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Roles of U4 and U6 snRNAs in the assembly of splicing complexes

Pierre Vankan[1], Caroline McGuigan and Iain W.Mattaj

EMBL, Meyerhofstrasse 1, D-6900 Heidelberg, FRG

[1]Present address: Botanisches Institut der Ludwig-Maximillians Universität München, Menzingerstrasse 67, D-8000 München 19, FRG

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A series of U4 and U6 snRNA mutants was analysed in *Xenopus* oocytes to determine whether they block splicing complex assembly or splicing itself. All the U4 and U6 mutants found to be inactive in splicing complementation resulted in defects in assembly of either U4/U6 snRNP or of splicing complexes. No mutants were found to separate the entry of U5 and U6 snRNAs into splicing complexes and neither of these RNAs was able to associate with the pre-mRNA in the absence of U4. In the absence of U6 snRNA, however, U4 entered a complex containing pre-mRNA as well as the U1 and U2 snRNAs. U6 nucleotides whose mutation resulted in specific blockage of the second step of splicing in *Saccharomyces cerevisiae* are shown not to be essential for splicing in the oocyte assay. The results are discussed in terms of the roles of U4 and U6 in the assembly and catalytic steps of the splicing process.

Key words: RNA processing/U4 snRNA/U6 snRNA/U snRNPs

Introduction

Splicing of mRNA precursors occurs in a multicomponent complex, the spliceosome. This complex consists of the pre-mRNA together with snRNPs and protein factors (for reviews see Ruby and Abelson, 1991; Lührmann *et al.*, 1990). Five snRNAs have been shown to be essential for pre-mRNA splicing. These are U1, U2, U4, U5 and U6. Their functional roles have been defined to variable extents. During the splicing reaction U1 binds to the 5′ splice site and U2 to the branchpoint by interactions involving both RNA–RNA and protein–RNA contacts (see reviews cited above for references). Furthermore, interactions betwen U1 snRNP and the branchpoint region of the intron have been shown to be important for early steps in splicing complex assembly both in yeast (Legrain *et al.*, 1988; Ruby and Abelson, 1988; Séraphin and Rosbash, 1989, 1991) and in vertebrates (Barabino *et al.*, 1990). An interaction of U5 snRNP with the 3′ splice site has been suggested (Chabot *et al.* 1985) and, more recently, U5 has been shown to influence the site of cleavage at the 5′ splice site (Newman and Norman, 1991).

U5 is thought to enter the spliceosome as part of a multi snRNP complex which also contains U4 and U6 snRNAs (Ruby and Abelson, 1991; Behrens and Lührmann; 1991, Séraphin *et al.*, 1991; Lamm *et al.* 1991). A base pairing interaction between U6 and U2 snRNAs which is important for splicing has been identified (Hausner *et al.*, 1990; Datta and Weiner, 1991; Wu and Manley, 1991). However, there is as yet no evidence that U4 or U6 interact directly within the pre-mRNA, or that they influence the accuracy of splice site selection. A better definition of the roles of U4 and U6 in splicing is therefore desirable.

Based on available evidence, a model for U4/U6 function has been proposed (Brow and Guthrie, 1989; Guthrie, 1991). The information used in formulating this model can be summarized as follows. Group II autocatalytic introns undergo splicing via the same chemical pathway as nuclear pre-mRNA introns (Cech 1986). Thus, RNA is capable of adopting a structure which can catalyse these reactions, and may do so in pre-mRNA splicing. U6 is the most conserved of the snRNAs (Guthrie and Patterson, 1988) and is therefore a good choice for a putative *trans*-acting 'catalytic' component. Furthermore, while U6 exists in a stable snRNP with U4 (U4/U6), it has been known for some time that the interaction between U4 and U6 is destabilized concomitantly with the appearance of splicing intermediates in the spliceosome (Pikielny *et al.* 1986; Cheng and Abelson, 1987; Lamond *et al.*, 1988; Blencowe *et al.*, 1989), suggesting that U6 might be released from U4 in order to carry out its function in splicing.

Examination of fungal snRNA genes led to the discovery that introns were present in the U6 genes of some species (Tani and Ohshima, 1989, 1991). The sporadic occurrence of these introns suggested that they had been generated by insertion into the U6 genes. It was proposed that this could have occurred via insertion of the introns into U6 snRNA in the spliceosome by reversal of the splicing process followed by reverse transcription and integration of the cDNA copy into the genome (Brow and Guthrie, 1989). The fact that the insertion positions of the first two introns discovered corresponded to extremely highly conserved regions of U6 led to the proposal that these regions might be positioned at the 'catalytic centre' of the spliceosome (Brow and Guthrie, 1989; Guthrie, 1991). Evidence from the analysis of the phenotypes of yeast U6 mutants in splicing either *in vitro* or *in vivo* (Fabrizio and Abelson, 1990; Madhani *et al.*, 1990) provided some support for this idea. Point mutation in positions adjacent to the two intron insertion positions discussed above, resulted in the unusual phenotype of causing accumulation of splicing intermediates, i.e. of uncoupling the two steps of the splicing reaction. The more recent finding that a third fungal species has a U6 gene which contains four introns has been interpreted as further support for the model (Tani and Ohshima, 1991). However, since some of these introns are inserted at positions whose mutation had no effect on splicing (Madhani *et al.*, 1990; Fabrizio and Abelson, 1990; Vankan *et al.*, 1990) these data could also indicate that at least some introns have been

inserted into U6 genes by a mechanism not involving reversal of splicing.

An additional series of U6 mutants, and fewer U4 mutants, have also been tested in various splicing assays *in vitro* and *in vivo* (Fabrizio *et al.*, 1989, 1990; Madhani *et al.*, 1990; Vankan *et al.*, 1990; Wersig and Bindereif, 1990; Shannon and Guthrie, 1991). The extensive nature of many of these mutations and the lack of information on their effects on either snRNP or splicing complex assembly has led us to use a splicing complementation assay in *Xenopus* oocytes (Hamm *et al.*, 1989) in order to define better the roles of U4 and U6, and their component domains, in splicing. The effect of mutation on U4/U6 snRNP and splicing complex assembly has also been tested. The significance of the results for models of U4 and U6 function in splicing is discussed.

Results

Localization of regions responsible for U4 mutant phenotypes

In a previous mutational analysis of the U4/U6 snRNP in *Xenopus* (Vankan *et al.*, 1990), three U4 mutants, U4.2, U4.3 and U4.4 (Figure 1A), were all found to be negative in splicing but were apparently unaffected in U4/U6 snRNP assembly. The mutations were all extensive, U4.2 and U4.4 being deletions and U4.3 an alteration of six bases. Before attempting to characterize the effects of the mutants further, we checked whether more subtle mutations in the same regions led to the same phenotype.

Mutants U4.11−U4.20 (Figure 1A) were generated by site-directed mutagenesis within the sequences deleted in

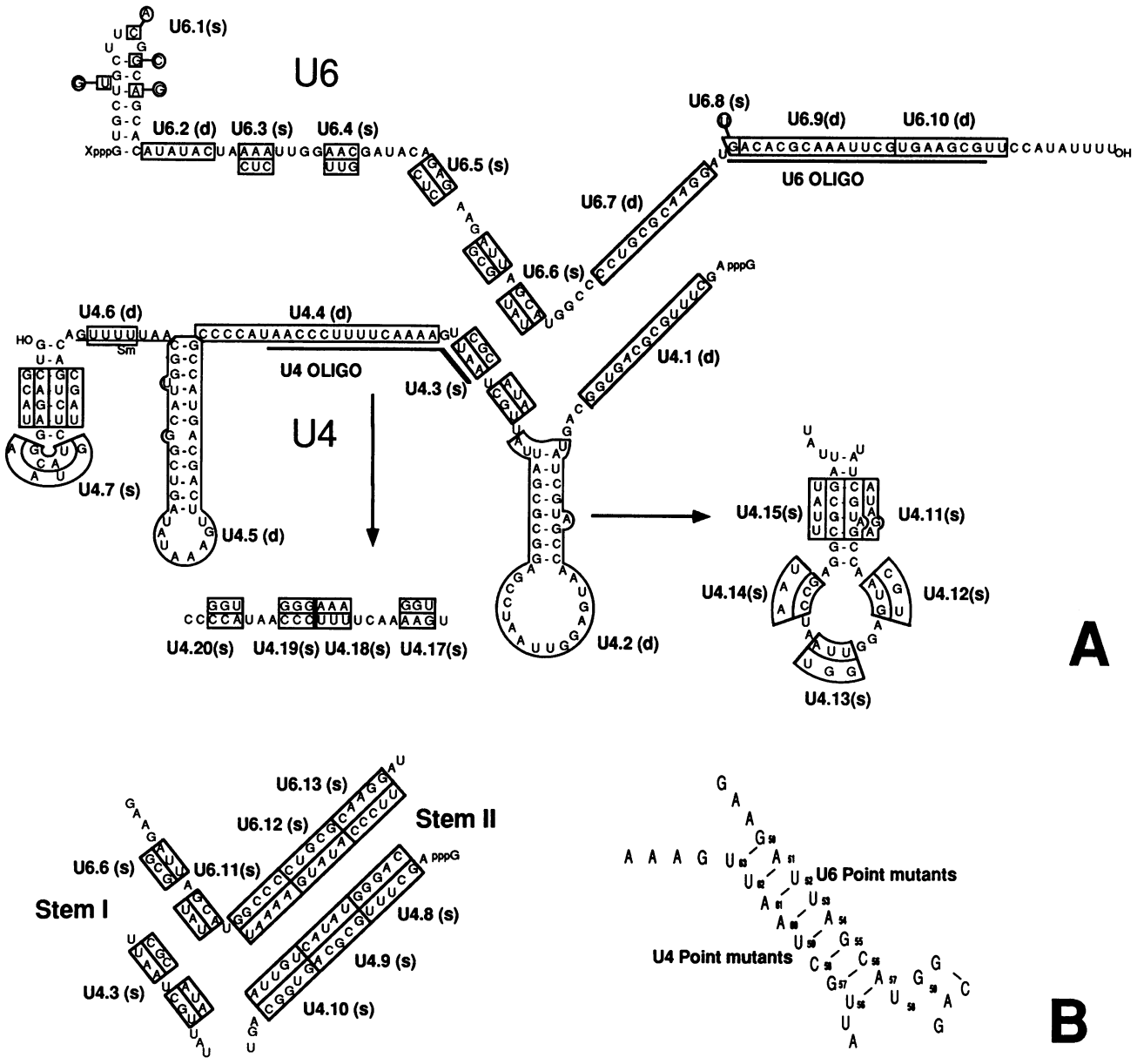

Fig. 1. Sequences of wt and mutant U4 and U6 snRNAs. (**A**) Substitution mutants are indicated with (s) and deletion mutants with (d). Bases used for substitution are indicated in boxes or circles next to the wt position. Target sites of DNA oligonucleotides normally used for RNase H-mediated cleavage are indicated with a line. The structural model for U4 and U6 snRNA is that of Brow and Guthrie (1988). (**B**) Complementary mutations in stems I and II of the U4−U6 interaction domain and positions of point mutations in stem I. Note that U6.6(s) and U4.3(s) are both mutated in two groups of three nucleotides separated by an unmutated position.

U4.2 and U4.4. Mutants U4.11 and U4.15 disrupted the stem of the 5′ hairpin of U4, while U4.16 combined these two mutations to restore pairing. The activity of all the mutants was tested in *Xenopus* oocytes in a splicing complementation assay (Hamm *et al.*, 1989). The precursor tested, pBSAd1, is efficiently spliced in *Xenopus* oocytes to produce the intron lariat (I) and spliced exon (E) products (Figure 2A, lane 12). Destruction of endogenous U4 by oligonucleotide-directed RNase H cleavage inhibits splicing (Figure 2A, lane 1). Activity is restored by coinjection of DNA encoding U4 RNA (lane 10) but not U2 RNA (lane 11). A Northern blot (Figure 2B) of RNA from the injected oocytes confirmed the destruction of the endogenous U4 (lane 1) and transcription of the injected DNA templates (lanes 2−11). Note that similar controls were performed for all the splicing complementation experiments presented in this paper, but are not shown. The level of accumulation of transcripts from injected genes varies in different experiments (see also Vankan *et al.*, 1990), but is routinely higher than that of the endogenous U4 (or U6) RNA. We have not seen evidence for concentration-dependent effects in complementation, but no careful titration experiments have been carried out.

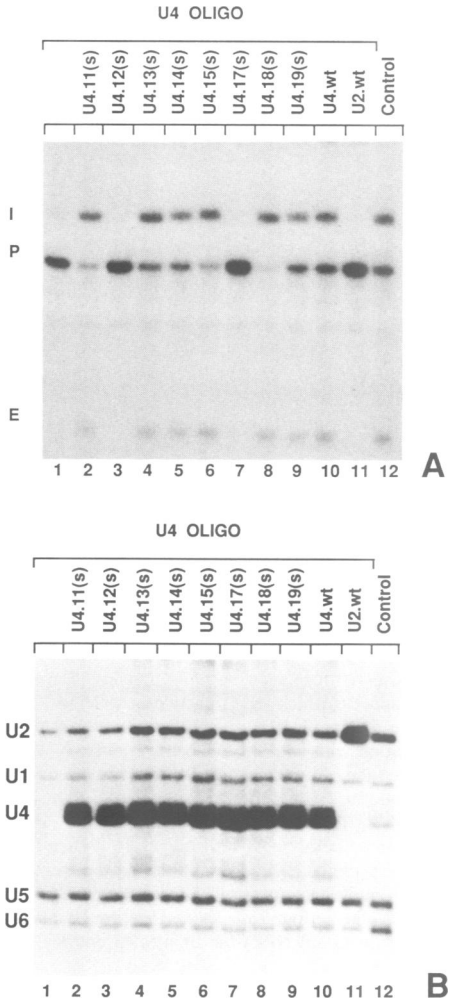

Fig. 2. Splicing complementation by U4 mutants. Oocytes were injected with a DNA oligonucleotide complementary to part of the U4 sequence together with a plasmid containing a wt or mutant U4 gene as indicated. Control oocytes were not injected. After an overnight incubation a labelled adenovirus pre-mRNA was injected into the same oocytes. (**A**) After 90 min incubation, total RNA was prepared and splicing was analysed on a denaturing polyacrylamide gel. I. intron lariat; P, precursor (pBSAd1); E, spliced exons. (**B**) The destruction of the endogenous U4 and transcription of the injected genes was monitored on a Northern blot probed with antisense RNA corresponding to U1, U2, U4, U5 and U6.

Of the mutants in the 5′ hairpin only U4.12 (Figure 2A, lane 3) was incapable of complementing splicing. Disruption of the stems of the hairpin in mutants U4.11 and U4.15 had no detectable effect on splicing complementation, indicating that a stable stem structure in the 5′ hairpin is not essential for the function of U4 in this assay. Of the four three-base mutants in the central single-stranded region of U4, deleted in U4.4, only one, U4.17, was negative in splicing complementation (Figure 2A, lane 7). U4.18, U4.19 (lanes 8 and 9) and U4.20 (data not shown) behaved like wild-type (wt) U4. Mutants U4.2 and U4.4 were previously shown to be capable of assembling into U4/U6 snRNPs (Vankan *et al.*, 1990). Not surprisingly, all of the U4.11−U4.20 mutants were also positive in this assay (data not shown).

Thus, mutation of three bases in either the loop of the 5′ hairpin of U4 (U4.12) or in the single-stranded stretch 3′ of the interaction helices (U4.17) led to the same mutant phenotype as the U4.2 and U4.4 deletions. It is probable that these mutations disrupt essential interactions made by U4 at some step(s) in the splicing pathway.

Point mutations in stem I of the interaction domain

The U4 strand of interaction helix I (Figure 1B) is required for splicing complementation but not for U4/U6 assembly (Hamm and Mattaj, 1989; Bindereif *et al.*, 1990; Wersig and Bindereif, 1990; Vankan *et al.*, 1990). We wished to determine whether this phenotype could also be further localized. A collection of seven point mutations in U4 at positions 56−63 (Figure 1B) was generated and tested in the splicing complementation assay. No mutant was recovered at position 58. With one exception, U4.U63-G (Figure 3A, lane 7), the U4 point mutants tested all behaved like wt U4 (Figure 3A, lanes 1−8 and Figure 3B, lanes 10−12). U63 lies outside the sequence mutant in the original U4.3 construct (Figure 1B). Thus, while extensive mutation of the U4 strand of interaction stem I results in a defective U4/U6 snRNP, the identity of most individual bases in the strand, and thus base pairing with U6 snRNA at most positions, is not required for splicing.

Analogous point mutants in the U6 strand of interaction stem I of the U4/U6 snRNP have previously been analysed in yeast. Mutation at several positions led to defective splicing complementation *in vitro* (Fabrizio and Abelson, 1990) and to growth defects or lethality *in vivo* (Madhani *et al.*, 1990). Experiments with similar U6 mutants were carried out in the vertebrate complementation system. As previously observed (Vankan *et al.*, 1990), the RNase H mediated destruction of U6 snRNA is not always completely effective and residual splicing activity is sometimes seen after oligonucleotide injection. (Compare Figure 3A, lane 9 with Figure 3B, lanes 2 and 6.) U6 RNAs mutant at individual positions between G50 and G58 (Figure 1B) were tested. No mutants were recovered at positions 55 and 56, and the only mutant in which A57 was altered was a double mutation in which both A57 and G50 were changed to C.

Like the U4 point mutants, many of the U6 mutants behaved like wt U6 (Figure 3A, lanes 9−16 and Figure 3B,

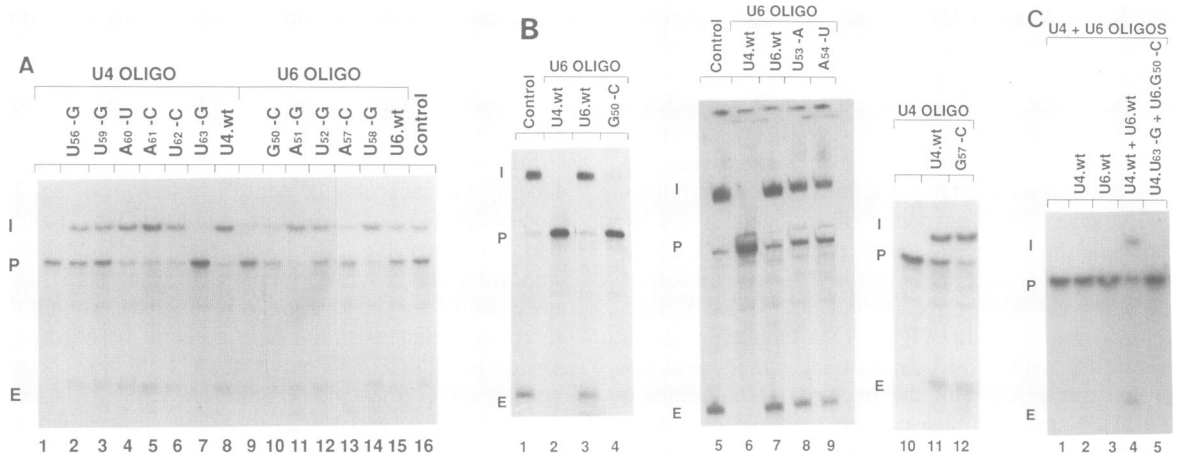

Fig. 3. Splicing complementation by U4 and U6 point mutants. (**A** and **B**). The point mutants were analysed as described in Figure 2, after injection of either an anti-U4 or an anti-U6 oligonucleotide as indicated. The position and also the change in sequence of each mutant is indicated above the lanes. I, intron lariat; P, pre-mRNA; E, spliced exons. (**C**) Splicing complementation of complementary mutants in stem I. Oocytes were injected with oligonucleotides to destroy both U4 and U6. Wild-type and mutant U4 and U6 DNAs, either alone or in combination, were tested for complementation of splicing. I, intron lariat; P, pre-mRNA; E, spliced exons.

lanes 5–9). The two exceptions were G50-C and the double mutant G50-C, A57-C (Figure 3A, lanes 10 and 14; Figure 3B, lane 4) which in repeated experiments showed strongly reduced or no activity in the splicing complementation assay. The double mutant will be referred to as U6.A57-C. Since the G50-C alteration is present in both mutants, it would be sufficient to explain the splicing defect in both cases. Further results presented below suggest, however, that the A57-C mutation has an effect on U4/U6 snRNP assembly.

U6.G50 is positioned in interaction helix I opposite U4.U63, suggesting that the defects in the two point mutants might be due to a disruption of their potential for base pairing. This was tested by introducing the two complementary mutants simultaneously into the splicing complementation assay. First U4 and U6 RNAs were both destroyed by oligonucleotide injection (Figure 3C, lane 1). Splicing was not restored by injection of either U4 or U6 DNA alone, but was corrected by simultaneous injection of both wt DNAs (Figure 3C, lanes 2–4). Simultaneous injection of both U4.U63-G and U6.G50-C failed to complement splicing activity. Thus, the identity of these two nucleotides, not the fact that they can form a base pair, is apparently a requirement for splicing in this assay.

Effect of defective point mutants on U4/U6 snRNP assembly

To begin to define the step at which the defective U4 and U6 point mutants had their effects, we analysed their ability to assemble in U4/U6 snRNPs. After injection of the anti-U4 oligonucleotide, neither endogenous U6 nor transcripts of an injected U6 wt gene are immunoprecipitable with Y12 (Lerner *et al.*, 1981) anti-Sm antibodies (Figure 4, lanes 5 and 6). Transcripts of both U4 wt and the point mutant which is negative in splicing complementation, U4.U63-G, restore U4/U6 assembly as measured by immunoprecipitation of U6 by anti-Sm antibodies (Figure 4, lanes 7 and 8). To test the U6 mutants, anti-U6 oligonucleotide was injected together with DNA encoding both U4 and U6 transcripts (the U4 template is injected in order to increase the amount of U4/U6 snRNP assembly possible), followed by immunoprecipitation. All the U6 mutants were transcribed to similar levels

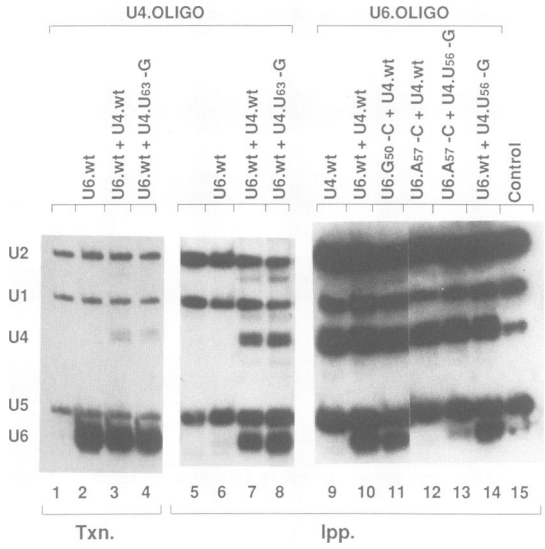

Fig. 4. U4/U6 snRNP formation by point mutants. The ability of point mutants of U4 and U6 which were inactive in splicing complementation to form U4/U6 snRNPs was tested by immunoprecipitation with anti-Sm monoclonal antibody Y12 (Lerner *et al.*, 1981). For the U4 mutant, oocytes were injected with an anti-U4 oligonucleotide in the presence or absence of U6 wt DNA and U4 wt or mutant DNA. Transcript accumulation (lanes 1–4) and immunoprecipitation (lanes 5–8) were tested 16 h later. For the U6 mutants an anti-U6 oligonucleotide was injected together with wt or mutant U4 DNA and wt or mutant U6 DNA. Immunoprecipitation (lanes 9–14) was carried out 16 h later. The control lane shows immunoprecipitation of snRNPs from uninjected oocytes.

(data not shown). In combination with wt U4, both wt U6 and the defective U6.G50-C mutant formed Sm-immunoprecipitable snRNPs (Figure 4, lanes 10 and 11). On the other hand, the splicing-negative U6 double mutant, U6.A57-C, did not (lane 12). The assembly defect in U6.A57-C could be corrected to a limited extent by coinjection of the U4 mutant U4.U56-G, which restores base pairing at this position (Figure 4, lane 13). U4.U56-G could also assemble with wt U6 (lane 14). Thus, while combinations of A–U, A–G or G–C residues at U6 position 57

and U4 position 56 (Figure 1B) were tolerated, juxtaposition of C with U appeared to have a considerable effect on U4/U6 snRNP assembly or stability. In the case of the A−G combination, bulging U4.G57 would allow U4.G56 and U55 to pair with U6.C56 and A57. This might explain why the A−G combination is tolerated. Since the U6.A57-C mutation is not available separately from G50-C we have not been able to determine whether the effect on U4/U6 snRNP assembly results from a synergistic effect of the two mutations.

In vivo spliceosome assembly

The next goal was to determine the step of splicing at which U4 and U6 mutants capable of snRNP assembly had their effects. It is thought that the U4/U6 snRNP forms a triple snRNP complex with U5 before entering the spliceosome (see Introduction). Despite trying many assays which have previously been utilized to examine the U4/U6/U5 snRNP in extracts of mammalian or yeast cells, we have thus far failed to detect this complex in oocytes (see Discussion). The formation of pre-splicing and splicing complexes was therefore analysed. Biotin−streptavidin based affinity chromatography methods (Grabowski and Sharp, 1986; Bindereif and Green, 1987) were adapted to study the ability of the U4 and U6 mutants to enter splicing complexes.

After injection of biotinylated pBSAd1 transcripts, complexes assemble and can be retained on streptavidin−agarose beads. These complexes contain U1, U2, U4, U5 and U6 snRNAs (Figure 5, lane 1). When no precursor (lane 2) or a non-biotinylated precursor (data not shown) are injected, only background levels of U snRNAs are precipitated. To verify the specificity of the precipitation further, mutant versions of the Ad1 precursor were used (Hamm and Mattaj, 1990). When the 5′ splice site was either deleted (Figure 5, lane 3) or mutated (lane 5) so as to leave a precursor containing only a functional branchpoint/3′ splice site region, a specific association of U2 snRNA with the precursor was seen. When the branchpoint/3′ splice site region was removed, U1 was specifically bound (Figure 5, lane 4). This binding was abolished when the 5′ splice site was mutated (lane 6). Binding of U4, U5 and U6 required the presence of functional 5′ splice site and branchpoint/3′ splice site sequences (lanes 3−6).

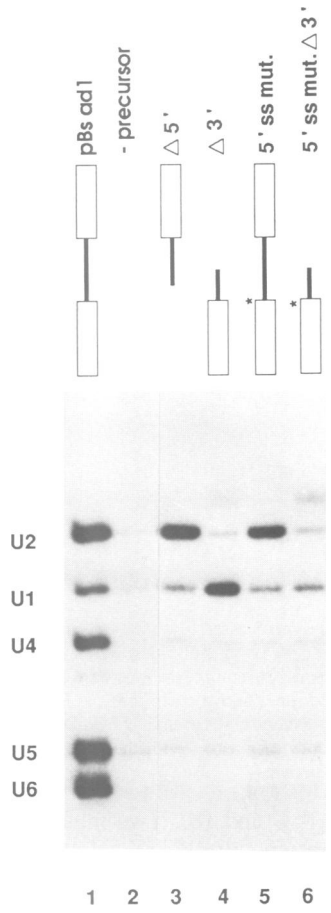

Fig. 5. Affinity selection of *in vivo* assembled spliceosomes. Oocytes were injected with biotinylated pre-mRNA. Following incubation the oocytes were disrupted and the pre-mRNA and components associated with it were selected using streptavidin beads. Lane 1, pre-mRNA with consensus splice sites (pBSAd1). Lane 2, background binding to streptavidin beads without injection of pre-mRNA. Lanes 3 and 4, pre-mRNAs containing a truncated 5′ or 3′ end, respectively. Lane 5, pre-mRNA containing a mutated 5′ splice site. Lane 6, pre-mRNA containing both a truncated 3′ end and a mutated 5′ splice site. The band migrating above U2 in lanes 4 and 6 is caused by cross-hybridization with the pre-mRNA.

Complex formation with U6 mutants

To test the ability of the U6 mutants to enter splicing complexes in oocytes the various mutant DNAs were coinjected with the anti-U6 oligonucleotide. Sixteen hours later, biotinylated Ad1 precursor was injected and complex formation was assayed after streptavidin precipitation. Unexpectedly, in the absence of U6 snRNA, U4 was seen to enter a complex with the precursor which also contained U2 and U1 RNA. (Figure 6A and B, lanes 1). This complex also formed in the presence of a variety of U6 mutants which were inactive in the splicing complementation assay (Figure 6A, lanes 7−11, 13 and 14; 6B, lane 3). Precipitation of U1, U2 and U4 after RNase H-mediated destruction of U6 required the presence of biotinylated precursor RNA (data not shown).

For the U6 mutants tested, there was a striking correlation between splicing complementation (Vankan *et al.*, 1990; Figure 3) and entry into complexes with the pre-mRNA. Mutants U6.1−U6.3 and U6.8 are all active and entered splicing complexes (lanes 3−5 and 12). Mutant U6.4, which complements to an intermediate level, allowed formation of a low level of complexes containing U5 and U6.4 in this and other experiments (lane 6 and unpublished data). The other mutants were negative for both complex formation and functional complementation (Figure 6A, lanes 7−11, 13 and 14; 6B lane 3). In all cases where a U6 mutant could enter the splicing complex, U5 was also present, whereas U5 was never detected in the absence of U6 RNA (Figure 6A and B).

Complex formation with U4 mutants

After oligonucleotide-mediated destruction of U4, only U1 and U2 RNAs associate with precursor (Figure 6B, lane 4; Figure 7, lane 1). Complex formation with all five U snRNAs is restored in the presence of wt U4 transcripts (Figure 6B, lane 5; Figure 7, lane 2). When the U4 mutants were tested, a tight correlation between splicing complementation and complex formation was also observed. Mutants U4.7, 11 and 13, which are functionally active, allowed complex assembly with all five spliceosomal U snRNAs

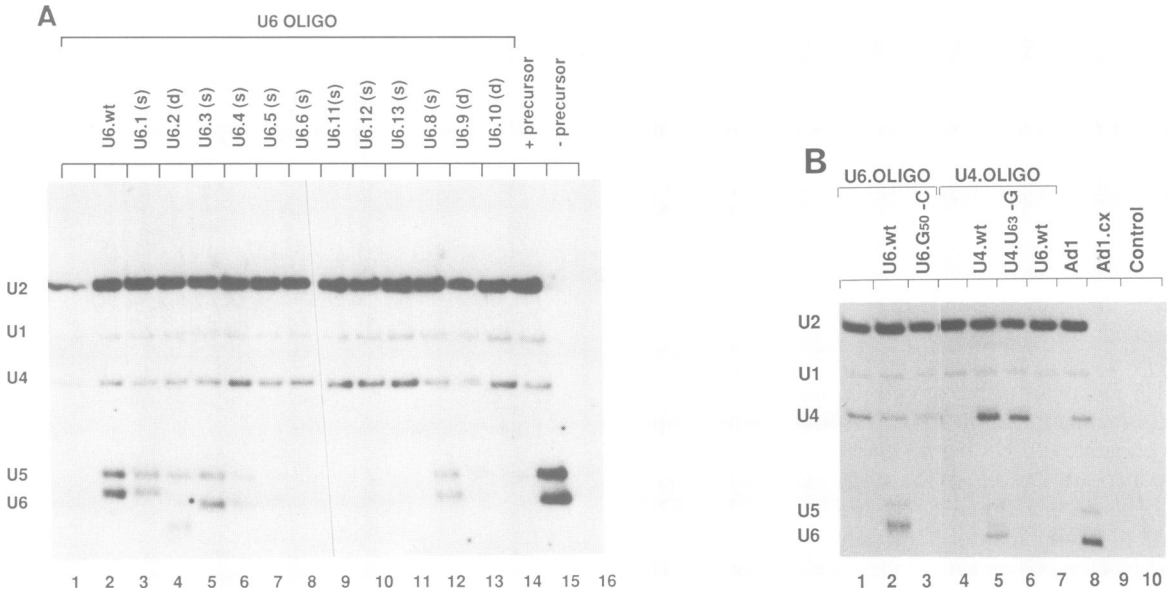

Fig. 6. *In vivo* spliceosome assembly with U6 mutants. Ten oocytes were injected with a DNA oligonucleotide to destroy U6 snRNA in combination with a gene for either wt or mutant U6 snRNA. After an overnight incubation, a biotin-labelled pre-mRNA (pBSAd1) was injected into the oocytes and selection of pre-mRNA complexes was carried out. (**A**) Analysis of deletion and substitution mutants of U6. The gene coinjected with the DNA oligonucleotide is indicated above each lane. + Precursor lane, oocytes injected with biotinylated pre-mRNA only. − Precursor lane, uninjected oocytes. (**B**) As (A) except that U6 and U4 point mutants were analysed following injection of either anti-U6 or anti U4 oligonucleotides respectively. Ad1, oocytes injected with the pBSAd1 precursor. Ad1cx, oocytes injected with the mutant Ad1 precursor shown in Figure 5, lane 6. Control, uninjected oocytes.

(Figure 7, lanes 8, 9 and 18). Two mutants which had an intermediate phenotype in splicing, U4.10 and U4.5 (which has an electrophoretic mobility slightly less than that of U5), were able to form complexes (Figure 7, lanes 6 and 14) but a reduced level of U5 and U6 RNAs were found in these complexes. In the case of U4.5 there is an apparent increase in U5 RNA. This is due to the fact that most of the U4 DNAs injected, including U4.wt, give rise to two closely spaced transcripts. In the case of U4.5 the upper transcript comigrates with U5 snRNA (see Figure 4 for well resolved doublets and Figure 2 of Vankan *et al.*, 1990).

The inactive U4 mutants could be divided into two classes. Mutant U4.2, which is slightly longer than U6; mutant U4.4, which is intermediate in mobility between U4 and U5; and mutants U4.8, U4.12, U4.17 together with the defective point mutant U4.U63-G formed one class. They could enter the complex consisting of U1, U2 and precursor, but U5 and U6 were not co-precipitated with these mutants (Figure 7, lanes 4, 7, 10−12 and 17; Figure 6B, lane 6). U4.4 precipitated only slightly in repeated experiments, but was detectable after long autoradiographic exposure. The second class contained two other mutants, U4.1, from which the U4 strand of interaction helix II had been deleted, and U4.9, in which the central five bases of this region were mutated (Figure 1). These RNAs did not enter the U1/U2/pre-mRNA/U4 complex. Note that in the absence of a functionally active U4 RNA, U5 and U6 did not associate with the pre-mRNA.

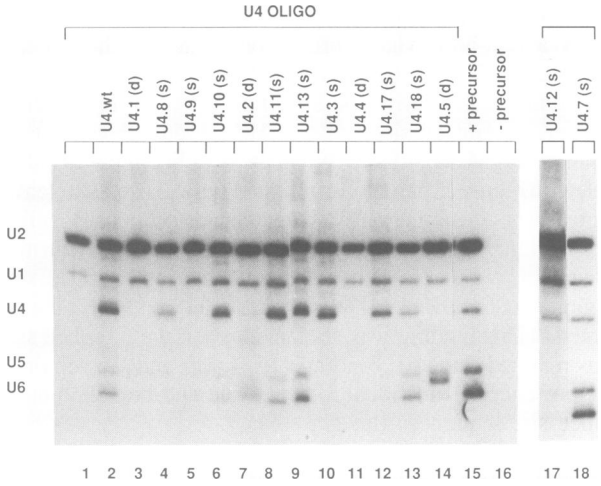

Fig. 7. *In vivo* spliceosome assembly with U4 deletion and substitution mutants. As Figure 6, using the U4 mutants indicated above each lane. Mutants U4.12 and U4.7 were tested in a separate experiment.

Discussion

Sequences in U4 snRNA required for splicing complementation

We have extended the analysis of the effects of mutation on the ability of U4 and U6 snRNAs to function in splicing in *Xenopus* oocytes. The results obtained in this and a previous study (Vankan *et al.*, 1990) are summarized in Table I. The sequence and structural requirements for U4 activity in splicing complementation have been narrowed down considerably. Outside the regions of base pairing with U6, three short regions of U4 have been shown to be required for splicing. The first was the Sm binding site, which is mutated in U4.6 (Vankan *et al.*, 1990). This site is required for association of U4 with the common U snRNP proteins and thus for snRNP formation, nuclear localization and cap trimethylation (for reviews see Mattaj, 1988; Zieve and Sauterer, 1990) although it is not required for splicing complex assembly *in vitro* (Wersig and Bindereif, 1990). The second is the sequence in the loop of the 5′ hairpin of

Table I. Summary of the effects of U4 and U6 mutants

	U4/U6 snRNP assembly	Splicing complex assembly	Splicing
U6.1	+	+	+
U6.2	+	+	+
U6.3	+	+	+
U6.4	+	±	±
U6.5	±	−	−
U6.6	±[b]	−	−
U6.7	−		−
U6.8	+	+	+
U6.9	+	−	−
U6.10	+	−	−
U6.11	−[b]	−	−
U6.12	−[b]	−	−
U6.13	−[b]	−	−
U6.G50-C	+	−	−
U6.A51-G	+		+
U6.U52-G	+		+
U6.U53-A	+		+
U6.A54-U	+		+
U6.A57-C[a]	−[b]	−	−
U6.U58-G	+		+
U4.1	−	−[c]	−
U4.2	+	−	−
U4.3	+	−	−
U4.4	+	−	−
U4.5	+	±	±
U4.6	−		−
U4.7	+	+	+
U4.8	±[b]	−	−
U4.9	−[b]	−[c]	−
U4.10	±[b]	±	±
U4.11	+	+	+
U4.12	+	−	−
U4.13	+	+	+
U4.14	+		+
U4.15	+		+
U4.17	+	−	−
U4.18	+	+	+
U4.19	+		+
U4.U56-G	+		+
U4.G57-C	+		+
U4.U59-G	+		+
U4.A60-U	+		+
U4.A61-C	+		+
U4.U62-C	+		+
U4.U63-G	+	−	−

This summarizes the data from both this study and from Vankan *et al.* (1990).
± indicates an intermediate phenotype.
[a]U6.A57-C is a double mutant which also contains a G50-C mutation.
[b]The snRNP assembly defect of these mutants can be corrected by a complementary mutation.
[c]Apart from U4.1 and U4.9, the U4 mutants can enter a complex containing U1, U2 and pre-mRNA.

U4 which is mutated in U4.12. Other mutations in the loop sequence or mutations which should destabilize the stem of the hairpin had no detectable effect on the function of U4 in splicing complementation. The overall conformation of this region therefore seems to be less important than the sequence of a short region in the loop. In the case of *S.cerevisiae* U4, the loop of the 5′ hairpin has been shown to be required for association of U4 with the essential splicing factor PRP4 (Bordonné *et al.*, 1990; Xu *et al.*, 1990). It may be that the mutation in U4.12, which alters sequences whose evolutionary conservation is high (Guthrie and Patterson, 1988), disrupts interaction with the *Xenopus* homologue of PRP4.

The third non-paired region of U4 required for splicing is defined by the U4.17 mutation. This is located adjacent to the essential U4.U63 and U6.G50 residues and close to the U6.5 mutation, which also causes loss of function. Thus the regions of U4 and U6 adjacent to interaction helix I are both required for functional activity. Recently, evidence for the existence of several U4/U6 snRNP proteins in HeLa cells has been presented (Okano and Medsger, 1991). It would be interesting to test whether the above mutations disrupt their interaction with U4 or U6 RNA. Other possible effects of the mutations are discussed below.

A role for U6 in catalysis?

The evidence which led to the hypothesis that U6 might be involved in the catalysis of splicing was discussed in the Introduction. The most direct experimental support for the idea came from the phenotypes of U6 point mutants in yeast splicing. Mutations in several nucleotides gave rise to the accumulation of splicing intermediates (Fabrizio and Abelson, 1990; Madhani *et al.*, 1990). Their positions were G50−G52, C58 and A59, which are equivalent to G45−G47, U53 and A54 of the *Xenopus* sequence (Figure 1). In the oocyte complementation assay, the effect of mutation at two of these positions was tested, U6.U53-A and A54-U (equivalent to the yeast U6.C58-A and A59-U mutants). Both had a wt phenotype. On the other hand, the only negative U6 point mutant in our study (U6.G50-C), which blocked both steps of splicing, is in a position whose alteration from G to A or U had little or no effect in the yeast *in vitro* experiments (Fabrizio and Abelson, 1990). The effects of changing G to C in the yeast system or of G to A or U in the *Xenopus* assays have not been tested.

The differences between the yeast and *Xenopus* systems could simply reflect the assays used. The two steps of the splicing reaction appear to be tightly coupled in the oocyte complementation assay, and only small amounts of intermediate are ever seen (Hamm *et al.*, 1989). It might therefore be that it is more difficult to observe uncoupling of the two steps of the splicing reaction in this assay. In fact, the phenotype of the *S.cerevisiae* G50-U mutant was different when assayed in extracts of a yeast strain containing the mutant U6 and when tested in the *in vitro* complementation assay from which endogenous U6 had been removed (Madhani *et al.*, 1990; Fabrizio and Abelson, 1990). Only in the former case was accumulation of intermediates observed. Thus, all we can say is that our results provide no support for, and no decisive evidence against, the argument that U6 may have a catalytic role. On the other hand, all of our data are consistent with the U4/U6 snRNP having a crucial structural role in the assembly of splicing complexes, as discussed below.

Roles for U4 and U6 in splicing complex assembly

A biotin−streptavidin based method for the analysis of association of U snRNPs with Ad1 pre-mRNA was employed. Although this method does not allow spliceosomes to be distinguished from pre- and post-splicing complexes it does give an indication of whether particular mutant U snRNAs affect association of snRNPs with the pre-mRNA. In addition, if a mutant is not present in the precipitate, we

cannot say whether it never associated with the pre-mRNA or was dislodged during the isolation procedure. Since both of these possibilities would result from an abnormal interaction between the snRNP and the pre-mRNA complex we will refer to such mutants as assembly defective.

Analysis of the behaviour of the U4 and U6 mutants in the complex formation assay led to several conclusions. The major one was that there was a perfect correlation between the abilities of both U4 and U6 mutants to function in splicing and their abilities to participate in complete (i.e. U1-, 2-, 4-, 5- and 6-containing) pre-mRNA complex formation. All splicing defective mutants had an assembly phenotype. Some of the deleterious mutations were already known to be unable to form U4/U6 snRNPs (Vankan *et al.*, 1990), and some others overlap with mutations shown to affect splicing complex assembly in HeLa cell nuclear extracts (Bindereif *et al.*, 1990; Wersig and Bindereif, 1990). Our studies, however, have allowed the separate examination of the effects of all the U4 and U6 mutants on snRNP formation, association with the pre-mRNA and on splicing *per se*. The results thus provide the strongest evidence to date for the importance of the structural integrity of the U4/U6 snRNP in spliceosome assembly. The mutations which affect complex assembly are widespread. They cover the 5′ half of the U4 sequence and the 3′ two-thirds of the U6 sequence. It is therefore likely that multiple possible sites of interaction with other components of the splicing machinery have been affected in different mutants. Much further work will be required to reveal the identity of these components, although several possible candidates have been identified both in yeast (Ruby and Abelson, 1991; Shannon and Guthrie, 1991) and in vertebrates (Okano and Medsger, 1991; Behrens and Lührmann, 1991). Available data suggest that some sequences in U6 are involved in more than one interaction in the course of the splicing process (discussed in Vankan *et al.*, 1990; Guthrie, 1991). This could provide an explanation for the extreme conservation of parts of the U6 sequence.

Complex formation on pre-mRNA

In the absence of U6, U4 was able to enter a complex containing pre-mRNA, U1 and U2. The U1/U2/U4/pre-mRNA complex was also seen in the presence of all U6 mutants which were negative in splicing complementation. On the contrary, U6 was not found in the complex in the absence of U4. Similarly, U5 was never present in the complex without both U6 and U4, in agreement with previous observations made in HeLa cell extracts (Barabino *et al.*, 1990). Since most cell types examined contain an excess of U6 over U4, there is probably not a large amount of free U4 snRNP in the nucleus. It is therefore unlikely that the U4-containing complex is an intermediate on the normal splicing pathway. However, the results suggest that the primary interaction of the U4/U6 (or U4/U6/U5) snRNP with the spliceosome could be through the U4 moiety.

The ability of U4 mutants to associate with the U1/U2/pre-mRNA complex indicated that the only U4 sequences essential for attachment are those mutated in U4.9 and deleted in U4.1 i.e. the central region of interaction stem II. In yeast, this region has been proposed to be involved in PRP4 binding (Xu *et al.*, 1990). Thus, a PRP4 homologue could play a role in U4 binding to the pre-mRNA complex.

We have thus far been unable to detect a U4/U6/U5 complex in oocytes, in spite of repeated attempts with methods and conditions which have been successful with extracts from mammalian cells. Methods used include native gel electrophoresis (Konarska and Sharp, 1986) density gradient centrifugation (Black and Pinto, 1989), 2′-*O*-methyl RNA oligonucleotide affinity chromatography with a biotinylated oligonucleotide directed against U6 (B.Blencowe, personal communication) and immunoprecipitation with a monoclonal antibody, H 386 (Behrens and Lührmann, 1991). It may be that the U4/U6/U5 complex is extremely short-lived *in vivo*, or that it is present at a level below our detection limits. Alternatively, it may be destroyed during the preparation of oocyte extracts. The possibility that U4/U6 and U5 might be capable of independent assembly into splicing complexes *in vivo* cannot be entirely ruled out, although recent *in vitro* results (Behrens and Lührmann, 1991; Séraphin *et al.*, 1991; Lamm *et al.*, 1991) make this unlikely.

The non-functional U6 mutants all prevent association of U6 with the pre-mRNA. Many of these mutants, U6.5, U6.6 and U6.11−13, are either unable to form U4/U6 snRNPs or exhibit a reduction in either the quantity or stability of the U4/U6 snRNPs formed (Vankan *et al.*, 1990; see also Bindereif *et al.*, 1990). Two exceptions, U6.9 and U6.10, are capable of efficient U4/U6 assembly (Vankan *et al.*, 1990), but nevertheless are not found in association with pre-mRNA. These deletions both result in the removal of parts of a region of U6 proposed to interact wth U2 RNA by base pairing (Hausner *et al.*, 1990; Datta and Weiner, 1991; Wu and Manley, 1991). It may therefore be that U6 needs to interact with both U4 and U2 RNAs before it can stably associate with the splicing complex. More detailed mutagenesis experiments might help to answer this question, although technical limitations currently make the analysis of U2 and U6 mutations simultaneously in the oocyte complementation assay very difficult. The U6.9 mutation also encompasses a region which, in *S.cerevisiae*, appears to be important for interaction between U6 and the essential splicing factor PRP24 (Shannon and Guthrie, 1991). This provides another possible explanation for the inactivity of this mutant. Further progress in understanding the defects in these and other mutants will require the development of assays which will allow the identification of the interactions within splicing complexes which are disturbed by the mutations.

Materials and methods

Microinjection, splicing complementation and Northern analysis were carried out exactly as described previously (Vankan *et al.*, 1990). In addition to the anti-U4 and -U6 oligonucleotides described in that study a second anti-U6 oligonucleotide, TAATCTTCTCTGTATCGTTC, was sometimes used. Neither U6 oligonucleotide resulted in quantitative destruction of U6 in a reproducible way, as measured by residual splicing activity. Methods for RNA synthesis (Hamm *et al.*, 1989) and incorporation of biotinylated UTP into the pBSAd1 precursor (Scherly *et al.*, 1989) have also been described. Site-directed mutagenesis was as described (Vankan *et al.*, 1990). The point mutants in the stems of interaction domain I were made by mixing 5% of each of the three alternative phosphoramidites with the correct one at the positions to be mutagenized.

Affinity selection of in vivo assembled spliceosomes

Ten oocytes were injected with oligonucleotides to destroy endogenous U4 or U6 snRNAs. A gene coding for a mutant or wt U4 or U6 snRNA was coinjected, such that transcripts of these genes replaced the endogenous snRNAs. After an overnight incubation a biotinylated pre-mRNA (pBSAd1;

Konarska and Sharp, 1987) was injected into the nucleus. After 7 min the oocytes were disrupted on ice in 300 μl of 1 × washing buffer (1 × WB) containing 350 mM KCl, 20 mM HEPES pH 7.9, 0.05% (v/v) NP40 and 5 units of RNAsin. 100 μl of this extract was removed and used for total RNA extraction to check transcription of the injected genes. To the remaining 200 μl, 800 μl of 2 × WB and 100 μl of preblocked beads were added. This mixture was revolved at 4°C for 45 min. The beads were prepared by adding 4 μl of tRNA (10 mg/ml) and 4 μl glycogen (10 mg/ml) to 100 μl of streptavidin–agarose beads (Sigma). After incubation for 30 min at 4°C, the beads were spun and washed twice for 10 min at 4°C in 1 × WB. After the last wash and prior to use, the volume was readjusted to the original 100 μl. After incubation with the disrupted oocytes, the beads were spun and washed twice for 10 min, and once for 1 min in 2 × WB at 4°C. After the last wash the supernatant was removed and 300 μl of elution buffer (100 mM NaCl, 10 mM Tris pH 7.6, 1 mM EDTA, 0.5% SDS, 100 μg/ml glycogen, 5 mg/ml proteinase K) was added. This was incubated at 65°C for 45 min and at 85°C for 10 min. The supernatant was extracted once with phenol–chloroform and the RNAs were precipitated with ethanol and subsequently analysed by Northern blotting. Time course experiments showed that there was little difference in the amount of snRNAs precipitated over the time course of the splicing reaction in oocytes (i.e. from 5 to 90 minutes after pre-mRNA injection).

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References

Barabino,S.M.L., Blencowe,B., Ryder,U., Sproat,B.S. and Lamond,A.I. (1990) *Cell*, **63**, 293–302.
Behrens,S.E. and Lührmann,R. (1991) *Genes Dev.*, **5**, 1439–1452.
Bindereif,A. and Green,M.R. (1987) *EMBO J.*, **6**, 2415–2424.
Bindereif,A., Wolff,T. and Green,M.R. (1990) *EMBO J.*, **9**, 251–255.
Black,D.L. and Pinto,A.L. (1989) *Mol. Cell. Biol.*, **9**, 3350–3359.
Blencowe,B.J., Sproat,B.S., Ryder,U., Barabino,S. and Lamond,A.I. (1989) *Cell*, **59**, 531–539.
Bordonné,R., Banroques,J., Abelson,J. and Guthrie,C. (1990) *Genes Dev.*, **4**, 1185–1196.
Brow,D.A. and Guthrie,C. (1988) *Nature*, **334**, 213–218.
Brow,D.A. and Guthrie,C. (1989) *Nature*, **337**, 14–15.
Cech,T.R. (1986) *Cell*, **44**, 207–210.
Chabot,B., Black,D.L., Le Master,D. and Steitz,J.A. (1985), *Science*, **230**, 1344–1349.
Cheng,S. and Abelson,J. (1987) *Genes Dev.*, **1**, 1014–1027.
Datta,B. and Weiner,A.M. (1991) *Nature*, **352**, 821–824.
Fabrizio,P. and Abelson,J. (1990) *Science*, **250**, 404–409.
Fabrizio,P., McPheeters,D.S. and Abelson,J. (1989) *Genes Dev.*, **3**, 2137–2150.
Grabowski,P.J., and Sharp,P.A. (1986) *Science*, **233**, 1294–1299.
Guthrie,C. (1991) *Science*, **253**, 157–163.
Guthrie,C. and Patterson,B. (1988) *Annu. Rev. Genet.*, **22**, 387–419.
Hamm,J. and Mattaj,I.W. (1990) *Cell*, **63**, 109–118.
Hamm,J. and Mattaj,I.W. (1989) *EMBO J.*, **8**, 4179–4187.
Hamm,J. Dathan,N.A. and Mattaj,I.W. (1989) *Cell*, **59**, 159–169.
Hausner,T.-P., Giglio,L.M. and Weiner,A.M. (1990) *Genes Dev.*, **4**, 2146–2156.
Konarska,M.M. and Sharp,P.A. (1986) *Cell*, **46**, 845–855.
Konarska,M.M. and Sharp,P.A. (1987) *Cell*, **49**, 763–774.
Lamm,G.M., Blencowe,B.J., Sproat,B.S., Iribarren,A.M., Ryder,U. and Lamond,A.I. (1991) *Nucleic Acids Res.*, **19**, 3193–3198.
Lamond,A.I., Konarska,M.M., Grabowski,P.J. and Sharp,P.A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 411–415.
Legrain,P., Séraphin,B. and Rosbash,M. (1988) *Mol. Cell. Biol.*, **8**, 3755–3760.
Lerner,E.A., Lerner,M.R., Janeway,C.A. and Steitz,J.A. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2737–2741.
Lührmann,R., Kastner,B. and Bach,M. (1990) *Biochim. Biophys. Acta.*, **1087**, 265–292.
Madhani,H.D., Bordonné,R. and Guthrie,C. (1990) *Genes Dev.*, **4**, 2264–2277.
Mattaj,I.W. (1988) In Birnstiel,M.L. (ed.), *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles*. Springer Verlag, Berlin, pp. 100–114.
Newman,A. and Norman,C. (1991) *Cell*, **65**, 115–124.
Okano,Y. and Medsger,T.A. (1991) *J. Immunol.*, **146**, 535–542.
Pikielny,C.W., Rymond,B.C. and Rosbash,M. (1986) *Nature*, **324**, 341–345.
Ruby,S.W. and Abelson,J. (1988) *Science*, **242**, 1028–1035.
Ruby,S.W. and Abelson,J. (1991) *Trends Genet.*, **7**, 79–85.
Scherly,D., Boelens,W., van Venrooij,W.J., Dathan,N.A., Hamm,J. and Mattaj,I.W. (1989) *EMBO J.*, **8**, 4163–4170.
Séraphin,B. and Rosbash,M. (1989) *Cell*, **59**, 349–358.
Séraphin,B. and Rosbash,M. (1991) *EMBO J.*, **10**, 1209–1216.
Séraphin,B., Abovich,N. and Rosbash,M. (1991) *Nucleic Acids Res.*, **19**, 3857–3860.
Shannon,K.W. and Guthrie,C. (1991) *Genes Dev.*, **5**, 773–785.
Tani,T. and Ohshima,Y. (1989) *Nature*, **337**, 87–90.
Tani,T. and Ohshima,Y. (1991) *Genes Dev.*, **5**, 1022–1031.
Vankan,P., McGuigan,C. and Mattaj,I.W. (1990) *EMBO J.*, **9**, 3397–3404.
Wersig,C. and Bindereif,A. (1990) *Nucleic Acids Res.*, **18**, 6223–6229.
Wu,J. and Manley,J.L. (1991) *Nature*, **352**, 818–821.
Xu,Y., Peterson-Bjørn,S. and Friesen,J.D. (1990) *Mol. Cell. Biol.*, **10**, 1217–1225.
Zieve,G.W. and Sauterer,R.A. (1990) *CRC Crit. Rev. Biochem. Mol. Biol.*, **25**, 1–26.

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