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Reconstituted mammalian U4/U6 snRNP complements splicing: a mutational analysis

Thorsten Wolff and Albrecht Bindereif[1]

Max-Planck-Institut für Molekulare Genetik, Otto-Warburg-Laboratorium, Ihnestrasse 73, D-1000 Berlin 33 (Dahlem), FRG

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[1]Corresponding author

We have developed an *in vitro* complementation assay to analyse the functions of U6 small nuclear RNA (snRNA) in splicing and in the assembly of small nuclear ribonucleoproteins (snRNPs) and spliceosomes. U6-specific, biotinylated 2'-OMe RNA oligonucleotides were used to deplete nuclear extract of the U4/U6 snRNP and to affinity purify functional U4 snRNP. The addition of affinity purified U4 snRNP together with U6 RNA efficiently restored splicing activity, spliceosome assembly and U4/U5/U6 multi-snRNP formation in the U4/U6-depleted extract. Through a mutational analysis we have obtained evidence for multiple sequence elements of U6 RNA functioning during U4/U5/U6 multi-snRNP formation, spliceosome assembly and splicing. Surprisingly, the entire 5' terminal domain of U6 RNA is dispensable for splicing function. In contrast, two regions in the central and 3' terminal domain are required for the assembly of a functional U4/U5/U6 multi-snRNP. Another sequence in the 3' terminal domain plays an essential role in spliceosome assembly; a model is strongly supported whereby base pairing between this sequence and U2 RNA plays an important role during assembly of a functional spliceosome.

Key words: reconstitution/snRNA/splicing/U4/U6 snRNP/U4/U5/U6 multi-snRNP

Introduction

U6 snRNA is one of the five spliceosomal small nuclear RNAs (U1, U2, U4, U5 and U6) that are essential for pre-mRNA splicing and, in the form of ribonucleoproteins (snRNPs) and together with non-snRNP protein factors, interact in a coordinated fashion to assemble the pre-mRNA to spliceosomes (reviewed by Green, 1986; Padgett *et al.*, 1986; Maniatis and Reed, 1987; Krainer and Maniatis, 1988; Steitz *et al.*, 1988; Bindereif and Green, 1990). Based on phylogenetic sequence comparisons, U6 RNA can be divided into several domains: the 5' terminal domain containing the 5' stem-loop; the central, single-stranded domain; the U4-U6 interaction region, which base pairs with U4 through the stem I and stem II regions; and the 3' terminal domain (Figure 1). With the exception of the 5' stem-loop of U6 RNA (Roiha *et al.*, 1990), the secondary structure of these sequence elements and the spacing between them have been strongly conserved, implying important functional constraints (Brow and Guthrie, 1988; Guthrie and Patterson, 1988).

U6 snRNA occurs in the form of several different snRNP complexes. In the U6 snRNP, it is bound by a protein required for U6 capping and transport (Hamm and Mattaj, 1989; Gröning *et al.*, 1991); in the U4/U6 snRNP, it interacts through base pairing with U4 RNA (Bringmann *et al.*, 1984; Hashimoto and Steitz, 1984; Rinke *et al.*, 1985); the form in which U6 most likely enters the spliceosome, is the U4/U5/U6 multi-snRNP (Bindereif and Green, 1987; Cheng and Abelson, 1987; Konarska and Sharp, 1987; Lossky *et al.*, 1987; Black and Pinto, 1989; Behrens and Lührmann, 1991; Lamm *et al.*, 1991; Séraphin *et al.*, 1991). The U4-U6 interaction appears to be destabilized during the splicing reaction, reflecting a conformational change of the spliceosome (Pikielny *et al.*, 1986; Cheng and Abelson, 1987; Lamond *et al.*, 1988; Blencowe *et al.*, 1989). Two additional base pairing interactions have been proposed, involving U2 and U6 RNAs (McPheeters *et al.*, 1989; Hausner *et al.*, 1990). One of these interactions, which was first supported by cross-linking data and phylogenetic sequence comparisons, has very recently been established as essential for splicing by a genetic approach (Datta and Weiner, 1991; Wu and Manley, 1991); it involves the 5' end of U2 RNA and a sequence element in the 3' terminal domain of U6 RNA (nucleotides 87-95 of the human U6 RNA; Hausner *et al.*, 1990). At which stage of the splicing reaction this base pairing interaction functions, is not known. In yeast, several splicing factors with sequence motifs of the ATP-dependent RNA helicase family have recently been identified, suggesting a mechanism of how ATP hydrolysis, through modulating RNA-RNA interactions, may drive the conformational transitions of the spliceosome during splicing (Burgess *et al.*, 1990; Dalbadie-McFarland and Abelson, 1990; Company *et al.*, 1991; Schwer and Guthrie, 1991; Strauss and Guthrie, 1991).

Interestingly, there is evidence that U6 RNA engages not only in multiple snRNP-snRNP interactions, but that it may also play a direct catalytic role in the splicing mechanism. First, in *Saccharomyces cerevisiae* several point mutations in the stem I region as well as in the adjacent conserved hexanucleotide sequence (ACAGAG, nucleotides 41-46 in the human U6 RNA) result in splicing inhibition or block the second step of the splicing reaction (Fabrizio and Abelson, 1990; Madhani *et al.*, 1990). Second, introns have been discovered in the U6 RNA genes of certain yeast species (Tani and Oshima, 1989; Reich and Wise, 1990; Tani and Oshima, 1991) that map to this splicing-essential region. As the intron insertion could result from an aberrant splicing event, this domain may be in close proximity to the reactive centre of the spliceosome.

In summary, U6 RNA acts at several stages of snRNP and spliceosome assembly as well as during the two steps of pre-mRNA splicing. This may also explain why U6 RNA is the most highly conserved of the spliceosomal RNAs, both in terms of RNA sequence and secondary structure (Brow and Guthrie, 1988; Guthrie and Patterson, 1988). To

investigate the domain structure of U6 RNA, mutational approaches have been employed in yeast (Fabrizio *et al.*, 1989; Fabrizio and Abelson, 1990; Madhani *et al.*, 1990), *Xenopus* (Vankan *et al.*, 1990) and mammalian systems (Pikielny *et al.*, 1989; Bindereif *et al.*, 1990). Although mammalian snRNPs are characterized best in terms of their assembly, protein composition and structural organization, no *in vitro* assay for the biochemical investigation of splicing domains of mammalian snRNPs has been described. With respect to U4 and U6 RNAs, only the U4–U6 interaction and the assembly of the U4/U6 snRNP into the spliceosome could so far be studied *in vitro* (Pikielny *et al.*, 1989; Bindereif *et al.*, 1990; Wersig and Bindereif, 1990).

In this paper we report the development of an *in vitro* splicing complementation system, which in combination with mutagenesis has enabled us for the first time to analyse biochemically the functions of U6 RNA in the mammalian splicing mechanism. This assay makes use of nuclear extract specifically depleted of the U4/U6 snRNP by a streptavidin–agarose affinity selection with biotinylated 2′-OMe RNA oligonucleotides. In addition, we describe a novel procedure for releasing functional U4 snRNP from affinity selected U4/U6 snRNP. The U4 snRNP and synthetic U6 RNA are reconstituted to a U4/U6 snRNP, which efficiently complements U4/U5/U6 multi-snRNP formation, spliceosome assembly and splicing activity in the U4/U6-depleted extract. The complementation analysis of U6 RNA mutant derivatives revealed, first, two sequence elements within the central and 3′ terminal domain that are essential for the formation of a functional U4/U5/U6 multi-snRNP, and second, that the proposed U6–U2 base pairing region in the 3′ terminal domain is essential for splicing *in vitro* and functions already at the stage of spliceosome assembly. In contrast, the entire 5′ terminal domain of U6 RNA was found to be dispensable for splicing.

Results

Reconstituted U4/U6 snRNP complements splicing in vitro

U4 and U6 RNAs are organized in several distinct domains (Figure 1; Guthrie and Patterson, 1988); to identify the role of each of these sequence elements in the splicing mechanism, we developed a functional complementation assay using reconstituted wild-type and mutant U4/U6 snRNPs. Our approach is schematically outlined in Figure 2: in principle, nuclear extract is first depleted specifically of the U4/U6 snRNP, resulting in a splicing-deficient extract. Second, the U4 snRNP is affinity purified from nuclear extract and added together with U6 RNA to this U4/U6-depleted extract. After a short incubation, during which U4/U6 snRNP reconstitution can take place, splicing of a ^{32}P-labelled pre-mRNA is assayed. The restoration of splicing activity reflects the functionality of the U4 snRNP and of U6 RNA.

To achieve inactivation of the endogenous U4/U6 snRNP present in nuclear extract, we first used RNase H cleavage directed by biotinylated DNA oligonucleotides. However, repeated attempts using this strategy were unsuccessful due to incomplete RNase H cleavage reactions. In addition, biotinylated oligonucleotides could not be quantitatively removed from extracts by streptavidin–agarose, most likely because DNA oligonucleotides were partially degraded in the extract (data not shown). In contrast, 2′-OMe RNA oligonucleotides are extremely stable to nucleases (Inoue *et al.*, 1987a,b; Sproat *et al.*, 1989) and have, in their biotinylated form and in combination with streptavidin–agarose, previously been used for the depletion and affinity selection of specific snRNPs (Barabino *et al.*, 1989, 1990; Gröning *et al.*, 1991; Palfi *et al.*, 1991). Incubation of nuclear extract with a biotinylated antisense 2′-OMe RNA

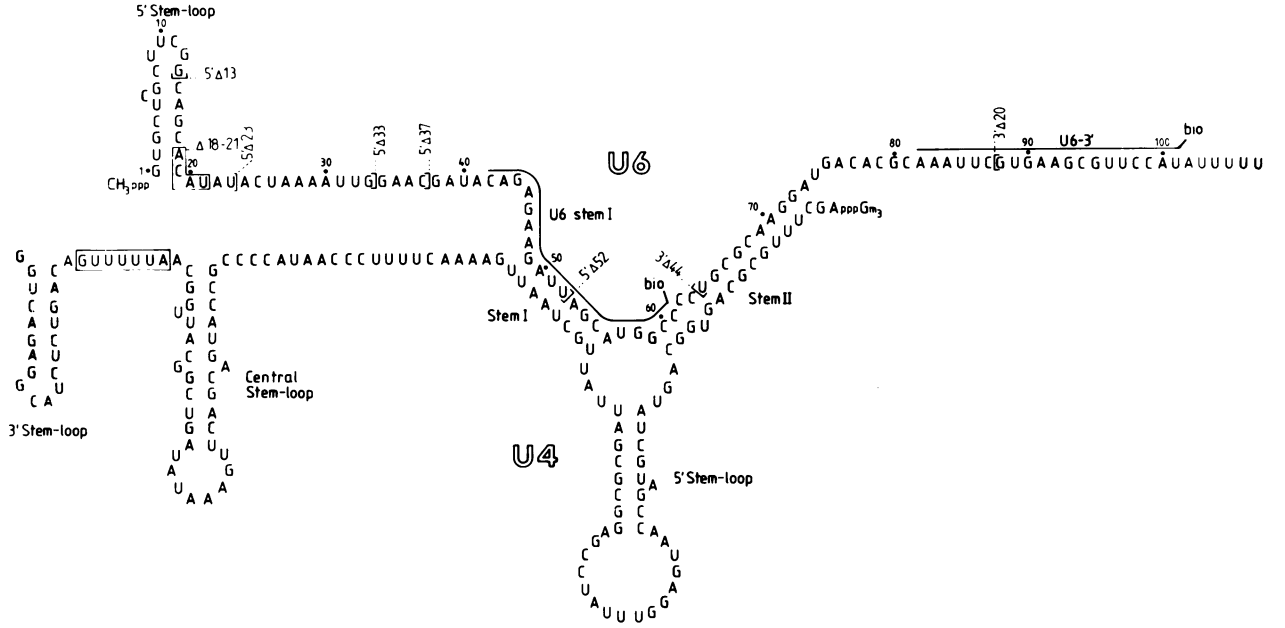


Fig. 1. Secondary structure model of the human U4 and U6 RNAs (Guthrie and Patterson, 1988). The endpoints of the terminal deletion derivatives of SP6 U6 used in this study are given; the Sm binding site of U4 RNA and the U6 sequence deleted in SP6 U6 Δ18–21 are indicated by boxes. The U6 sequences targeted by biotinylated antisense 2′-OMe RNA oligonucleotides (U6 stem I; U6-3′) are marked by bars.

oligonucleotide against the 3′ terminal domain of human U6 RNA (nucleotides 82–101; U6–3′; Figure 1), followed by a streptavidin–agarose affinity selection, efficiently codepleted U4 and U6 RNAs from the extract (Blencowe *et al.*, 1989; Figure 3A). Only a relatively minor amount of U1 RNA contaminated the affinity selected material (Figure 3A, lane S). The RNA analysis of the depleted extract (Figure 3A, lane D) and, most convincingly, the reduction of its splicing activity to less than 5% of the control (Figure 4A) proved that the U4/U6 snRNP depletion was nearly quantitative.

The U4 snRNP needed for our splicing complementation assay was affinity purified by the following procedure outlined schematically in Figure 2. The selection was monitored by RNA analysis (Figure 3B). First, the U4/U6 snRNP was affinity selected from nuclear extract with streptavidin–agarose and a biotinylated 2′-OMe RNA oligonucleotide directed against the stem I region of U6 RNA (nucleotides 42–61; U6 stem I; Figure 1). The RNA analysis demonstrated that U4 and U6 RNAs were coselected at an efficiency of ~50% (Figure 3B, compare lanes T, D and S). Secondly, after extensive washing the temperature was increased to 37°C, resulting in the release of the U4 snRNP from the affinity selected U4/U6 snRNP in a form not contaminated by other snRNAs (Figure 3B, lane R). The released U4 snRNP could be quantitatively immunoprecipitated by anti-Sm antibodies (data not shown), confirming that the Sm core proteins remain stably associated with U4 RNA through the entire procedure. Based on RNA analysis, the U4 snRNP could be released with a yield of ~40% (Figure 3B, compare lanes S and R). The U4 snRNP release appears to depend on the destabilization of the U4–U6 interaction due to binding of the U6 stem I oligonucleotide, since no U4 snRNP could be prepared when the same procedure was applied using the 3′ terminal U6 oligonucleotide (U6-3′; data not shown).

Figure 4 shows complementation of splicing in the U4/U6 snRNP-depleted nuclear extract by affinity purified U4 snRNP and U6 RNA. The concentrations of added U4 snRNP and U6 RNA were varied over a wide range, and the complementation levels obtained with endogenous, gel purified HeLa U6 RNA and with SP6-transcribed U6 RNA (SP6-U6) were compared with each other. Splicing activity in the codepleted extract was consistently reduced to less than 5% of that observed in a mock-treated extract (Figure 4A and C, compare lanes NE and NE ΔU4/U6 without added U4 snRNP and U6 RNA). Since the addition of either U4 snRNP or U6 RNA alone to codepleted extract did not restore splicing activity (Figure 4A, B and C), the depletion of both U4 and U6 RNAs must be nearly quantitative. Therefore, splicing complementation depended on both U4 snRNP and U6 RNA, indicating that both components were efficiently depleted. However, when increasing concentrations of HeLa or SP6 U6 RNA were added back in the presence of an excess of U4 snRNP, the splicing activity was efficiently restored to control levels (Figure 4A and B). This result implies that the U4 snRNP and U6 RNA reconstitute to a functional U4/U6 snRNP in the depleted extract. The requirement for U6 RNA is specific, since U1 or U5 RNAs could not substitute for U6 RNA (data not shown). Surprisingly, titration of added U6 RNA revealed a significant difference in splicing complementation activity between endogenous HeLa and synthetic SP6 U6 RNA

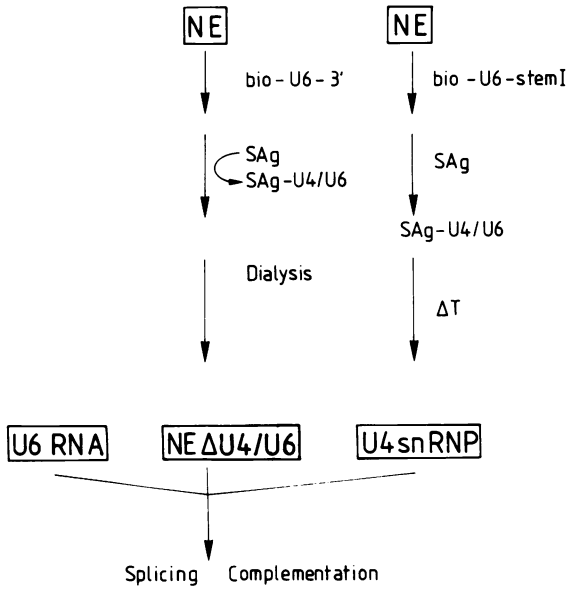


Fig. 2. Splicing complementation assay. On the left, the U4/U6 snRNP depletion of nuclear extract (NE) through a biotinylated 2′-OMe RNA oligonucleotide (bio-U6-3′) and streptavidin–agarose (SAg) is schematically represented. On the right, the affinity purification of the U4 snRNP through a biotinylated 2′-OMe RNA oligonucleotide (bio-U6-stemI) and streptavidin–agarose (SAg) is outlined. The U4 snRNP is released from affinity selected U4/U6 snRNP (SAg-U4/U6) by a temperature increase (ΔT).

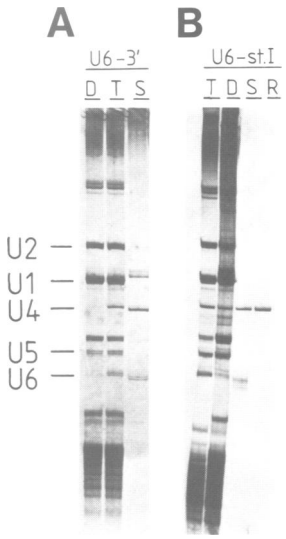


Fig. 3. U4/U6 snRNP depletion of nuclear extract (A) and affinity selection of the U4 snRNP (B). (**A**) Nuclear extract was depleted of the U4/U6 snRNP through the 2′-OMe RNA oligonucleotide U6-3′ as described in Materials and methods. Total RNA was analysed from equivalent aliquots (20 μl) of nuclear extract (total, T), U4/U6-depleted extract (depleted, D), and from the streptavidin–agarose beads (selected, S) by silver staining. (**B**) The U4 snRNP was affinity purified from the U4/U6 snRNP through the 2′-OMe RNA oligonucleotide U6 stem I as described in Materials and methods. RNA analysis by silver staining is shown for equivalent aliquots (20 μl) of nuclear extract (total T), U4/U6-depleted extract (depleted, D), and of RNA bound by the streptavidin–agarose beads (selected, S); for the released material (released, R), an aliquot corresponding to 100 μl of extract was analysed.

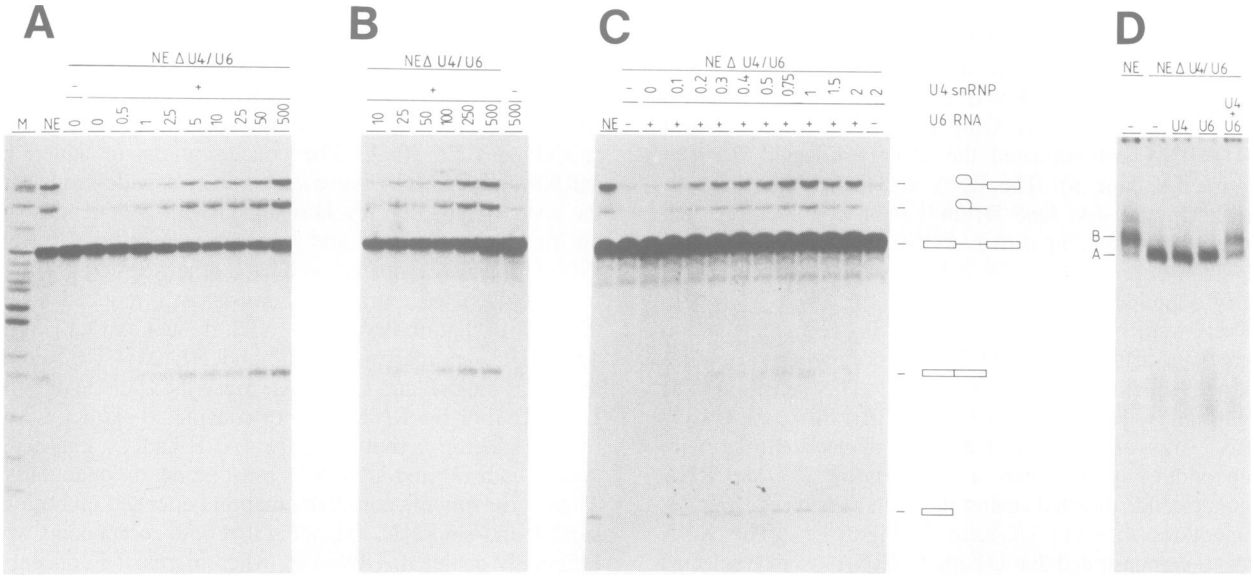


Fig. 4. Complementation of splicing and spliceosome assembly. Splicing complementation reactions were performed for 90 min in U4/U6-depleted nuclear extract (NE ΔU4/U6), using affinity purified U4 snRNP (2 μl) and varying amounts (0–500 ng) of HeLa U6 (**A**) or SP6 U6 RNA (**B**), or using 300 ng of SP6 U6 RNA and varying amounts (0–2 μl) of U4 snRNP (**C**). In control reactions, mock-treated nuclear extract (NE) was used, or U4 snRNP or U6 RNA were omitted (− U4 snRNP; − U6 RNA). The positions of pre-mRNA, splicing intermediates and products are indicated. M, pBR322 digested with *Hpa*II. (**D**) Spliceosome assembly of ^{32}P-labelled pre-mRNA was assayed after a 40 min incubation in U4/U6-depleted extract, complemented with either 2 μl of affinity purified U4 snRNP (U4), 300 ng SP6 U6 RNA (U6) or both (U4+U6). In control reactions, mock treated nuclear extract (NE) was used, or U4 snRNP and U6 RNA were omitted (−). The positions of A and B complexes are indicated.

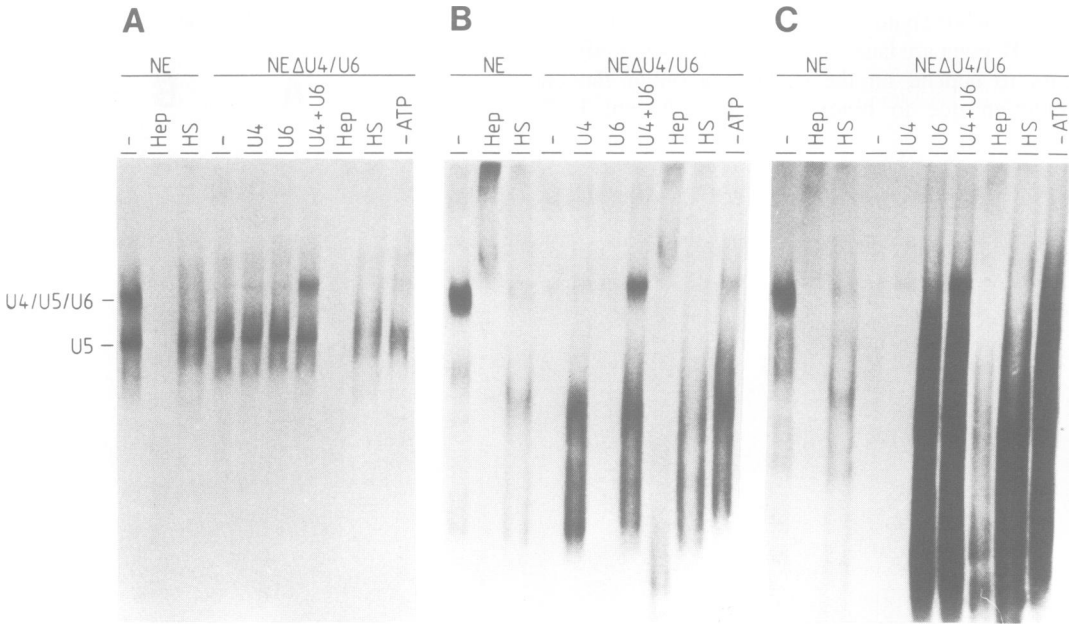


Fig. 5. Reconstitution of the U4/U5/U6 multi-snRNP. Reconstitution reactions were performed, under splicing complementation conditions in the absence of pre-mRNA, by incubating U4/U6-depleted extract (NE ΔU4/U6) without (−) or with U4 snRNP (U4), SP6 U6 RNA (U6) or both U4 snRNP and SP6 U6 RNA (U4+U6). As controls, reconstitution reactions containing U4 snRNP and SP6 U6 RNA were treated with heparin (Hep) or high salt (HS), or were carried out in the absence of ATP and creatine phosphate. For comparison, snRNP complexes in mock treated nuclear extract (NE) were analysed, without additions (−), or after heparin (Hep) or high salt (HS) treatment. snRNP complexes were separated by native gel electrophoresis and detected by Northern blot analysis with probes specific for U5 (panel **A**), U4 (panel **B**), or U6 RNA (panel *C*). The positions of the U4/U5/U6 multi-snRNP and the U5 snRNP are indicated on the left.

(Figure 4, compare panels A and B). Complementation of splicing activity at the level of the control reaction was achieved by the addition of 25 ng HeLa U6 RNA (Figure 4A), corresponding to a concentration of 1000 ng/ml in the splicing reaction. In the case of synthetic SP6 U6 RNA, however, a 4-fold higher concentration was required for the same effect (Figure 4B, lane 100 ng). When either HeLa or SP6 U6 RNA were added at significantly higher concen-

trations, splicing was further stimulated up to 2-fold higher levels (Figure 4A, lanes 50 and 500 ng; Figure 4B, lanes 250 and 500 ng). For comparison, the splicing reaction with mock-treated extract contains endogenous U6 RNA at a concentration of ~1000 ng/ml (data not shown). Some of the difference in splicing complementation between the natural and synthetic RNAs is most likely due to different stabilities of the two RNAs in extract, since we found by RNA analysis that most or all of the HeLa RNA, but only 10–20% of the SP6 U6 RNA, remained undegraded after the 15 min pre-incubation period (data not shown).

Splicing complementation was also assayed as a function of the concentration of added U4 snRNP. Figure 4C shows that the addition of as little as 0.4 μl of the U4 snRNP in the complementation reaction (corresponding to 60 ng/ml of U4 RNA) in the presence of an excess of U6 RNA resulted already in full splicing complementation activity. This U4 snRNP concentration corresponds to ~10% of the U4 snRNP concentration present in the splicing reaction using mock-treated nuclear extract (600 ng/ml). Addition of the U4 snRNP at concentrations higher than those needed for full splicing complementation did not further elevate splicing activity (Figure 4C). In summary, both affinity purified U4 snRNP and HeLa- and SP6-derived U6 RNAs are capable of fully complementing splicing activity in the U4/U6-depleted nuclear extract.

In addition to splicing we assayed for spliceosome assembly under *in vitro* splicing complementation conditions (Figure 4D). Mock-treated nuclear extract supported the assembly of ^{32}P-labelled pre-mRNA into A and B complexes, which could be detected by native gel electrophoresis. The A complex contains the U2 snRNP, the B complex the U2, U4/U6 and U5 snRNPs (Konarska and Sharp, 1987); at the time point of the splicing reaction shown in Figure 4D, most of the pre-mRNA was already in the form of B complexes (lane NE). In contrast, in the U4/U6-depleted extract, spliceosome assembly was blocked at the stage of the A complex (Figure 4D, lane NE ΔU4/U6, −), confirming the observation reported by Barabino *et al.* (1990). The addition of either the U4 snRNP or U6 RNA alone did not release this block of assembly (Figure 4D, lanes U4 and U6). Only when both U4 snRNP and U6 RNA were added to the depleted extract, the pre-mRNA was assembled into A and B complexes (Figure 4D, lane U4+U6).

Since the U4/U5/U6 multi-snRNP most likely represents an obligatory intermediate in spliceosome assembly, we also investigated the assembly of wild-type U6 RNA and mutant derivatives into multi-snRNP complexes. Figure 5 demonstrates how multi-snRNP formation was assayed. Under splicing complementation conditions and in the absence of pre-mRNA, U4 snRNP and wild-type SP6 U6 RNA were added to U4/U6-depleted nuclear extract; snRNP complexes formed were separated by native gel electrophoresis and, after electrophoretic transfer, detected by Northern blot analysis, using antisense probes specific for U4, U5 and U6 RNAs. In mock-treated nuclear extract, both the U5 snRNP and the U4/U5/U6 multi-snRNP were detected by the respective probes; both complexes were sensitive to treatment with heparin, and furthermore, the multi-snRNP and most of the U5 snRNP was unstable to increased ionic strength (0.5 M KCl) (Figure 5, lanes NE, compare panels A, B and C). This behaviour agrees well with the reported salt and heparin sensitivity of the U4/U5/U6 multi-snRNP (Cheng and Abelson, 1987; Lossky *et al.*, 1987; Behrens and Lührmann, 1991). As expected, no multi-snRNP was detectable in the U4/U6 snRNP-depleted extract; the U5 snRNP, however, was still present (Figure 5, lanes NEΔU4/U6, no addition). The addition of either the U4 snRNP or of U6 RNA alone did not change this; however, when both the U4 snRNP and U6 RNA were incubated together under splicing complementation conditions, the appearance of an snRNP complex was readily detected by all three probes (Figure 5, lanes U4, U6 and U4+U6). When probing for U6, an excess of partially assembled or degraded U6 RNP complexes migrating below the multi-snRNP band became apparent upon the addition of U6 RNA (Figure 5C, lane U6). To verify further the identity of the reconstituted U4/U5/U6 multi-snRNP, we applied the two additional criteria of heparin and high-salt instability: as the endogenous multi-snRNP, the reconstituted complex was heparin and high-salt sensitive (Figure 5, compare NE and NEΔU4/U6, lanes Hep and HS). Finally, we found that U4/U5/U6 multi-snRNP reconstitution is ATP dependent, consistent with an earlier study on the ATP requirement of the interaction between the U4/U6 and U5 snRNPs in HeLa nuclear extract (Black and Pinto, 1989). In the absence of ATP and creatine phosphate, the U4 snRNP and U6 RNA formed multi-snRNPs only at a very low level, which may be due to some residual endogenous ATP in the extract (Figure 5, compare lanes U4+U6 and −ATP). In summary, based on several criteria we have demonstrated that the U4/U5/U6 multi-snRNP can be efficiently reconstituted *in vitro*.

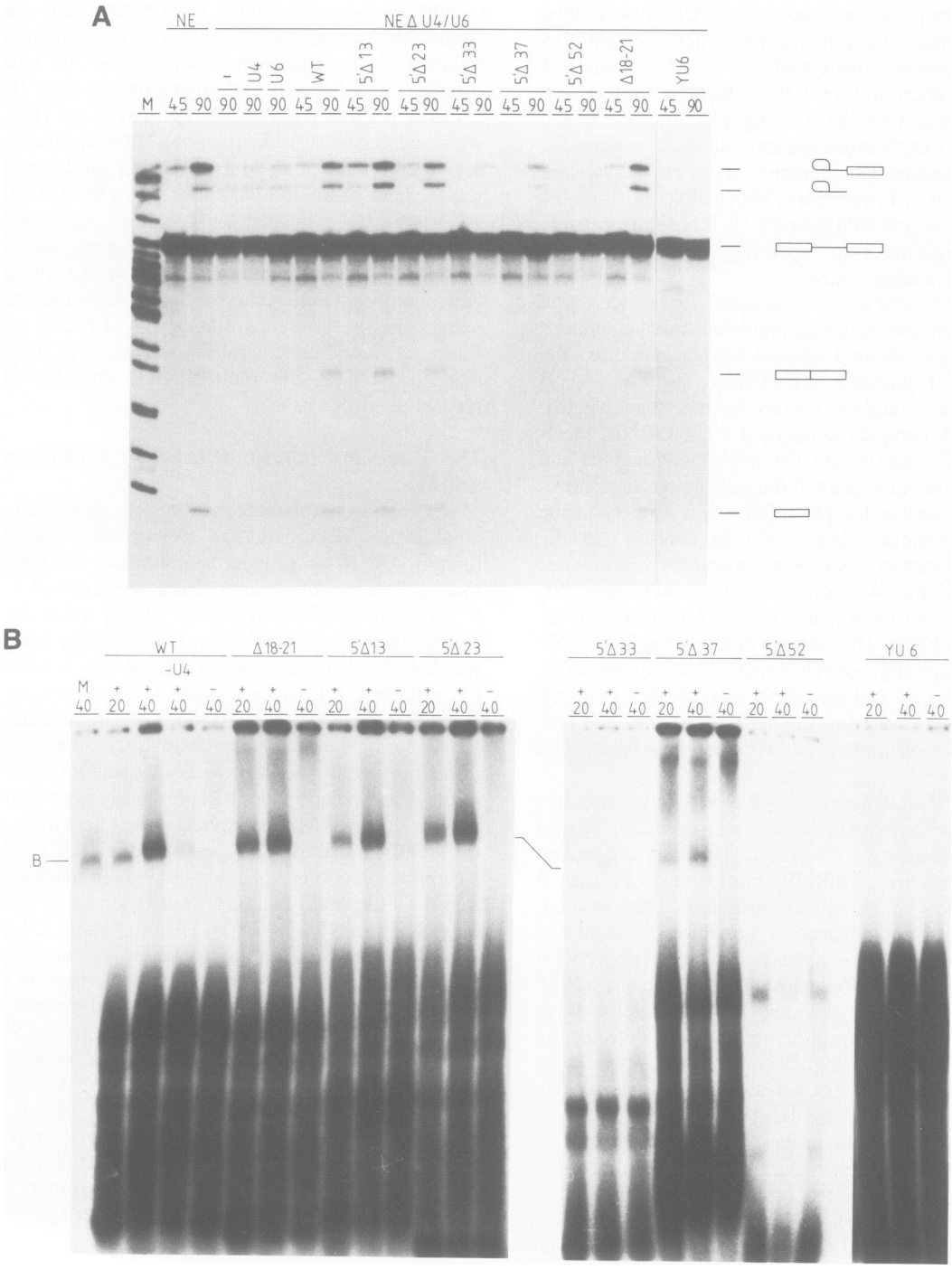
The 5′ terminal domain of U6 RNA is dispensable for splicing

The splicing complementation system described above in combination with extensive mutagenesis allowed us to identify U6 RNA sequences important for splicing in a mammalian *in vitro* system. First, we assayed a series of 5′ terminal deletion derivatives of U6 RNA for splicing complementation (Figure 6A). Each of these mutant RNAs was tested at a molar concentration at which wild-type SP6 U6 exhibited at least the control level of splicing activity, together with an excess of U4 snRNP. The stabilities of these RNAs during the complementation assay did not significantly differ from wild-type SP6 U6 RNA (data not shown). Some of the deletion derivatives had previously been characterized in U4/U6 snRNP and spliceosome assembly, using a different reconstitution procedure (Bindereif *et al.*, 1990). In control reactions, splicing activities in mock-treated and in U4/U6-depleted extracts were determined (Figure 6A, lanes NE and NEΔU4/U6−). Adding either U4 snRNP or U6 RNA alone did not complement splicing (Figure 6A, NEΔU4/U6, lanes U4 and U6); complementing with both U4 snRNP and SP6 U6 RNA restored control levels of splicing (Figure 6, lanes WT). Deletion of the 5′ terminal 13 nt or 23 nt of U6 RNA did not change splicing complementation activity (Figure 6, lanes 5′Δ13 and 5′Δ23). Similarly, deleting the highly conserved ACAU sequence (nucleotides 18–21; Figure 1), which is important for binding of a U6 snRNP-specific protein (Hamm and Mattaj, 1989; Gröning *et al.*, 1991) and for efficient U6 RNA capping (Singh *et al.*, 1990), did not alter splicing activity (Figure 6, lanes Δ18–21).

We also assayed these U6 mutant derivatives for their ability to form stable, heparin-resistant B-type splicing complexes (Konarska and Sharp, 1987; Bindereif *et al.*, 1990). Using the U4/U6-depleted nuclear extract, we assembled U4/U6 snRNPs from ^{32}P-labelled wild-type and mutant U6 RNAs and the affinity purified U4 snRNP under the conditions of splicing complementation. Unlabelled splicing substrate was then added and the assembly of the ^{32}P-labelled U4/U6 snRNP into spliceosomes after 20 and 40 min was monitored by the appearance of radioactive label in the B complex. As Figure 6B shows, wild-type SP6 U6 RNA was efficiently asembled into spliceosomes under the conditions of splicing complementation. As expected, spliceosome assembly depended on the addition of both splicing substrate and the U4 snRNP (lanes WT). Deleting 13 or 23 nt from the 5′ terminus of U6 RNA or deleting the ACAU sequence did not affect spliceosome assembly (Figure 6B, lanes 5′Δ13, 5′Δ23, and Δ18−21).

Finally, the same U6 mutant derivatives were tested for U4/U5/U6 multi-snRNP assembly. Both SP6-U6 5′Δ13, 5′Δ23, and Δ18−21 RNAs formed, under the splicing complementation conditions, multi-snRNP complexes indistinguishable from those formed with wild-type SP6-U6 (Figure 6C, lanes WT, 5′Δ13, 5′Δ23 and Δ18−21).

In summary, the entire 5′ terminal domain of human U6 RNA (nucleotides 1−23) including the conserved ACAU sequence is dispensable for U4/U5/U6 multi-snRNP formation, spliceosome assembly, and splicing. To test for a poten-



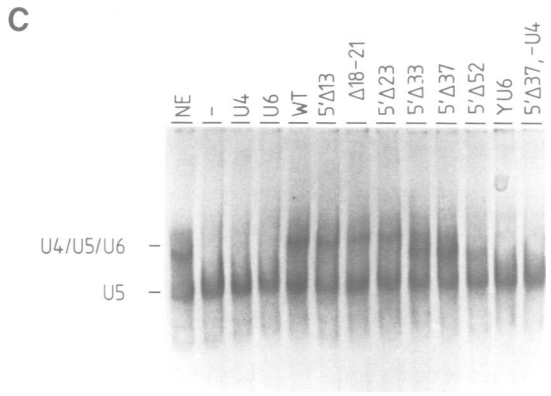


Fig. 6. Splicing complementation and assembly of spliceosomes and multi-snRNPs by 5′ terminal deletion derivatives of human U6 RNA and yeast U6 RNA (YU6). (**A**) Splicing complementation assays were performed in U4/U6-depleted nuclear extract (NE ΔU4/U6) for the times indicated (45 and 90 min), complementing with U4 snRNP and the RNAs as indicated. On the left end of the panel, control reactions are shown using mock treated nuclear extract (NE), U4/U6-depleted extract without complementation (−), with U4 snRNP alone (U4) and with SP6 U6 RNA alone (U6). The positions of pre-mRNA, splicing intermediates and products are indicated to the right. M, pBR322 digested with *Hpa*II. (**B**) Spliceosome assembly of ^{32}P-labelled U6 RNAs was analysed in U4/U6-depleted extract under complementation conditions by native gel electrophoresis. For each RNA, spliceosome assembly was assayed at two time points (20 and 40 min), and in the presence (+) or absence (−) of added splicing substrate. At the left side of the panel, assembled ^{32}P-labelled pre-mRNA marks the position of the B complex. In a control reaction, ^{32}P-labelled wild-type SP6 U6 RNA (WT) was assembled in the absence of added U4 snRNP (−U4). (**C**) U4/U5/U6 multi-snRNP reconstitution was assayed by native gel electrophoresis, followed by Northern blot analysis with a ^{32}P-labelled U5-specific probe. As controls, snRNPs present in mock treated nuclear extract (NE) were analysed, as well as snRNPs from reconstitution reactions without (−), with U4 snRNP alone (U4), with SP6 U6 RNA alone (U6) and with SP6 U6 5′Δ37 RNA alone (5′Δ37, −U4). The positions of the U4/U5/U6 multi-snRNP and the U5 snRNP are indicated on the left.

tial conservation of the splicing function of U6 RNA, we also determined the assembly and splicing activities of synthetic *S.cerevisiae* U6 RNA (T7-YU6) in the mammalian splicing complementation assay. We found that the yeast U6 RNA, which efficiently complements *in vitro* splicing in yeast extract (Fabrizio *et al.*, 1989), is inactive in splicing complementation and did not detectably form multi-snRNPs or spliceosomes in the mammalian system (Figure 6, lanes YU6). Finally, as demonstrated by anti-Sm immunoprecipitation, the yeast U6 RNA did not interact with the mammalian U4 snRNP (data not shown).

A sequence in the central domain is required for the assembly of a functional U4/U5/U6 multi-snRNP

After no splicing-essential function could be assigned to the 5′ terminal domain, we extended our complementation analysis to the central domain of U6 RNA. First, splicing complementation activities of SP6 U6 5′Δ33, 5′Δ37 and 5′Δ52 RNAs were assayed and found to be reduced to less than 10%, indicating that there is a sequence element important in splicing between nucleotides 23 and 33 of human U6 RNA (Figure 6A, lanes 5′Δ33, 5′Δ37 and 5′Δ52).

To differentiate between whether this effect is on the level of splicing or assembly, these mutant derivatives were assayed for spliceosome assembly and multi-snRNP formation. SP6 U6 5′Δ33, 5′Δ37 and 5′Δ52, resulted in a dramatic decrease of spliceosome assembly to levels of less than 10% of wild-type SP6 U6 RNA (Figure 6B, lanes 5′Δ33, 5′Δ37 and 5′Δ52). In the case of the SP6 U6 5′Δ33 and 5′Δ52 RNAs, we observed reproducibly that less RNA than for most other mutant derivatives migrated in the lower half of the gel. As expected, spliceosome assembly depended on the addition of added splicing substrate (Figure 6B, lanes −).

When multi-snRNP formation of SP6-U6 5′Δ33, 5′Δ37 and 5′Δ52 RNAs was tested, an interesting mutant phenotype became apparent. Both SP6-U6 5′Δ33 and 5′Δ37 RNAs assembled to complexes with an altered electrophoretic mobility, migrating between the positions of the normal U4/U5/U6 and the U5 snRNPs; these mutant derivatives formed multi-snRNPs of the normal electrophoretic behaviour only to a low extent (Figure 6C, lanes 5′Δ33 and 5′Δ37). In addition to U5, this mutant multi-snRNP complex contains U4 and U6 RNAs, as shown by sequential Northern blot analysis with U4 and U6 probes (data not shown); also the formation of the mutant complex with SP6 U6 5′Δ37 RNA depended on the addition of the U4 snRNP (Figure 6C, lane 5′Δ37, −U4). In contrast to multi-snRNP formation, the ability of U6 RNA to interact with the U4 snRNP was not significantly affected by deleting up to 37 nt from the 5′ end (data not shown). SP6 U6 5′Δ52 RNA very inefficiently formed multi-snRNPs that migrated as the mutant complexes of SP6 U6 5′Δ33 and 5′Δ37 RNAs (Figure 6C, lane 5′Δ52); this defect in multi-snRNP formation can be explained by the very low efficiency of U4−U6 interaction with SP6 U6 5′Δ52 RNA (data not shown).

We therefore conclude that a sequence element, mapping between nucleotides 23 and 33 within the central domain of U6, is essential for the assembly of a functional U4/U5/U6 multi-snRNP. This sequence is not required for U4−U6 interaction (Bindereif *et al.*, 1990; data not shown) nor for the stable interaction of U4, U5 and U6 RNAs; however, it is essential for functionality of the multi-snRNP, as the mutant U4/U5/U6 snRNP complexes formed by SP6 U6 5′Δ33 and 5′Δ37 RNAs are only very inefficiently incorporated into the spliceosome (for a summary of these data, see Figure 9).

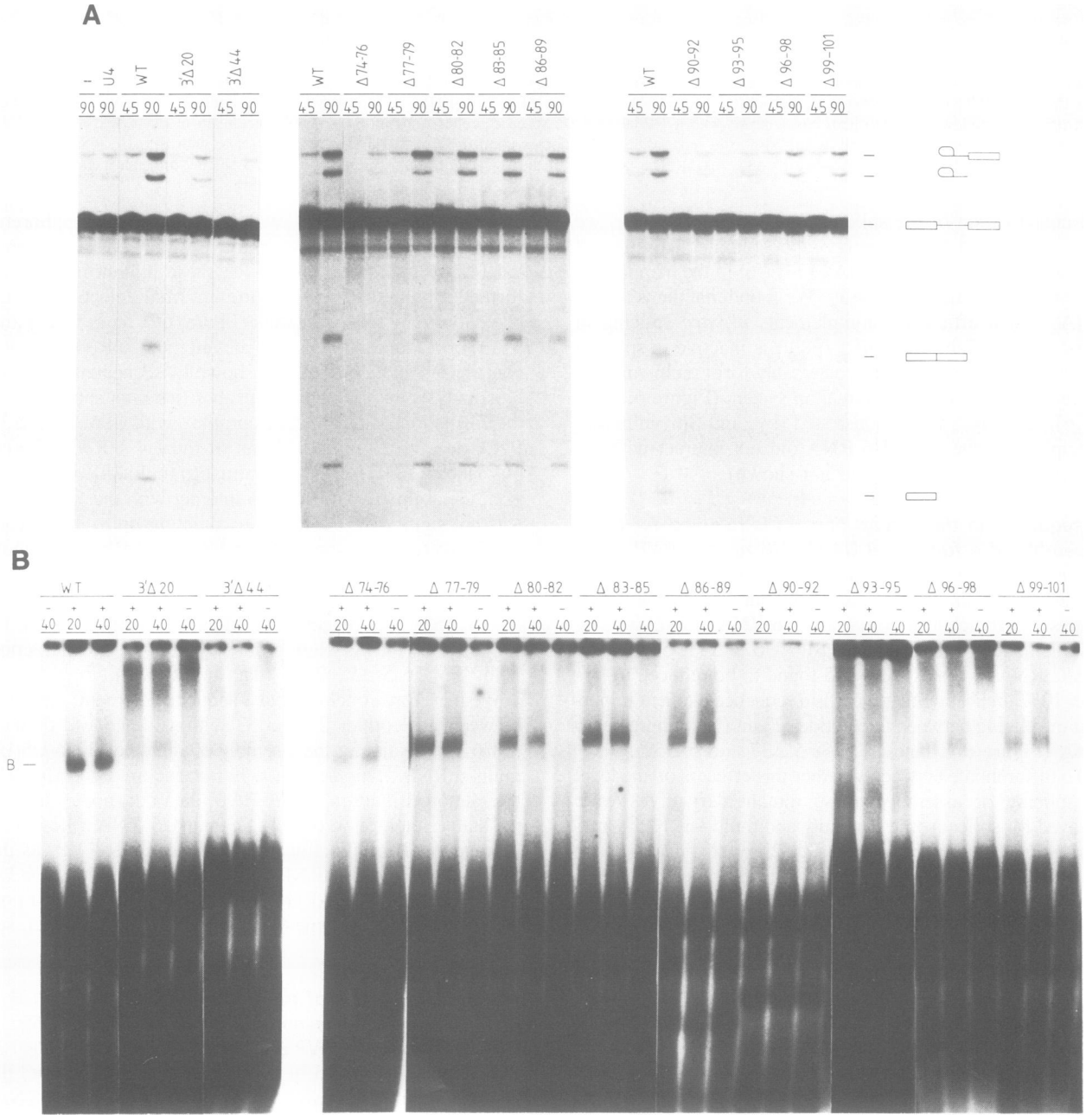
Mutational analysis of the 3′ terminal domain of U6 RNA: two splicing-essential regions are required for U4/U5/U6 multi-snRNP and spliceosome assembly

Similarly as with the 5′ terminal deletions, we assayed the splicing activities of two 3′ terminal deletions of U6 RNA:

when 20 or 44 nt were deleted from the 3′ end, less than 10% of wild-type splicing activity or background levels remained, respectively (Figure 7A, compare lanes 3′Δ20 and 3′Δ44 with lanes −, U4 and WT). Both spliceosome and multi-snRNP assembly of the SP6 U6 3′Δ20 and 3′Δ44 RNAs were decreased to background levels (Figure 7B and C, lanes 3′Δ20 and 3′Δ44), and U4/U6 snRNP formation to very low and undetectable levels, respectively (data not shown).

These results indicated that there are functionally important sequences within the 3′ terminal domain of U6 RNA. To delineate the fine structure of this region, a detailed mutational analysis was undertaken by using a series of contiguous small deletions, each spanning three or four nucleotides (Figure 9). The stabilities of these mutant RNA derivatives in U4/U6-depleted extract did not significantly differ from wild-type SP6-U6 RNA (data not shown). *In vitro* splicing complementation assays with these mutant U6 RNAs revealed two distinct, splicing-essential sequence elements within the 3′ terminal domain (Figure 7). U6 mutant RNAs with deletions of either nucleotides 74−76, 90−92, or 93−95 gave no splicing activity above background levels (Figures 7A, compare lanes Δ74−76, Δ90−92 and Δ93−95). In contrast, four deletions which map in between these two elements were fully active in splicing (Figure 7A, lanes Δ77−79, Δ80−82, Δ83−85 and Δ86−89). Interestingly, for one of these deletions, SP6 U6 Δ77−79, the ratio of splicing products to intermediates was reproducibly less than that of wild-type U6 RNA, indicating an effect of this deletion specifically on the second step of the splicing reaction (Figure 7A, compare lanes Δ77−79 and WT).

To distinguish between whether the reduced splicing activities of these mutant derivatives were due to defects at the level of splicing or at preceding stages, we next assayed them for spliceosome and multi-snRNP assembly. Deletion



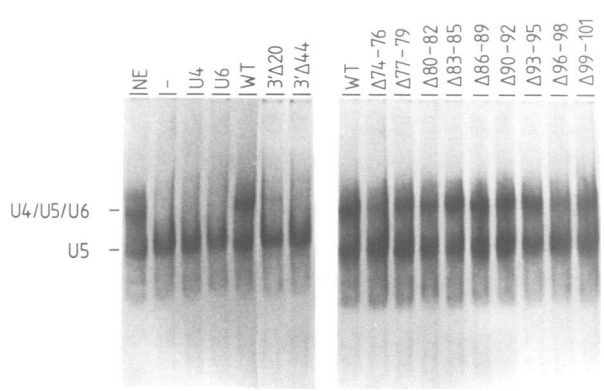


Fig. 7. Splicing complementation and assembly of spliceosomes and multi-snRNPs by derivatives of human U6 RNA containing deletions in the 3′ terminal domain. (**A**) Splicing complementation assays were performed in U4/U6-depleted nuclear extract for the times indicated (45 and 90 min), complementing with U4 snRNP and the RNAs as indicated. On the left end of the panel, control reactions are shown using U4/U6-depleted extract without complementation (−) and with U4 snRNP alone (U4). For comparison, splicing was also complemented by wild-type SP6 U6 RNA (WT). The positions of pre-mRNA, splicing intermediates, and products are indicated to the right. (**B**) Spliceosome assembly of ^{32}P-labelled U6 RNAs was analysed in U4/U6-depleted extract under complementation conditions by native gel electrophoresis. For each RNA, spliceosome assembly was assayed at two points (20 and 40 min), and in the presence (+) or absence (−) of added splicing substrate. To the left, the position of the B complex is indicated. (**C**) U4/U5/U6 multi-snRNP reconstitution was assayed by native gel electrophoresis, followed by Northern blot analysis with a ^{32}P-labelled U5-specific probe. As controls, snRNPs present in mock treated nuclear extract (NE) were analysed, as well as snRNPs from reconstitution reactions without (−), with U4 snRNP alone (U4) or with SP6 U6 RNA alone (U6). The positions of the U4/U5/U6 multi-snRNP and the U5 snRNP are indicated on the left.

of the first region (nucleotides 74−76) strongly reduced both multi-snRNP formation and spliceosome assembly, to levels of less than 10% of the control (Figure 7B and C, lane Δ74−76). Deletions within the second region (nucleotides 90−95) reduced spliceosome assembly also to very low or background levels; in contrast, multi-snRNP assembly was not affected (Figure 7B and C, lanes Δ90−92 and Δ93−95). With the exception of SP6 U6 Δ80−82, which was reduced in spliceosome assembly to a level of ~50%, small deletions in the region between these two important elements did not affect multi-snRNP and spliceosome assembly (Figure 7B and C, lanes Δ77−79, Δ80−82, Δ83−85 and Δ86−89). Finally, deletions of nucleotides 96−98 and 99−101 reduced splicing activity to ~50% and spliceosome assembly to ~10 and 30%, respectively; multi-snRNP formation of SP6 U6 Δ96−98 proceeded with an efficiency of ~20%, and SP6 U6 Δ99−101 had full wild-type efficiency (Figure 7, lanes Δ96−98 and Δ99−101). None of these small internal deletions within the 3′ terminal domain showed significantly reduced U4/U6 snRNP formation (data not shown).

In summary, this deletion analysis suggested that two sequence elements within the 3′ terminal domain of human U6 RNA are functionally important. The first maps between nucleotides 74 and 76, which abuts stem II, and acts already at the stage of multi-snRNP formation. The second spans nucleotides 90−95 and is clearly essential at the spliceosome assembly stage, but not during multi-snRNP formation. Significantly, the latter element covers most of the U6−U2 base pairing region, which comprises $C_{87}G_{88}$ and GAAGCG (nucleotides 90−95), separated by an unpaired position, U_{89} (Hausner *et al.*, 1990; Figure 9).

To evaluate further whether this region functions through U6−U2 base pairing, we constructed a series of U6 mutant derivatives with either an increased or decreased U2 base pairing potential. When incubated in U4/U6-depleted extract, these mutant RNAs were as stable as wild-type SP6 U6 RNA (data not shown). SP6-U6 X2203 carries five nucleotide substitutions ($A_{84}G$, $U_{85}G$, $U_{86}C$, $U_{89}A$ and $U_{96}A$); as a result, the potential U6−U2 interaction is extended to 16 nt and should be dramatically stabilized. Splicing complementation activity of this mutant U6 RNA was reduced to background levels (Figure 8A, lanes X2203). When we assayed the SP6 U6 X2203 RNA for spliceosome assembly, we found to our surprise that it very efficiently formed a complex which migrated slightly ahead of the B complex and which formed independently of the addition of splicing substrate (Figure 8B, compare lanes WT and X2203, + and −). We tested whether this novel complex might represent a U6−U2 snRNP by using a monoclonal antibody, 4g3, which specifically recognizes the B″ protein of U2 snRNP (Habets *et al.*, 1989). Independently of the addition of the U4 snRNP, ^{32}P-labelled SP6 U6 X2203 RNA could be efficiently immunoprecipitated by the U2 snRNP-specific antibody, but not by a control monoclonal antibody; in contrast, wild-type SP6 U6 was not immunoprecipitable by the U2-specific antibody. In addition, we have observed that an excess of SP6 U6 X2203 RNA can inhibit splicing in nuclear extract, presumably by sequestering the U2 snRNP in a splicing-inactive U6 X2203−U2 snRNP complex (data not shown).

To avoid such severe effects on snRNP−snRNP interactions, we next introduced 1 and 2 nt substitutions in the proposed U6−U2 base pairing region. By replacing the unpaired position U_{89} with an A ($U_{89}A$) this base pairing interaction could be extended to 9 nt (nucleotides 87−95; Figure 9). Compared with wild-type U6 RNA, spliceosome assembly and splicing activities of this mutant U6 RNA were not significantly changed (Figure 8A and B, compare lanes WT and U89A). Six other U6 RNA derivatives bearing either single point mutations ($A_{91}C$, $A_{92}U$, $G_{93}C$ and $C_{94}G$) or 2 nt substitutions ($A_{91}A_{92}\rightarrow CU$ and $G_{93}C_{94}\rightarrow CG$) should be weakened in their potential interaction with U2 RNA. Surprisingly, each of these mutations resulted in a reduction of spliceosome assembly to levels below 10% and in undetectable or very low splicing activities (Figure 8A and B; for a summary, see Table I). In addition, we assayed their

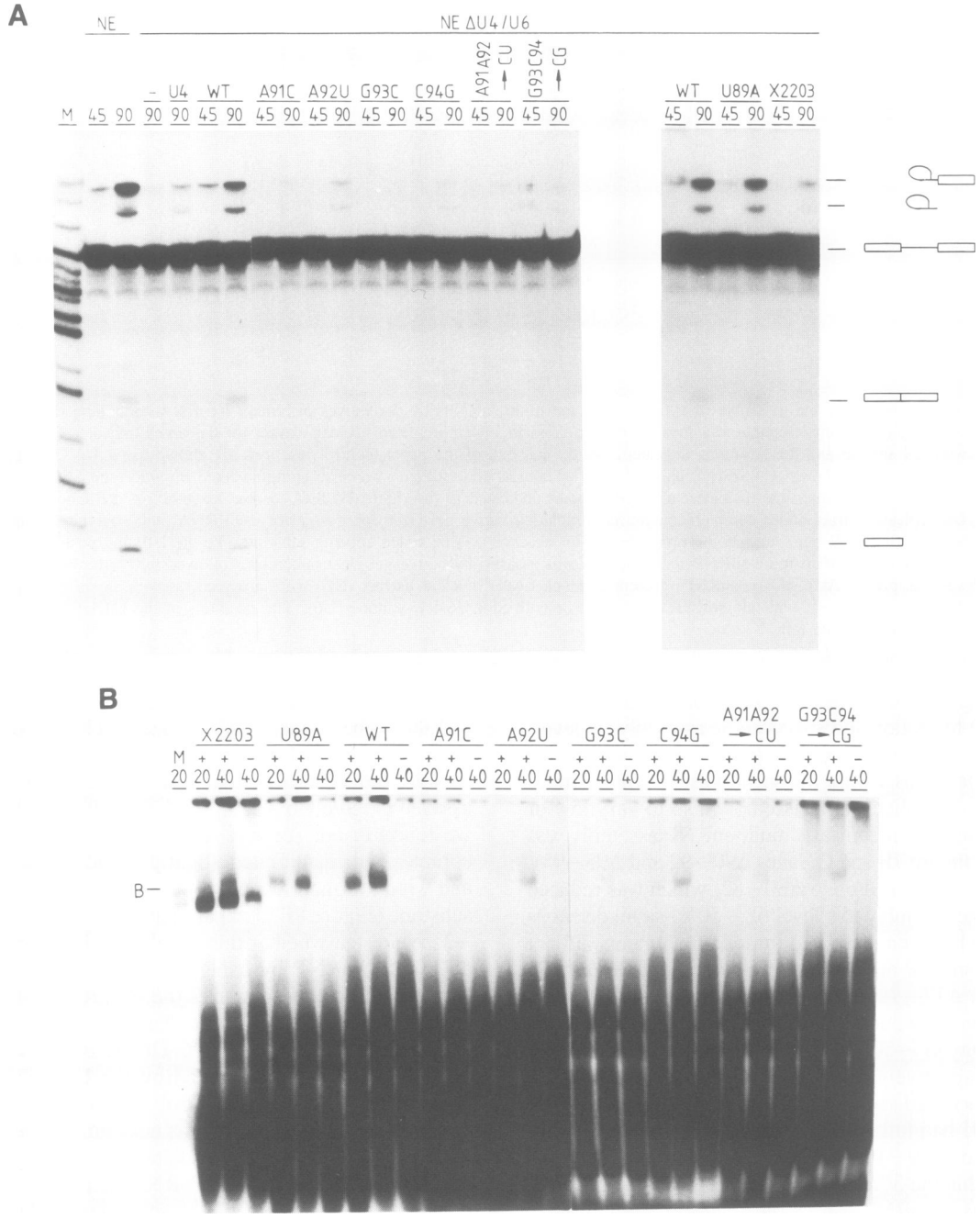


Fig. 8. Splicing complementation and spliceosome assembly by derivatives of human U6 RNA containing substitutions in the U6–U2 base pairing region. (**A**) Splicing complementation assays were performed in U4/U6-depleted nuclear extract (NE ΔU4/U6) for the times indicated (45 and 90 min), complementing with U4 snRNP and wild-type SP6 U6 (WT) or mutant U6 RNAs as indicated. On the left end of the panel, control reactions are shown using mock treated nuclear extract (NE), U4/U6-depleted extract without complementation (–), and with U4 snRNP alone (U4). The positions of pre-mRNA, splicing intermediates and products are indicated to the right. M, pBR322 digested with *Hpa*II. (**B**) Spliceosome assembly of ^{32}P-labelled U6 RNAs were analysed in U4/U6-depleted extract under complementation conditions by native gel electrophoresis. For each RNA, spliceosome assembly was assayed at two time points (20 and 40 min), and in the presence (+) or absence (–) of added splicing substrate. At the left side of the panel, assembled ^{32}P-labelled pre-mRNA marks the position of the B complex.

ability to assemble into U4/U5/U6 multi-snRNPs; each of the 1 and 2 nt substitutions in the U6–U2 base pairing region allowed U4/U5/U6 multi-snRNP formation at wild-type efficiency (data not shown), as expected from the full activities of SP6 U6 Δ90–92 and Δ93–95 in snRNP assembly (see above and Figure 7C).

We conclude that the two splicing-essential elements of the 3′ terminal domain of U6 RNA function at two different assembly stages; the region between nucleotides 74 and 76 is important already at the stage of multi-snRNP formation; in the U6–U2 base pairing region, only nucleotides G_{90} to G_{95}, but not the flanking positions C_{87} and G_{88}, are splicing-essential and play a critical role during spliceosome assembly.

Discussion

We have reported a mutational analysis of the domain structure of human U6 RNA using a mammalian *in vitro* splicing complementation assay. This system has made it possible

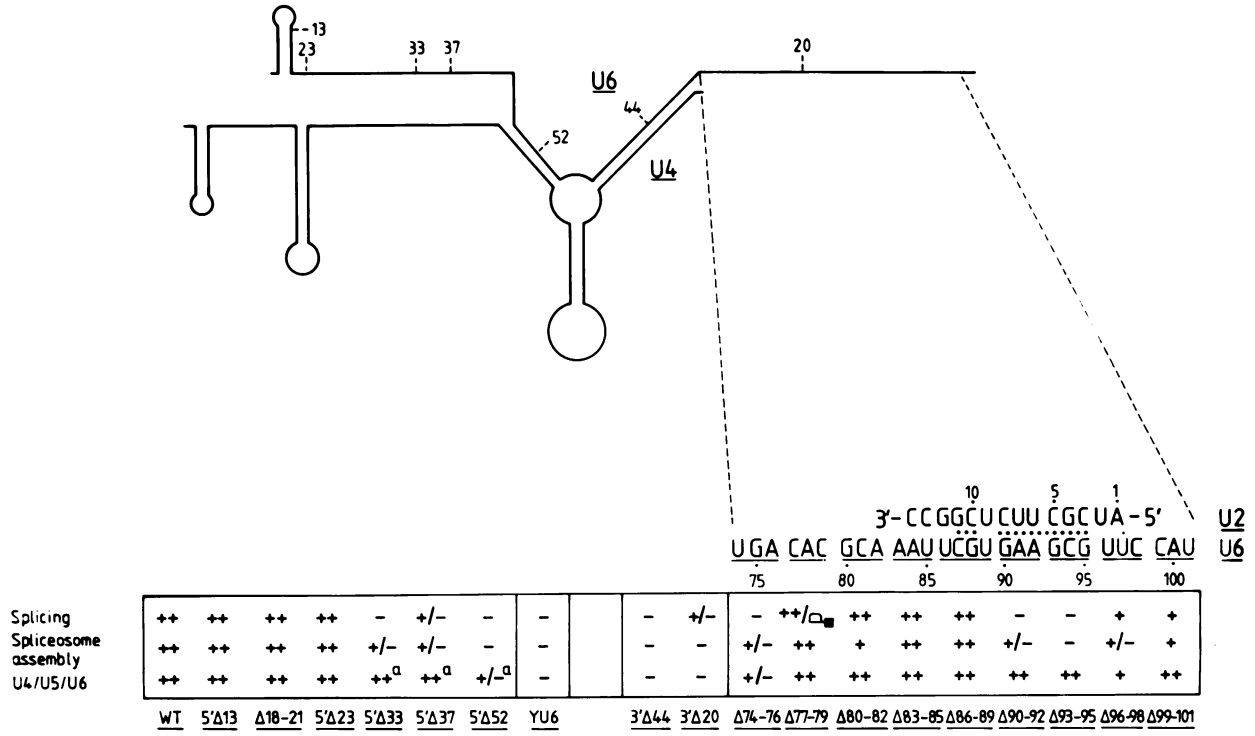


Fig. 9. Summary of splicing activities, spliceosome assembly and multi-snRNP formation of U6 mutant derivatives. The secondary structure of U4 and U6 RNAs with the endpoints of the mutant derivatives is schematically represented. Below, the RNA sequence of the 3′ terminal domain of U6 RNA is given (nucleotides 74−101), and the sizes of the internal deletions are shown by underlining. The 5′ end of U2 RNA (nucleotides 1−14) is written above the U6 RNA sequence, with the U6−U2 complementarity indicated by the dotted line between. The efficiencies of U6 mutant derivatives in splicing complementation, spliceosome assembly and multi-snRNP formation are compared with those of wild-type SP6 U6 and expressed as ++ (50−100%), + (10−50%), ± (<10%, but above background level) and − (background level). In the case of SP6 U6 Δ77−79, the second step of splicing is partially blocked.

for the first time to study the splicing functions of human U6 RNA as well as snRNP and spliceosome assembly. We have also described a novel method for the affinity purification of a functional mammalian U4 snRNP, based upon the release of the U4 snRNP from the affinity selected U4/U6 snRNP. The same principle should also be applicable to the affinity purification of U4 snRNPs from extracts of other species.

We detected full complementation of splicing activity at a U4 snRNP concentration of only ~10% of that present in the control reaction. This correlates well with previous studies, since we know that at least 90% of snRNPs have to be depleted before a reduction of splicing activity can be detected (see, for example, Barabino *et al.*, 1990). Our quantitative complementation assays are therefore consistent with the majority or all of the affinity purified U4 snRNP being functionally active. In contrast, to achieve complementation of splicing at the level of the control reaction, we had to add HeLa U6 RNA at a concentration present in the control splicing reaction; using SP6 U6 RNA required ~4-fold higher levels. In part, this difference betweeen the natural and synthetic RNAs in splicing complementation can be accounted for by the lower stability of synthetic U6 RNA in the extract. In addition, some of the difference in splicing activity may be attributed to the presence of internal nucleotide modifications, to the cap structure of the natural RNA, or, less likely, to the few extra 5′ and 3′ terminal nucleotides of SP6 U6 RNA.

Since U6 RNA has been highly conserved in evolution, we also assayed the *S.cerevisiae* U6 RNA for complementation in the mammalian system. Functionality of the yeast

Table I. Spliceosome assembly and splicing properties of substitution derivatives of human U6 RNA.

	Spliceosome assembly	Splicing
SP6 U6	++	++
X2203 ($A_{84}G$, $U_{85}G$, $U_{86}C$, $U_{80}A$, $U_{96}A$)	N.A.	−
$U_{89}A$	++	++
$A_{91}C$	+/−	−
$A_{92}U$	+/−	+/−
$G_{93}C$	+/−	−
$C_{94}G$	+/−	−
$A_{91}A_{92}$→CU	+/−	−
$G_{93}C_{94}$→CG	+/−	−

The spliceosome assembly and splicing activities of all substitution derivatives of SP6 U6 are listed. The efficiencies are compared with those of wild-type SP6 U6 and expressed as ++ (50−100%), + (10−50%), +/− (<10%, but above background level) and − (background level). N.A., not applicable.

U6 RNA in such a heterologous system would require yeast U6 RNA to interact with mammalian splicing factors including the U4 snRNP. Yeast−mammalian cross complementation has in fact recently been demonstrated in yeast, using the human U2 RNA (Shuster and Guthrie, 1990). However, in the mammalian *in vitro* system the yeast U6 RNA proved non-functional by both splicing, spliceosome assembly and multi-snRNP formation assays. This negative result can be explained by the failure of yeast U6 RNA to interact with the mammalian U4 snRNP. Similarly, in the reciprocal experiment, the mammalian U6 RNA did not complement *in vitro* splicing in yeast (Fabrizio

et al., 1989). Complementation experiments using the U4/U6-depleted mammalian extract in combination with purified yeast U4 snRNP and yeast U6 RNA should yield more insight in the functional conservation of snRNP–snRNP interactions.

We investigated the domain structure of U6 RNA through assaying wild-type and mutant RNAs first for splicing complementation, then for spliceosome and U4/U5/U6 multi-snRNP assembly. Our earlier work had revealed that the U4–U6 interaction domain consisting of the stem I and stem II regions suffices for U4/U6 snRNP formation; for spliceosome assembly, however, additional U6 sequences flanking the interaction domain were required (Bindereif *et al.*, 1990). In that study we had exchanged endogenous U6 RNA present in the U4/U6 snRNP with ^{32}P-labelled mutant U6 RNAs under conditions of high temperature and increased ionic strength. In comparison with the complementation system, the exchange reaction imposed less stringent requirements on the U4/U6 snRNP reconstitution, since under conditions of high temperature and increased ionic strength a requirement of additional factors for the U4–U6 interaction might be circumvented; furthermore, weakly bound U4 snRNP proteins might stay associated with U4 RNA during the exchange reaction in nuclear extract. In contrast, in complementation assays a functional U4/U6 snRNP has to assemble from affinity purified U4 snRNP and U6 RNA in a U4/U6-depleted extract. Therefore it is not surprising that by using splicing complementation we now find more extensive requirements for spliceosome assembly within the 5′ and 3′ terminal domains. For example, spliceosome assembly required only sequences downstream of nucleotide 37 when tested in the exchange reaction (Bindereif *et al.*, 1990), while in the complementation assay, additional sequences in the central domain up to nucleotide 24 were necessary (this study). Similarly, a large 3′ terminal deletion (SP6 U6 3′△20) reduced U4/U6 snRNP formation only when tested under splicing complementation conditions; this may be related to the different ionic conditions during reconstitution or to a disturbed overall secondary structure.

Through mutational analysis we identified splicing-essential regions of human U6 RNA and investigated whether they are required for spliceosome or multi–snRNP assembly. Surprisingly, we found the entire 5′ terminal domain of U6 RNA (nucleotides 1–23) to be dispensable for both splicing activity and spliceosome and snRNP assembly. This U6 domain is the only region of U6 RNA that, despite the conservation of its stem–loop structure, varies considerably in size between different species (Guthrie and Patterson, 1988; Roiha *et al.*, 1989). Significantly, this region includes the ACAU sequence (nucleotides 18–21), which is required for binding of a U6 snRNP-specific protein and which appears to play a role during U6 RNA capping and U6 snRNP transport (Hamm and Mattaj, 1989; Singh *et al.*, 1990; Gröning *et al.*, 1991). Therefore, binding of the U6-specific protein is probably not necessary for splicing *in vitro*. The 5′ terminal domain of U6 RNA has been suggested to be functionally analogous to the Sm domain of the other spliceosomal RNAs (Hamm *et al.*, 1990; Gröning *et al.*, 1991), which is thought to be needed for cap trimethylation and snRNP transport (Mattaj and DeRobertis, 1985; Mattaj, 1986). Further support of this functional analogy was provided by our recent demonstration that Sm protein binding of U4 RNA is not essential for splicing *in vitro* (C.Wersig and A.Bindereif, manuscript submitted).

In contrast to the 5′ terminal domain, the central region of U6 RNA contains splicing-essential elements, as suggested by earlier experiments, which showed that an antisense 2′-OMe RNA oligonucleotide against most of the central domain of human U6 RNA (nucleotides 21–40) was able to inhibit splicing (Blencowe *et al.*, 1989). In addition, several conserved nucleotide positions support a potential functional role of this region (Guthrie and Patterson, 1988). Our mutational analysis indicated that sequences between nucleotides 23 and 33 are essential for efficient splicing complementation and spliceosome assembly. Interestingly, one intron insertion of *Rhodotorula hasegawae* has been mapped in this region of U6 RNA (Tani and Oshima, 1991). snRNP assembly assays revealed that the splicing defect of derivatives lacking this region is caused by the formation of a non-functional U4/U5/U6 snRNP complex. Since this mutant U4/U5/U6 complex migrates on native gels considerably faster than the normal multi-snRNP, the most likely interpretation is that it lacks one or more protein components that are not necessary for the stable interaction between the three snRNPs, but are essential for the stable incorporation of the U4/U5/U6 complex into the spliceosome. It is conceivable that the lacking factor represents one or more of the recently identified U4/U6- or U4/U5/U6-specific proteins (Behrens and Lührmann, 1991; Okano and Medsger, 1991). The altered electrophoretic mobility might also be explained by different conformations of the normal and the mutant multi-snRNP complexes. In yeast, some internal U6 mutations in the corresponding region had no splicing-defective phenotypes (Fabrizio and Abelson, 1990; Madhani *et al.*, 1990), similarly as in *Xenopus* (Vankan *et al.*, 1990). However, in yeast the *in vivo* analysis of this region for splicing defects is complicated because of the presence of an intragenic promoter element (Brow and Guthrie, 1990). The external location of the U6 promoter element in higher eukaryotes may reflect a requirement to accommodate more functional RNA sequences within the U6 coding sequence.

In addition to the 5′ terminal and central U6 domains, we have studied possible functions of the 3′ terminal domain of human U6 RNA. Within the snRNP, the latter region is accessible to hybridization (Black and Steitz, 1986) and thus represents a potential site of interaction with other splicing factors. In yeast (Fabrizio *et al.*, 1989; Madhani *et al.*, 1990) and in *Xenopus* (Vankan *et al.*, 1990), only a few mutations in this region have been characterized: some mutations of the highly conserved UGA sequence (nucleotides 74–76 in the human U6 RNA; Figure 1) as well as extensive deletions resulted in lethal or splicing-defective phenotypes. In the mammalian system, binding of an antisense 2′-OMe RNA oligonucleotide to 3′ terminal sequences (nucleotides 82–101) inhibited splicing (Blencowe *et al.*, 1989). Even a shorter antisense oligonucleotide against nucleotides 91–101 still exhibited the same effect (T.Wolff and A.Bindereif, unpublished results). Recently, a U6–U2 base pairing interaction within the 3′ terminal domain has been detected by psoralen crosslinking (nucleotides 85–106 of human U6 RNA); specifically, the 'core' sequence GAAGCG (nucleotides 90–95) and, separated by a single mismatch position, nucleotides C_{87} and G_{88}, can possibly

base pair with U2 RNA (Hausner *et al.*, 1990). Genetic evidence for an essential function of the U6–U2 base pairing interaction in splicing has been derived from studying U6 and U2 RNAs with compensatory mutations in mammalian cells (Datta and Weiner, 1991; Wu and Manley, 1991).

As a result of an extensive mutational analysis of the entire 3′ terminal domain and a search for potential functions in splicing, spliceosome assembly or snRNP formation, we have identified two elements; first, the UGA sequence (nucleotides 74–76), and secondly, the sequence between nucleotides 90 and 95 (Figure 9). The first element is critical at the stage of multi-snRNP formation, whereas the second element functions in the assembly of a stable spliceosome. Interestingly, the second sequence element coincides with the core of the U6–U2 base pairing region. Our results therefore strongly support a model whereby the U6–U2 interaction is essential for splicing; in addition, our data provide experimental evidence that only the core sequence (nucleotides 90–95) is functionally important, but not the two adjacent nucleotides C_{87} and G_{88}. More importantly, our analysis strongly suggests that the U6–U2 interaction functions already at the stage of spliceosome assembly and is essential for spliceosome stability. It is also possible that the U6–U2 base pairing induces a conformational switch of the spliceosome, converting an unstable conformational state, which our native gel assay would not detect, into a stable one. Finally, we should consider the possibility that, in addition to their involvement in spliceosome assembly, these regions may function at the level of splicing. In this context our observation is of interest that a deletion of nucleotides 77–79, adjacent to one of the splicing-essential regions (nucleotides 74–76), results in a partial block in the second step of the splicing reaction.

In conclusion, U6 RNA is clearly a multifunctional splicing factor. Multiple sequence elements are organized in a very compact manner and play specific roles during capping, snRNP transport, the formation of the U4/U6 and the U4/U5/U6 snRNP, spliceosome assembly and in the mechanism of the cleavage–ligation reactions. To investigate mechanistically important interactions of U6 RNA with other components of the splicing machinery, it will be necessary to extend the complementation analysis to the other spliceosomal RNAs. In combination with reconstituted mutant snRNPs this approach should reveal new insights into structural transitions the spliceosome undergoes during the ordered excision of introns.

Materials and methods

DNA and 2′-OMe RNA oligonucleotides

DNA oligonucleotides. dT43, 5′-GCGAATTCGATTTAGGTGACACTATAG-3′; 489, 5′-CGAATTCGATTTAGGTGACACTATAGAAGTGCCTTCGGCAGCATACTAAAATTGGAACG-3′; 490, 5′-GCGAATTCGATTTAGGTGACACTATAGAACTAAAATTGGAACG-3′; 869, 5′-CGGGATCCGAAAAATATGGAACGCTTCACGAATTTGCGTGTCCTTGCGCAGGGG-3′; 870, 5′-CGGGATCCGAAAAATATGGAACGCTTCACGATGCGTGTCATCCTTGCGCAGGGG-3′; 871, 5′-CGGGATCGAAAAATATGGAACGCTTCACGAATTGTGTCATCCTTGCGCAGGGG-3′; 872, 5′-CGGGATCCGAAAAATATGGAACGCTTCACGAATTTGCTCATCCTTGCGCAGGGG-3′; 1134, 5′-GCGGATCCGAAAAATATGGAACGCTTCACG-3′; 2198, 5′-CGGGATCCGAAAAATATGGAACGCTTCATTTGCGTGTCATCCTTGCGC-3′; 2199, 5′-CGGGATCCGAAAAATATGGAACGCACGAATTTGCGTGTCATCCTTGCGC-3′; 2200, 5′-CGGGATCCGAAAAATATGGAATTCACGAATTTGCGTGTCATCCTTGCGC-3′; 2201, 5′-CGGGATCCGAAAAATATGCGCTTCACGAATTTGCGTGTCATCCTTGCGC-3′; 2202, 5′-CGGGATCCGAAAAATGAACGCTTCACGAATTTGCGTGTCATCCTTGCGC-3′; 2203, 5′-CGGGATCCGAAAAATATGGATCGCTTCTCGGCCTTGCGTGTCATCCTTGCGC-3′; 1221, 5′-CGGGATCCGAAAAATATGGAACGCTTCTCGAATTTGCGTGTC-3; 1222, 5′-CGGGATCCGAAAAATATGGAACGCTGCACGAATTTGCGTG-3′; 1223, 5′-CGGGATCCGAAAAATATGGAACGCATCACGAATTTGCTG-3′; 1224, 5′-CGGGATCCGAAAAATATGGAACGGTTCACGAATTTGCG-3′; 1225, 5′-CGGGATCCGAAAAATATGGAACCCTTCACGAATTTGCG-3′; 1226, 5′-CGGGATCCGAAAAATATGGAACGCAGCACGAATTTGCGTG-3′; 1227, 5′-CGGGATCCGAAAAATATGGAACCGTTCACGAATTTGCG-3.

2′-OMe RNA oligonucleotides. U6 stem I, 5′-UXXXXIICCAUICUAAUCUUCUCUG-3′; U6-3′, 5′-UXXXXAUGGAACGCUUCACGAAUUU-3′ (X denoting a biotinylated 2′-deoxycytidine; Sproat *et al.*, 1989).

Mutagenesis and in vitro *transcription*

Mutant derivatives of SP6 U6 (Bindereif *et al.*, 1990) were generated by polymerase chain reaction (PCR) methods, using SP6 U6 as a template and the following pairs of oligonucleotides: SP6 U6 5′ Δ23, 490/1134; SP6 U6 Δ18–21, 489/1134; SP6 U6 Δ74–76, dT43/869; SP6 U6 Δ77–79, dT43/872; SP6 U6 Δ80–82, dT43/871; SP6 U6 Δ83–85. dT43/870; SP6 U6 Δ86–89, dT43/2198; SP6 U6 Δ90–92, dT43/2199; SP6 U6 Δ96–98, dT43/2201; SP6 U6 Δ99–101, dT43/2202; SP6 U6 X2203, dT43/2203; SP6 U6 U_{89}A,dT43/1221; SP6 U6 A_{91}C, dT43–1222; SP6 U6 A_{92}U, dT43/1223; SP6 U6 G_{93}C, dT43/1224; SP6 U6 C_{94}G, dT43/1225; SP6 U6 $A_{91}A_{92}$→CU, dT43/1226; SP6 U6 $G_{93}C_{94}$→CG, dT43/1227. For each derivative, the amplified DNA fragment was cut with *Eco*RI and *Bam*HI and cloned into pUC19. SP6 U6 △93–95 was generated from SP6 U6 by PCR amplification with oligonucleotides dT43 and 2200, followed by a filling in reaction with Klenow fragment, digestion with *Bam*HI, and cloning into pUC19 cut with *Bam*HI and *Hinc*II.

U6 RNA derivatives were transcribed by SP6 or T7 RNA polymerase as described in the presence of GpppG cap analogue, MINX RNA by SP6 RNA polymerase in the presence of m^7GpppG cap analogue (Bindereif and Green, 1987). The following DNAs were used as transcriptional templates: SP6 U6 and derivatives cut with *Bam*HI; T7–U6/*Dra*I (Fabrizio and Abelson, 1989; in this study called T7–YU6), MINX/*Bam*HI (Zillman *et al.*, 1988) and the U6 mutant templates SP6 U6 5′ Δ13, 5′ Δ33, 5′ Δ37, 5′ Δ52, 3′ Δ20 and 3′ 44 (Bindereif *et al.*, 1990), which have been previously described. All the new U6 RNA derivatives carry an additional GGA at their 5′ end (except for SP6 U6 5′ Δ23 which has an additional GC) and a GC or CGGAUC at their 3′ end, depending on whether the *Bam*HI run-off site is transcribed through.

Affinity purification of the U4 snRNP

1 ml of HeLa nuclear extract (Dignam *et al.*, 1983) was adjusted to 400 mM KCl and incubated with 10 μg of biotinylated 2′-OMe RNA oligonucleotide U6 stem I for 15 min at 30°C. Streptavidin–agarose (Sigma) was blocked (Barabino *et al.*, 1989) and washed three times, each for 5 min with four volumes of BB400 (20 mM HEPES, pH 8.0, 400 mM KCl, 0.5 mM DTT, 0.01% NP40). The oligonucleotide-tagged U4/U6 snRNP was bound to 125 μl pre-blocked streptavidin–agarose during a 1 h incubation at 4°C. The streptavidin–agarose was pelleted and washed three times, each with 750 μl of BB400 for 15 min at 4°C. To release the U4 snRNP from the affinity selected U4/U6 snRNP, 200 μl of prewarmed elution buffer EB25 (20 mM HEPES, pH 8.0, 25 mM KCl, 0.1 mg/ml bovine serum albumin, 1000 U/ml RNasin, 4 μg/ml leupeptin) was added to the streptavidin–agarose beads and incubated for 20 min at 37°C. The supernatant was recovered by centrifugation (10 s, 13 000 *g*) and contained the released U4 snRNP at a concentration corresponding to ~4 μg/ml of U4 RNA. After the addition of glycerol to 10%, it was stored at −20°C. RNAs bound by streptavidin–agarose through 2′-OMe RNA oligonucleotides were released by incubation in two volumes of PK buffer (100 mM Tris–HCl, pH 7.5, 12.5 mM EDTA, 150 mM NaCl, 1% SDS and 0.5 μg/μl proteinase K) for 45 min at 55°C followed by 15 min at 85°C. Released RNAs were purified and analysed on 10% denaturing polyacrylamide–urea gels by silver staining.

U4/U6 snRNP depletion of HeLa nuclear extract

After adjusting to 600 mM KCl, HeLa nuclear extract (Dignam *et al.*, 1983) was incubated for 1 h at 30°C with 10 μg/ml of a biotinylated 2′-OMe RNA oligonucleotide complementary to nucleotides 82–101 of human U6 RNA (U6–3′; Figure 1). As a control, nuclear extract was incubated without oligonucleotide. Streptavidin–agarose was blocked (Barabino *et al.*, 1989) and washed three times at 4°C, each for 5 min with two volumes of BB600

(20 mM HEPES, pH 8.0, 600 mM KCl, 0.5 mM DTT, 0.01% NP40). The oligonucleotide-tagged U4/U6 snRNP was removed from the extract by two sequential incubations, each for 1 h at 4°C with gentle agitation and with one-third volume of streptavidin–agarose. The streptavidin–agarose beads were pelleted by centrifugation in a table centrifuge for 2 min at 3000 *g*. After the second selection the extract was dialysed against buffer D (Dignam *et al.*, 1983) for 5 h and stored at −70°C. RNAs bound by streptavidin–agarose through 2′-OMe RNA oligonucleotides were released as described above.

Splicing complementation

To assay for splicing complementation, the affinity purified U4 snRNP and wild-type or mutant U6 RNAs were first pre-incubated for 15 min at 30°C in a 24 μl reaction containing 7.5 μl U4/U6-depleted extract, 5.5 μl buffer D, 2 μl affinity purified U4 snRNP (corresponding to ~8 ng U4 RNA), 3.2 mM $MgCl_2$, 0.5 mM ATP, 20 mM creatine phosphate, 1600 U/ml RNasin (Promega), 40 μg/ml yeast tRNA and 300 ng unlabelled SP6 U6 RNA or equimolar amounts of U6 mutant derivatives. tRNA was added to suppress RNase activity in the extract (data not shown). Splicing was initiated by the addition of 1 ng of ^{32}P-labelled MINX pre-mRNA, followed by incubation at 30°C for the times indicated. For RNA analysis, splicing products were purified and analysed on denaturing 13% polyacrylamide–urea gels; spliceosome assembly was analysed after the addition of heparin to a final concentration of 1 mg/ml by native acrylamide–agarose gel electrophoresis as described (Nelson and Green, 1988).

Spliceosome assembly

To determine the assembly of ^{32}P-labelled U6 RNA and mutant derivatives in spliceosomes, assays were performed under splicing complementation conditions, using 20 ng of ^{32}P-labelled U6 RNA per 1×reaction (25 μl). After a 15 min incubation at 30°C, 75 ng of unlabelled MINX pre-mRNA was added and incubation continued at 30°C. At the times indicated, heparin was added to a final concentration of 1 mg/ml and the formation of B complexes assayed on native acrylamide–agarose gel as described (Nelson and Green, 1988).

U4/U5/U6 multi-snRNP assembly

The assembly of wild-type SP6 U6 RNA or mutant derivatives to U4/U5/U6 multi-snRNP complexes was assayed by native gel electrophoresis followed by Northern blot analysis. In a 1× reaction, 100 ng SP6 U6 wild-type or equimolar amounts of mutant U6 RNA were incubated in U4/U6-depleted nuclear extract in the absence of pre-mRNA with 4 μl affinity purified U4 snRNP under the conditions of splicing complementation for 15 min at 30°C. To control reactions, heparin or KCl were added after reconstitution to final concentrations of 1 mg/ml or 500 mM, respectively. snRNP complexes were separated in a composite 3.5% acrylamide (acrylamide:bisacrylamide ratio of 80:1)–0.5% agarose gel, using 50 mM Tris–50 mM glycine (pH 9.0) buffer system (Konarska and Sharp, 1987) and electroblotted in 10 mM Tris, 5 mM NaAc, 0.5 mM EDTA, pH 7.8, to a Hybond N membrane (Amersham). To detect snRNP complexes, the membrane was sequentially probed with ^{32}P-labelled antisense probes specific for U4, U5 and U6 RNAs. As a U4-specific probe, a 2′-OMe RNA oligonucleotide complementary to nucleotides 64–83 was used. The U5 probe was a DNA oligonucleotide complementary to nucleotides 65–88. A U6 probe was obtained by *in vitro* T7 transcription of SP6 U6 5′E4 (unpublished), resulting in an RNA complementary to nucleotides 15–107 of human U6 RNA. When probing for U4 or U5, blots were prehybridized in a buffer containing 5×SSPE (50 mM sodium phosphate, pH 7.7, 900 mM NaCl, 0.5 mM Na_2EDTA) 5×Denhardt's solution, 0.1% SDS, 50 μg/ml yeast tRNA, 200 μg/ml sonicated salmon sperm DNA) for 5 h at 38°C. ^{32}P-labelled oligonucleotides were added to an activity of $1-2\times10^5$ c.p.m./ml, and hybridization was continued for 8–16 h at 38°C. Blots were washed once in 5×SSPE, 0.1% SDS and twice in 2×SSPE, 0.1% SDS at 38°C for 30 min each. When probing for U6, a similar protocol was followed, except that the hybridization buffer contained 25% formamide and no SDS; all steps were performed at 42°C, and after hybridization, the blots were washed once in 5×SSPE, 0.1% SDS, once in 2×SSPE, 0.1% SDS, and once in 0.1×SSPE, 0.1% SDS for 30 min each.

Immunoprecipitation analysis of snRNP–snRNP interactions

To assay for the U6–U2 interaction, 25 ng of ^{32}P-labelled SP6 U6 RNA was incubated with or without 4 μl of affinity purified U4 snRNP for 40 min at 30°C in a 50 μl reaction containing 30% U4/U6-depleted nuclear extract, 22% buffer D (Dignam *et al.*, 1983), 3.2 mM $MgCl_2$, 0.5 mM ATP, 20 mM creatine phosphate, 1600 U/ml RNasin and 40 μg/ml yeast tRNA. 10% of the reaction was used for the analysis of total RNA. The rest was incubated on ice for 1 h with 50 μl U2 snRNP-specific monoclonal antibody 4g3 (Habets *et al.*, 1989) and 3 μl RNasin (40 U/μl). After the addition of 700 μl G buffer (20 mM HEPES pH 8.0, 150 mM KCl, 1.5 mM $MgCl_2$, 4 μg/ml leupeptin, 0.5 mM DTT) immunocomplexes were precipitated at 4°C for 15 h by 20 μl protein A–Sepharose (Pharmacia) to which 5 μg of goat anti-mouse IgG1 (Sigma) had been coupled during an overnight incubation at 4°C in PBS, pH 8.0, 0.05% NaN_3. The immunoprecipitate was washed three times with 750 μl G buffer containing 1 mg/ml heparin. C383 was used as a control monoclonal antibody (Schwedler-Breitenreuther *et al.*, 1986). Immunoprecipitated RNAs were recovered by phenol extraction and ethanol precipitation.

The interaction of U6 RNAs with the U4 snRNP was determined by anti-Sm immunoprecipitation. 10 ng of ^{32}P-labelled U6 RNA was incubated for 15 min with 2 μl of U4 snRNP in U4/U6-depleted extract under splicing conditions. After removing 10% of the reaction mixture for total RNA analysis, the remainder was incubated for 2 h at 4°C with 20 μl of protein A–Sepharose (Pharmacia) pre-coated with 2 μl of anti-Sm serum in 700 μl of NET–500 buffer (50 mM Tris–HCl, pH 8.0, 500 mM KCl, 0.01% NP40). The beads were washed three times with NET–500 buffer and immunoprecipitated RNAs were recovered by phenol extraction and ethanol precipitation.

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