snRNA (Datta and Weiner, 1991; Wu and Manley, 1991; Madhani and Guthrie, 1992). A requirement of base modification for a stable U2/branch site interaction is consistent with the inability of the synthetic U2 snRNA to support splicing complex A formation. Modified bases could influence this interaction either directly or indirectly, by stabilizing an active conformation of the U2 snRNP. It is particularly noteworthy that the interaction of the U2 snRNP with the pre-mRNA has been shown to be highly dependent upon the formation of a particular U2 snRNP conformational state (Zavanelli and Ares, 1991). Mutational analyses of the yeast U2 snRNA indicate that competing, mutually exclusive conformations of the U2 snRNA exist (Zavanelli *et al.*, 1994). These conformations are distinguished by the presence (in the case of the functional form) or absence of stem–loops IIa and IIb. Normally, formation of the functionally active conformation of U2 is favoured. The absence of pseudouridines could conceivably shift this equilibrium such that the formation of an inactive conformation would be favoured. The latter could in turn inhibit the formation of the 17S U2 snRNP. Recent genetic studies by Wells and Ares (1994) suggest that the functional association of those yeast U2-specific proteins required for the incorporation of U2 into the spliceosome (i.e. PRP 9, 11 and 21) is dependent upon the stable formation of stem–loop IIa. Although the precise interaction sites of the mammalian homologues of these U2-specific proteins (i.e. the 60, 66 and 110 kDa proteins) is not known, they appear to interact with the 5′ half of the U2 snRNP (Behrens *et al.*, 1993b; Brosi *et al.*, 1993). Thus, the association of these proteins may also be sensitive to the conformation of stem–loop II of the mammalian U2 snRNA. Although base modification (at least pseudouridine formation) did not appear to be required for U5 snRNA function, it should be noted that the U2 snRNA is modified to a much greater extent than U5 and, thus, it would not be surprising if the apparent lack of base modification were to inhibit U2 snRNA function. Experiments are now in progress to generate synthetic U2 snRNAs modified *in vitro* and to test them in our reconstitution/complementation system.

The ability to reconstitute splicing active U5 snRNPs from synthetic U5 snRNA will facilitate the analysis of both structural and functional aspects of the U5 snRNP. In addition to establishing whether or not synthetic U5 and U2 snRNA bases are methylated during our *in vitro* reconstitution/complementation assays, future studies, using various U5 snRNA mutants, will be aimed at investigating which regions of the mammalian U5 snRNA are essential for spliceosome formation and splicing proper. In addition, the incorporation of the photoactivateable nucleoside, 4-thiouridine, should enable the detailed investigation of U5 snRNA interactions during splicing. Further, immunodepletion of a given U2- or U5-specific protein from our U2- or U5-depleted extracts should potentially allow the future investigation of the role of mammalian snRNP particle-specific proteins in pre-mRNA splicing.

Materials and methods

Preparation of snRNPs, snRNAs and native snRNP proteins

Nuclear and S100 extracts were prepared from HeLa cells (Computer Cell Culture Center, Mons) as described by Dignam *et al.* (1983). A mixture of U1, U2, U5 and U4/U6 snRNPs was isolated from HeLa nuclear extract by anti-m_3G immunoaffinity chromatography (Bach *et al.*, 1990). Purified 12S U1 or U2 snRNPs were isolated from the immunoaffinity purified snRNPs by Mono Q chromatography (Bach *et al.*, 1990). 10S (core) U5 snRNPs were also isolated by Mono Q chromatography as described previously (Kastner *et al.*, 1990). HeLa U2 and U5 snRNAs, as well as native, RNA-free, snRNP proteins, were isolated as described by Sumpter *et al.* (1992). Radiolabelled and non-radiolabelled synthetic U2 snRNA were prepared by *in vitro* transcription of *Sma*I linearized pMRGU2-27 (Jacobson *et al.*, 1993) with T7 polymerase. The synthesis of U5 snRNA was performed with a plasmid which contains the gene encoding the human U5a species. Transcriptions were performed essentially as described by Fischer *et al.* (1991). Chemically synthetized ApppG, m^7GpppG and m_3GpppG were added to the transcription reaction as described (Fischer *et al.*, 1991). ^{32}P-labelled U2 and U5 snRNAs, with a specific activity of 1.9×10^6 c.p.m./pmol and 1.3×10^6 c.p.m./pmol, were transcribed in the presence of [^{32}P]UTP (Amersham). Synthetic U5 and U2 snRNAs possess five and three additional nucleotides at their 5′ end, respectively.

In vitro splicing and preparation of snRNP-depleted extract

U5- and U2-depleted nuclear extracts were prepared by affinity selection with complementary 2′-*O*-alkyl (for U5) or 2′-*O*-methyl (for U2) biotinylated oligonucleotides and streptavidin–agarose beads essentially as described by Lamond and Sproat (1994), except the KCl concentration was increased to 800 mM. The following oligonucleotides have been used: for U5 depletion, an oligonucleotide complementary to nucleotides 36–47 of the human U5 snRNA, 5′-dC*dC*dC*dC* UZGUZZZZG-GCGdC*dC*dC*dC*dT-3′(where * denotes a biotinylated 2′-deoxycytidine, Z a 2′-*O*-alkyl-2-aminoadenosine, and U, G, C represent 2′-*O*-alkyl-ribonucleotides) (Lamm *et al.*, 1991), and for U2 depletion, an oligonucleotide complementary to nucleotides 1–20 of human U2 snRNA, 5′-CCAAAAGGCCGAGAAGCGAUdC*dC*dC*dT-3′ (where * denotes a biotinylated 2′-deoxycytidine and A, U, G, C represent 2′-*O*-methyl-ribonucleotides). The concentration of oligonucleotide required for optimum depletion was determined empirically and found in both cases to be 4.8 nmol/ml of HeLa nuclear extract. Mock-depleted extracts were handled in a manner identical to that of the depleted extracts, except oligonucleotide was omitted. Splicing reactions (12.5 μl) were incubated for 90 min at 30°C with 1 ng of ^{32}P-labelled MINX pre-mRNA (Zillmann *et al.*, 1988) and the splicing reactants and products were isolated and analysed essentially as described by Wolff and Bindereif (1992). Radiolabelled MINX pre-mRNA was prepared by *in vitro* transcription of *Bam*HI linearized pMINX with T7 polymerase as described by (Wolff and Bindereif, 1992).

In vitro reconstitution

U5 or U2 snRNP reconstitution was carried out in two steps. In the first step, endogenous or *in vitro* transcribed U2 or U5 snRNA (final concentration 0.64 and 1.0 μM, respectively) and purified native snRNP proteins (TPs) (final concentration 1.85 μM) were incubated for 30 min at 30°C and 15 min at 37°C in reconstitution buffer: 20 mM HEPES–KOH, pH 7.9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, pH 8.0, 2 mM ATP, and 4 U/μl RNasin (Promega). In the second step, the reconstitution mixture (2.5 μl) was added to a splicing reaction (total volume 12 μl) containing 30% depleted extract, 10 mM HEPES–KOH, pH 7.9, 52 mM KCl, 3.2 mM $MgCl_2$, 0.1 mM EDTA, pH 8.0, 0.5 mM ATP, 20 mM creatine phosphate, 40 μg/ml yeast tRNA, 10% glycerol and 1.6 U/μl RNasin, and then incubated for 30 min at 0°C. Splicing was performed directly in the reconstitution mixture by adding 0.5 μl (1 ng) pre-mRNA (see above).

Immunoprecipitation of reconstituted snRNPs

U5 and U2 snRNAs were 3′-end-labelled with [^{32}P]pCp (Amersham), using the method of England and Uhlenbeck (1978), and were gel purified (on 10% polyacrylamide–7 M urea gels) before reconstitution. Immunoprecipitations with the monoclonal antibodies H20 (Bochnig *et al.*, 1987) and Y12 (Lerner and Steitz, 1979) were performed as previously described (Hackl *et al.*, 1994). Immunoprecipitated RNA was extracted with phenol/chloroform, precipitated with ethanol, and fractionated on a 10% polyacrylamide–7 M urea gel (Bringmann and Lührmann, 1986).

Pseudouridine modification assay

[^{32}P]UTP-labelled U2 or U5 snRNA was incubated under standard reconstitution conditions (see above) or, for the positive control, in the presence of S100 and nuclear extract as described by Patton (1991). Subsequent to reconstitution, samples were incubated for 0, 30, 60 or 90 min at 30°C (i.e. under splicing conditions, except pre-mRNA was not added). To enrich for U2 or U5 snRNA which could potentially partake in the splicing reaction, reconstituted particles were isolated by immunoprecipitation with Y12 as described above. The immunoprecipitated RNA was isolated by phenol/chloroform extraction and precipitated with ethanol. Pseudouridine formation was assayed essentially as described by Patton (1991). Briefly, precipitated RNA was redissolved in buffer containing 20 mM sodium acetate (pH 5.2) and nuclease P1 at a final concentration of 100 μg/ml, and incubated at 37°C for 60 min The hydrolysed samples were chromatographed on cellulose TLC PEI plates (Macherey–Nagel) in buffer containing isopropanol/HCl/$H_2$0 (70:15:15, v/v/v). The plates were subsequently air dried and subjected to autoradiography.

Acknowledgements

We would like to thank Silke Börner, Michaela Wicke, Irene Öchsner, Stefan Becker and Axel Badouin for excellent technical assistance and the remaining members of our laboratory, especially Berthold Kastner, for advice and discussions. We are grateful to Albrecht Bindereif for the human U5a construct and for providing us with U2-depleted extracts in the early phase of the project. We also thank Joan Steitz and Iain Mattaj for their generous gift of monoclonal antibody Y12 and Mitsuo Sekine for kindly providing m_3GpppG cap. This work was supported by the Deutsche Forschungsgemeinschaft SFB 272. V.S. was supported by a fellowship from the Alexander von Humboldt Stiftung.

References

Bach,M., Winkelmann,G. and Lührmann,R. (1989) 20S small nuclear ribonucleoprotein U5 shows a surprisingly complex protein composition. *Proc. Natl Acad. Sci. USA*, **86**, 6038–6042.

Bach,M., Bringmann,P. and Lührmann,R. (1990) Purification of small nuclear ribonucleoprotein particles with antibodies against modified nucleotides of small nuclear RNAs. *Methods Enzymol.*, **181**, 232–257.

Barabino,S.M.L., Blencowe,B.J., Ryder,U., Sproat,B.S. and Lamond,A.I. (1990) Targeted snRNP depletion reveals an additional role for mammalian U1 snRNP in spliceosome assembly. *Cell*, **63**, 293–302.

Beggs,J.D. (1993) Yeast protein factors involved in nuclear pre-mRNA splicing. *Mol. Biol. Rep.*, **18**, 99–103.

Behrens,S.-E. and Lührmann,R. (1991) Immunoaffinity purification of a [U4/U6.U5]tri-snRNP from human cells. *Genes Dev.*, **5**, 1439–1452.

Behrens,S.-E., Galisson,F., Legrain,P. and Lührmann,R. (1993a) Evidence that the 60-kDa protein of 17S U2 small nuclear ribonucleoprotein is immunologically and functionally related to the yeast PRP9 splicing factor and is required for the efficient formation of prespliceosomes. *Proc. Natl Acad. Sci. USA*, **90**, 8229–8233.

Behrens,S.-E., Tyc,K., Kastner,B., Reichelt,J. and Lührmann,R. (1993b) Small nuclear ribonucleoprotein (RNP) U2 contains numerous additional proteins and has a bipartite RNP structure under splicing conditions. *Mol. Cell. Biol.*, **13**, 307–319.

Blencowe,B.L., Carmo-Fonseca,M., Behrens,S.-E., Lührmann,R. and Lamond,A.I. (1993) Interaction of the human autoantigen p150 with splicing snRNPs. *J. Cell Sci.*, **105**, 685–697.

Bochnig,P., Reuter,R., Bringmann,P. and Lührmann,R. (1987) A monoclonal antibody against 2,2,7-trimethylguanosine that reacts with intact U snRNPs as well as with 7-methylguanosine-capped RNAs. *Eur. J. Biochem.*, **168**, 461–467.

Branlant,C., Krol,A., Ebel,J.P., Lazar,E., Haendler,B. and Jacob,M. (1982) U2 RNA shares a structural domain with U1, U4 and U5 RNAs. *EMBO J.*, **1**, 1259–1265.

Bringmann,P. and Lührmann,R. (1986) Purification of the individual snRNPs U1, U2, U5 and U4/U6 from HeLa and characterization of their protein constituents. *EMBO J.*, **5**, 3509–3516.

Brosi,R., Gröning,K., Behrens,S.-E., Lührmann,R. and Krämer,A. (1993) Interaction of mammalian splicing factor SF3a with U2 snRNP and relation of its 60-kD subunit to yeast PRP9. *Science*, **262**, 102–105.

Datta,B. and Weiner,A.M. (1991) Genetic evidence for base pairing between U2 and U6 snRNA in mammalian mRNA splicing. *Nature*, **352**, 821–824.

Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.*, **11**, 1475–1489.

England,T.E. and Uhlenbeck,O.C. (1978) 3′-Terminal labelling of RNA with T4 RNA ligase. *Nature*, **275**, 560–561.

Fabrizio,P., McPheeters,D.S. and Abelson,J. (1989) *In vitro* assembly of yeast U6 snRNP: a functional assay. *Genes Dev.*, **3**, 2137–2150.

Fischer,U. and Lührmann,R. (1990) An essential signaling role for the m_3G cap in the transport of U1 snRNP to the nucleus. *Science*, **249**, 786–790.

Fischer,U., Darzynkiewicz,E., Tahara,S.M., Dathan,N.A., Lührmann,R. and Mattaj,I.W. (1991) Diversity in the signals required for nuclear accumulation of U snRNPs and variety in the pathways of nuclear transport. *J. Cell Biol.*, **113**, 705–714.

Fischer,U., Sumpter,V., Sekine,M., Satoh,T. and Lührmann,R. (1993) Nucleo-cytoplasmic transport of UsnRNPs: definition of a nuclear location signal in the Sm core domain that binds a transport receptor independently of the m_3G cap. *EMBO J.*, **12**, 573–583.

Gozani,O., Patton,J.G. and Reed,R. (1994) A novel set of spliceosome-associated proteins and the essential splicing factor PSF bind stably to pre-mRNA prior to catalytic step II of the splicing reaction. *EMBO J.*, **13**, 3356–3367.

Green,M.R. (1991) Biochemical mechanisms of constitutive and regulated pre-mRNA splicing. *Annu. Rev. Cell Biol.*, **7**, 559–599.

Hackl,W., Fischer,U. and Lührmann,R. (1994) A 69-kD protein that associates reversibly with the Sm core domain of several spliceosomal snRNP species. *J. Cell Biol.*, **124**, 261–272.

Hamm,J., Dathan,N.A. and Mattaj,I.W. (1989) Functional analysis of mutant *Xenopus* U2 snRNAs. *Cell*, **59**, 159–169.

Hamm,J., Darzynkiewicz,E., Tahara,S.M. and Mattaj,I.W. (1990a) The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite nuclear targeting signal. *Cell*, **62**, 569–577.

Hamm,J., Dathan,N.A., Scherly,D. and Mattaj,I.W. (1990b) Multiple domains of U1 snRNA, including U1 specific protein binding sites, are required for splicing. *EMBO J.*, **9**, 1237–1244.

Hermann,H., Fabrizio,P., Raker,V., Foulaki,K., Hornig,H., Brahms,H. and Lührmann,R. (1995) snRNP Sm proteins share two evolutionarily conserved sequence motifs which are involved in Sm protein–protein interactions. *EMBO J.*, **14**, 2076–2088.

Hinterberger,M., Pettersson,I. and Steitz,J.A. (1983) Isolation of small ribonucleoproteins containing U1, U2, U4, U5 and U6 RNAs. *J. Biol. Chem.*, **258**, 2604–2613.

Jacobson,M.R., Rhoadhouse,M. and Pederson,T. (1993) U2 small nuclear RNA 3′ end formation is directed by a critical internal structure distinct from the processing site. *Mol. Cell. Biol.*, **13**, 1119–1129.

Jarmolowski,A. and Mattaj,I.W. (1993) The determinants for Sm protein binding to *Xenopus* U1 and U5 snRNAs are complex and non-identical. *EMBO J.*, **12**, 223–232.

Kastner,B., Bach,M. and Lührmann,R. (1990) Electron microscopy of small nuclear ribonucleoprotein (snRNP) particles U2 and U5: evidence for a common structure-determining principle in the major U snRNP family. *Proc. Natl Acad. Sci. USA*, **87**, 1710–1714.

Lamm,G.M., Blencowe,B.J., Sproat,B.S., Iribarren,A.M., Ryder,U. and Lamond,A.I. (1991) Antisense probes containing 2-aminoadenosine allow efficient depletion of U5 snRNP from HeLa splicing extracts. *Nucleic Acids Res.*, **19**, 3193–3198.

Lamond,A.I. and Sproat,B.S. (1994) Isolation and characterization of ribonucleoprotein complexes. In Rickwood,D. and Hames,B.D. (eds), *RNA Processing. A Practical Approach*. Oxford University Press, Oxford, pp. 103–140.

Lehmeier,T., Foulaki,K. and Lührmann,R. (1990) Evidence for three distinct D proteins, which react differentially with anti-Sm autoantibodies, in the cores of the major snRNPs U1, U2, U4/U6 and U5. *Nucleic Acids Res.*, **18**, 6475–6484.

Lehmeier,T., Raker,V., Hermann,H. and Lührmann,R. (1994) cDNA cloning of the Sm proteins D2 and D3 from human snRNPs: evidence

for a direct D1–D2 interaction. *Proc. Natl Acad. Sci. USA*, **91**, 12317–12321.

Lerner,M.R. and Steitz,J.A. (1979) Antibodies to small nuclear RNAs complexed with proteins are produced by patients with systemic lupus erythematosus. *Proc. Natl Acad. Sci. USA*, **76**, 5495–5499.

Lührmann,R. (1988) snRNP proteins. In Birnstiel,M.L. (ed.), *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles*. Springer-Verlag, Berlin, pp. 71–99.

Lührmann,R., Kastner,B. and Bach,M. (1990) Structure of spliceosomal snRNPs and their role in pre-mRNA splicing. *Biochim. Biophys. Acta*, **1087**, 265–292.

Madhani,H.D. and Guthrie,C. (1992) A novel base-pairing interaction between U2 and U6 snRNAs suggests a mechanism for the catalytic activation of the spliceosome. *Cell*, **71**, 803–817.

Mattaj,I. (1986) Cap trimethylation of U snRNA is cytoplasmic and dependent on U snRNP protein binding. *Cell*, **46**, 905–911.

Mattaj,I.W. and De Robertis,E.M. (1985) Nuclear segregation of U2 snRNA requires binding of specific snRNP proteins. *Cell*, **40**, 111–118.

McPheeters,D.S., Fabrizio,P. and Abelson,J. (1989) *In vitro* reconstitution of functional yeast U2 snRNPs. *Genes Dev.*, **3**, 2124–2136.

Moore,M.J., Query,C.C. and Sharp,P.A. (1993) Splicing of precursors to messenger RNAs by the spliceosome. In Gestland,R.F. and Atkins,J.F. (eds), *RNA World*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1–30.

Nelissen,R.L.H., Will,C.L., van Venrooij,W.J. and Lührmann,R. (1994) The association of the U1-specific 70K and C proteins with U1 snRNPs is mediated in part by common U snRNP proteins. *EMBO J.*, **13**, 4113–4125.

Newman,A. and Norman,C. (1991) Mutations in yeast U5 snRNA alter the specificity of 5′ splice-site cleavage. *Cell*, **65**, 115–123.

Newman,A.J. and Norman,C. (1992) U5 snRNA interacts with exon sequences at 5′ and 3′ splice sites. *Cell*, **68**, 743–754.

Pan,Z.-Q. and Prives,C. (1988) Assembly of functional U1 and U2 human-amphibian hybrid snRNPs in *Xenopus laevis* oocytes. *Science*, **241**, 1328–1331.

Pan,Z.-Q. and Prives,C. (1989) U2 snRNA sequences that bind U2-specific proteins are dispensable for the function of U2 snRNP in splicing. *Genes Dev.*, **3**, 1887–1898.

Parker,R., Siliciano,P.G. and Guthrie,C. (1987) Recognition of the TACTAAC box during mRNA splicing in yeast involves base pairing to the U2-like snRNA. *Cell*, **49**, 229–239.

Patton,J.R. (1991) Pseudouridine modification of U5 RNA in ribonucleoprotein particles assembled *in vitro*. *Mol. Cell. Biol.*, **11**, 5998–6006.

Patton,J.R. (1994) Formation of pseudouridine in U5 small nuclear RNA. *Biochemistry*, **33**, 10423–10427.

Patton,J.R., Jacobson,M.R. and Pederson,T. (1994) Pseudouridine formation in U2 small nuclear RNA. *Proc. Natl Acad. Sci. USA*, **91**, 3324–3328.

Pikielny,C.W., Bindereif,A. and Green,M.R. (1989) *In vitro* reconstitution of snRNPs: a reconstituted U4/U6 snRNP participates in splicing complex formation. *Genes Dev.*, **3**, 479–487.

Plessel,G., Fischer,U. and Lührmann,R. (1994) m_3G cap hypermethylation of U1 small nuclear ribonucleoprotein (snRNP) *in vitro*: evidence that the U1 small nuclear RNA-(guanosine-*N*2)-methyltransferase is a non-snRNP cytoplasmic protein that requires a binding site on the Sm core domain. *Mol. Cell. Biol.*, **14**, 4160–4172.

Reddy,R. and Busch,H. (1988) Small nuclear RNAs: RNA sequences, structure, and modifications. In Birnstiel,M.L. (ed.), *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles*. Springer-Verlag, Berlin, pp. 1–37.

Rymond,B.C., Rokeach,L.A. and Hoch,S.O. (1993) Human snRNP polypeptide D1 promotes pre-mRNA splicing in yeast and defines nonessential yeast Smd1p sequences. *Nucleic Acids Res.*, **21**, 3501–3505.

Séraphin,B. (1995) Sm and Sm-like proteins belong to a large family: identification of proteins of the U6 as well as the U1, U2, U4 and U5 snRNPs. *EMBO J.*, **14**, 2089–2098.

Singh,R. and Reddy,R. (1989) Gamma-monomethyl phosphate: a cap structure in spliceosomal U6 snRNA. *Proc. Natl Acad. Sci. USA*, **86**, 8280–8283.

Sumpter,V., Kahrs,A., Fischer,U., Kornstädt,U. and Lührmann,R. (1992) *In vitro* reconstitution of U1 and U2 snRNPs from isolated proteins and snRNA. *Mol. Biol. Rep.*, **16**, 229–240.

Szkukalek,A., Myslinski,E., Mougin,A., Lührmann,R. and Branlant,C. (1995) Phylogenetic conservation of modified nucleotides in the terminal loop 1 of the spliceosomal U5 snRNA. *Biochimie*, **77**, 16–21.

Wassarman,D.A. and Steitz,J.A. (1992) Interactions of small nuclear RNAs with precursor messenger RNA during *in vitro* splicing. *Science*, **257**, 1918–1925.

Wells,S.E. and Ares,M.,Jr (1994) Interactions between highly conserved U2 small nuclear RNA structures and Prp5p, Prp9p, Prp11p, and Prp21p proteins are required to ensure integrity of the U2 small nuclear ribonucleoprotein in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **14**, 6337–6349.

Wersig,C. and Bindereif,A. (1990) Conserved domains of human U4 snRNA required for snRNP and spliceosome assembly. *Nucleic Acids Res.*, **18**, 6223–6229.

Wersig,C. and Bindereif,A. (1992) Reconstitution of functional mammalian U4 small nuclear ribonucleoprotein: Sm protein binding is not essential for splicing. *Mol. Cell. Biol.*, **12**, 1460–1468.

Will,C.L., Behrens,S.-E. and Lührmann,R. (1993) Protein composition of mammalian spliceosomal snRNPs. *Mol. Biol. Rep.*, **18**, 121–126.

Wolff,T. and Bindereif,A. (1992) Reconstituted mammalian U4/U6 snRNP complements splicing: a mutational analysis. *EMBO J.*, **11**, 345–359.

Wolff,T. and Bindereif,A. (1993) Conformational changes of U6 RNA during the spliceosome cycle: an intramolecular helix is essential both for initiating the U4-U6 interaction and for the first step of splicing. *Genes Dev.*, **7**, 1377–1389.

Wu,J. and Manley,J.L. (1989) Mammalian pre-mRNA branch site selection by U2 snRNP involves base pairing. *Genes Dev.*, **3**, 1553–1561.

Wu,J. and Manley,J.L. (1991) Base pairing between U2 and U6 snRNAs is necessary for splicing of a mammalian pre-mRNA. *Nature*, **352**, 818–821.

Zavanelli,M.I. and Ares,M.,Jr (1991) Efficient association of U2 snRNPs with pre-mRNA requires an essential U2 RNA structural element. *Genes Dev.*, **5**, 2521–2533.

Zavanelli,M.I., Britton,J.S., Igel,A.H. and Ares,M.,Jr (1994) Mutations in an essential U2 small nuclear RNA structure cause cold-sensitive U2 small nuclear ribonucleoprotein function by favoring competing alternative U2 RNA structures. *Mol. Cell. Biol.*, **14**, 1689–1697.

Zhuang,Y. and Weiner,A.M. (1989) A compensatory base change in human U2 snRNA can suppress a branch site mutation. *Genes Dev.*, **3**, 1545–1552.

Zillmann,M., Zapp,M.L. and Berget,S.M. (1988) Gel electrophoretic isolation of splicing complexes containing U1 small nuclear ribonucleoprotein particles. *Mol. Cell. Biol.*, **8**, 814–821.

Received on April 5, 1995; revised on 15th May, 1995