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Domains of U4 and U6 snRNAs required for snRNP assembly and splicing complementation in *Xenopus* oocytes

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Structure-function relationships in the vertebrate U4-U6 snRNP have been analysed by assaying the ability of mutant RNAs to form U4-U6 snRNPs and to function in splicing complementation in *Xenopus* oocytes. The mutants define three categories of domain within the RNAs. First, domains which are not essential for splicing. These include regions of U6 which have previously been implicated in the capping and transport to the nucleus of U6 RNA as well as, less surprisingly, regions of U4 and U6 which have been poorly conserved in evolution. Second, domains whose mutation reduces U4-U6 snRNP assembly or stability. This group includes mutations in both the proposed U4-U6 interaction domain, and also, in the case of U6, in a highly conserved sequence flanking stem I of the interaction domain. These mutants are all defective in splicing. Third, regions not required for U4-U6 assembly, but required for splicing complementation. This category defines domains which are likely to be required for specific contacts with other components of the splicing machinery. Combinations of mutants in the U4 and U6 interaction domain are used to show that there are not only requirements for base complementarity but also for specific sequences in these regions.

Key words: RNA processing / snRNP assembly / U4 snRNA / U6 snRNA

Introduction

Splicing occurs in a multicomponent complex containing the pre-mRNA, snRNPs and protein factors. The snRNPs (small nuclear ribonucleoprotein particles) consist of snRNAs complexed with a set of proteins. Some proteins are common and others are specific to one snRNP (Lührmann, 1988; Reddy and Busch, 1988). Five of the snRNAs have been shown to be essential for pre-mRNA splicing. These are U1, U2, U4, U5 and U6. It has been shown that during spliceosome assembly U1 interacts with the 5′ splice site (Black *et al.*, 1985; Chabot *et al.*, 1985; Zhuang and Weiner, 1986) and U2 with the branch site (Black *et al.*, 1985; Parker *et al.*, 1987; Wu and Manley, 1989; Zhuang and Weiner, 1989). Interaction of U5 with the 3′ splice site has been suggested (Chabot *et al.*, 1985). U4 and U6 are found in one particle, the U4-U6 snRNP (Hashimoto and Steitz, 1984; Bringmann *et al.*, 1984). The U4-U6 snRNP can associate with U5 into a U4-U5-U6 complex independent of spliceosome assembly (Cheng and Abelson, 1987; Konarska and Sharp, 1987; Lossky *et al.*, 1987).

Although it has been demonstrated that U4 and U6 are essential for splicing (Berget and Robberson, 1986; Black and Steitz, 1986), no interaction of this snRNP with the pre-mRNA has been detected. The nature of the U4-U6 interaction is dynamic. The particle undergoes a conformational change, measurable as a weakening of the U4-U6 interaction, concomitant with the appearance of intermediates of the splicing reaction (Pikielny *et al.*, 1986, Cheng and Abelson, 1987, Lamond *et al.*, 1988; Blencowe *et al.*, 1989). U6 is the most conserved of the snRNAs (Guthrie and Patterson, 1988) suggesting that it plays a key role in the splicing reaction, and leading to the proposal that U6 may function as a ribozyme in the catalysis of the transesterification reactions (Brow and Guthrie, 1989; Guthrie, 1989).

The U6 sequence has been divided into four domains on the basis of phylogenetic conservation and the secondary structure model of the U4-U6 snRNAs (Figure 1A, see Brow and Guthrie, 1988). These are the variable 5′ domain, containing a hairpin structure and a short single-stranded region. This domain has been implicated in two functions. The hairpin and a stretch of six bases at the 5′ end of the single-stranded region seem to be required for the capping of U6 with a γ-methyl triphosphate cap (Singh *et al.*, 1990). The six base motif has also been shown to be required for the movement of microinjected U6 RNA from the cytoplasm to the nucleus in *Xenopus* oocytes (Hamm and Mattaj, 1989). The next region, moving 5′ to 3′, is referred to as the central domain. This part of the molecule is thought to be single-stranded on the basis of its sensitivity to RNase H degradation (Guthrie and Patterson, 1988). This region is followed by the interaction domain that forms two contiguous stems with U4 snRNA (Figure 1A). The last region is the 3′ single-stranded domain that is variable in sequence but constant in length in the various species examined to date. The 5′ end of this domain appears to be required for efficient entry of U6 into spliceosomes in HeLa cell nuclear extracts (Bindereif *et al.*, 1990).

The U4 sequence is not as strongly conserved. The ability to form basepairs with the U6 interaction domain is highly conserved, as is the loop of the hairpin lying between these two helical stems. Additionally, the 5′ half of the single-stranded region downstream of the interaction domain shows phylogenetic conservation. The Sm binding site, the region of the RNA required for interaction with the common U snRNP proteins, is also conserved. It has been reported that the yeast protein PRP4 requires the 5′ portion of U4 (Xu *et al.*, 1990) or the conserved hairpin (Bordonné *et al.*, 1990) for binding. No U4-U6 snRNP-specific proteins have so far been found in vertebrates, although a protein that copurifies with the abundant free U6 snRNP in germ cells has been identified in *Xenopus* (Hamm and Mattaj, 1989). *In vitro* studies mostly based on the exchange of mutant U6 RNAs into U4/U6 snRNPs, have indicated that the stems in the interaction domain may be required for U4/U6 snRNP

formation (Hamm and Mattaj, 1989; Pikielny *et al.*, 1989; Bindereif *et al.*, 1990). However, no data concerning the activity of these mutants in splicing is available.

To gain insight into the role of the U4–U6 snRNP in splicing, knowledge of the structural requirements for function is essential. We have therefore carried out a structural analysis of U4 and U6 mutants by splicing complementation and snRNP assembly assays in *Xenopus* oocytes. The results define three categories of domain within the two molecules. Domains which are non-essential, domains which are required for U4–U6 snRNP formation, and thus for splicing, and domains which are required for splicing but not for U4–U6 snRNP formation. This last category is the most interesting, since it defines regions of U4 and U6 potentially required for interactions with other components of the splicing apparatus including, perhaps, the substrate pre-mRNA.

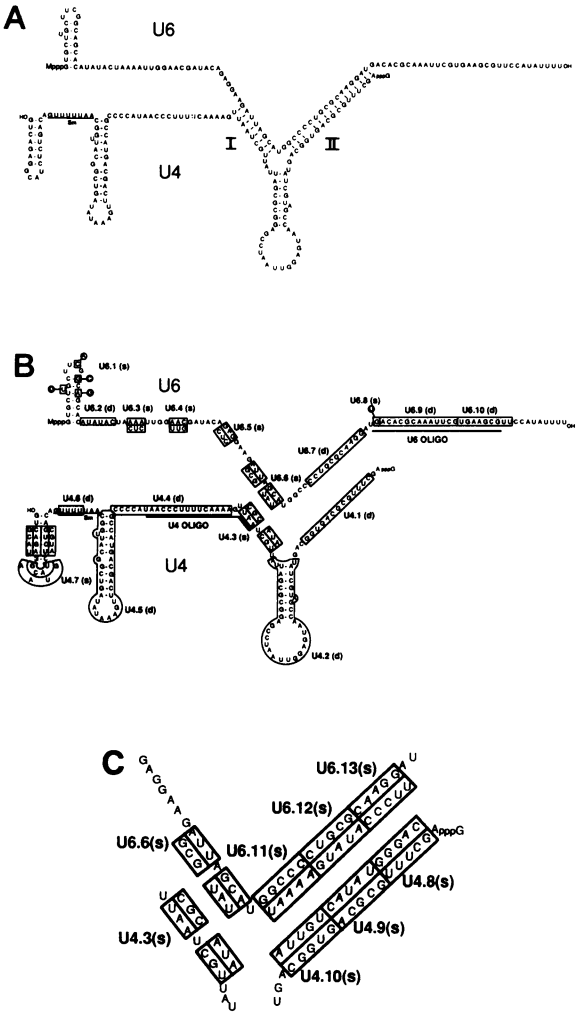

Fig. 1. Structures of wild-type and mutant U4 and U6 snRNAs. (**A**) Secondary structure model of U4 and U6 (Brow and Guthrie, 1988) Interaction stems I and II are indicated. (**B**) Mutations generated in U4 and U6. Boxes mark the position of mutants. Deletion mutants are indicated with (d), substitution mutants with (s). Bases substituted for the wild-type sequences are shown in boxes or circles next to the wild-type sequence. Sequences of U4 and U6 that are complementary to the oligodeoxyribonucleotides used for RNase H-mediated cleavage are indicated by lines. (**C**) Complementary mutations in interaction domains I and II. Note that U6.6(s) and U4.3(s) are both mutant in two groups of three nucleotides separated by an unmutated position.

Results

Splicing complementation with U4 and U6 mutants

U4 and U6 mutants were tested using an *in vivo* splicing complementation assay (Hamm *et al.*, 1989). Mutations in U4 were generated, using the Brow and Guthrie (1989) model as a guide, such that large single-stranded (ss) regions and complete hairpins were deleted or substituted scanning the complete molecule (Figure 1A and B). Deletions covered interaction stem II [U4.1(d)], the conserved hairpin between the interaction stems [U4.2(d)], the ss region 3′ of interaction stem I [U4.4(d)], the hairpin 5′ of the Sm binding site [U4.5(d)] and a stretch of four Us within the Sm binding site [U4.6(d)]. The 3′ hairpin was substituted with a hairpin of unrelated sequence, as deletion might have affected RNA stability [U4.7(s)]. Finally a mutant was tested in which six bases from interaction stem I were substituted [U4.3(s)].

The results of the splicing complementation assay are shown in Figure 2A. Microinjection of the pBSAdI precursor RNA (Konarska and Sharp, 1987) into control oocytes leads to the production of intron–lariat and spliced exon products (lane 1). Splicing is abolished by microinjection of an oligonucleotide complementary to part of U4 RNA (Figure 2, lane 2, the position of the oligonucleotide is shown in Figure 1B). The fact that the oligonucleotide results in the specific destruction of U4 RNA, and that the mutants are all transcribed and accumulated to similar extents, is shown in Figure 2B. Wild-type U4 (lane 10) and mutant U4.6(d) (lane 7), which is mutant in the Sm binding site and thus cannot re-enter the nucleus, serve as positive and negative controls for splicing complementation. The only mutants capable of efficiently complementing splicing are U4.5(d) and U4.7(s). This is in accordance with the observation that the hairpins altered in these two mutants are not conserved phylogenetically, whereas all other mutations affect regions that show strong structural or sequence conservation.

The mutations in U6 were also designed to scan the molecule for regions of importance (Figure 1B). This series contains two mutants that affect sequence elements reported to be required for capping (Singh *et al.*, 1990). These are U6.1(s), which affects the 5′ hairpin, and U6.2(d) which deletes the AUAUAC sequence 3′ of the hairpin. This latter sequence has also been shown to be required for transport of U6 RNA from the cytoplasm to the nucleus (Hamm and Mattaj, 1989). Mutant U6.6(s) was constructed such that it would restore basepairing in interaction stem I when used in combination with U4.3(s). In mutant U6.8(s) the conserved G_{76} is substituted by a U. This position has been reported to be important for function in a yeast splicing complementation assay (Fabrizio *et al.*, 1990). The results of the splicing complementation assays with the U6 mutants are shown in Figure 3A. All mutations 5′ of U6.4(s) complement splicing, whereas all mutations 3′ of U6.4(s), with the exception of the point mutant, U6.8(s), do not. Mutant U6.4(s) reproducibly has an intermediate effect (lane 11 and data not shown). All of the RNAs are transcribed and accumulate to a similar extent (Figure 3B).

U6 RNA remains in the nucleus after transcription

RNA polymerase II-transcribed snRNAs, following synthesis, are first exported to the cytoplasm and then re-imported into the nucleus. We had assumed that this would also be the case for U6 RNAs. However, mutant U6.2(d), which is unable to migrate to the nucleus from the cytoplasm,

restored splicing to the same level as U6 wild-type. To investigate the fate of this and other mutant RNAs after transcription, wild-type and mutant U6 genes were injected into the nucleus of *Xenopus* oocytes together with [α-^{32}P]GTP. After overnight incubation the nucleus and cytoplasm were separated and the labelled transcripts analysed on a polyacrylamide gel (Figure 4). U4 RNA, which was injected as a control, was found both in the nucleus and the cytoplasm (lanes 1 and 2) reflecting the dynamics of export and re-import of this RNA. All the U6 RNAs are found almost entirely in the nucleus (lanes 3, 5, 7, 9 and 11). In the case of U6.2(d), U6.6(s) and U6.7(d) a small quantity of RNA can be seen in the cytoplasmic fraction, possibly due to leakage from the nucleus. It is important to note that U6.2(d)RNA, unlike wild-type U6, cannot migrate from the cytoplasm to the nucleus (Hamm and Mattaj, 1989). If U6.2(d) left the nucleus it would therefore accumulate in the cytoplasm. Instead, like the other U6 transcripts, U6.2(d) is found almost entirely in the nucleus, showing that it never leaves this compartment. This result explains why U6.2(d) can be active in splicing complementation. Since U6 does not appear to leave the nucleus after transcription, the ability to re-accumulate in the nucleus is not required for function in the oocyte. The results suggest that U6 assembly with U4 snRNP takes place in the nucleus and are discussed further below.

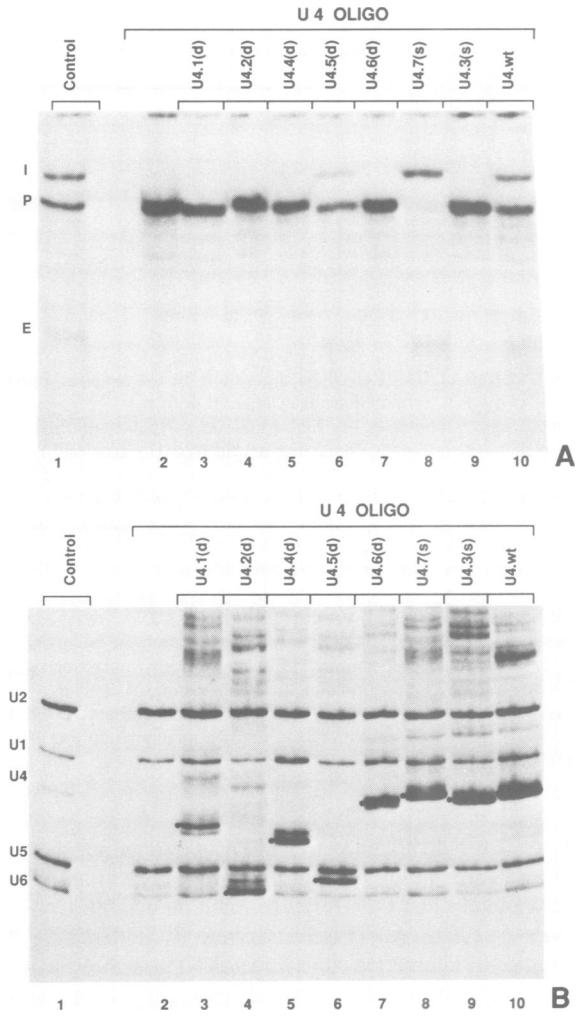

Fig. 2. Splicing complementation by U4 mutants. (**A**) Oocytes were injected with an oligodeoxyribonucleotide complementary to part of the U4 sequence together with a plasmid containing a wild-type or mutant U4 gene. After an overnight incubation a labelled adenovirus precursor mRNA was injected into the same oocytes. Control oocytes were not injected with the oligonucleotide. 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**) Total RNA prepared from the injected oocytes was separated by denaturing polyacrylamide gel electrophoresis, transferred to a nylon membrane, and hybridized with probes against U1, U2, U4, U5 and U6 to check destruction of endogenous U4, transcription of the microinjected U4 genes and to control that no other snRNAs were degraded. The positions of the various U4 transcripts are indicated by dots.

Many mutants affected in splicing complementation can form U4 – U6 snRNPs

The mutants that cannot complement splicing might either be affected in their ability to form U4–U6 snRNPs or in their interaction with other components of the spliceosome.

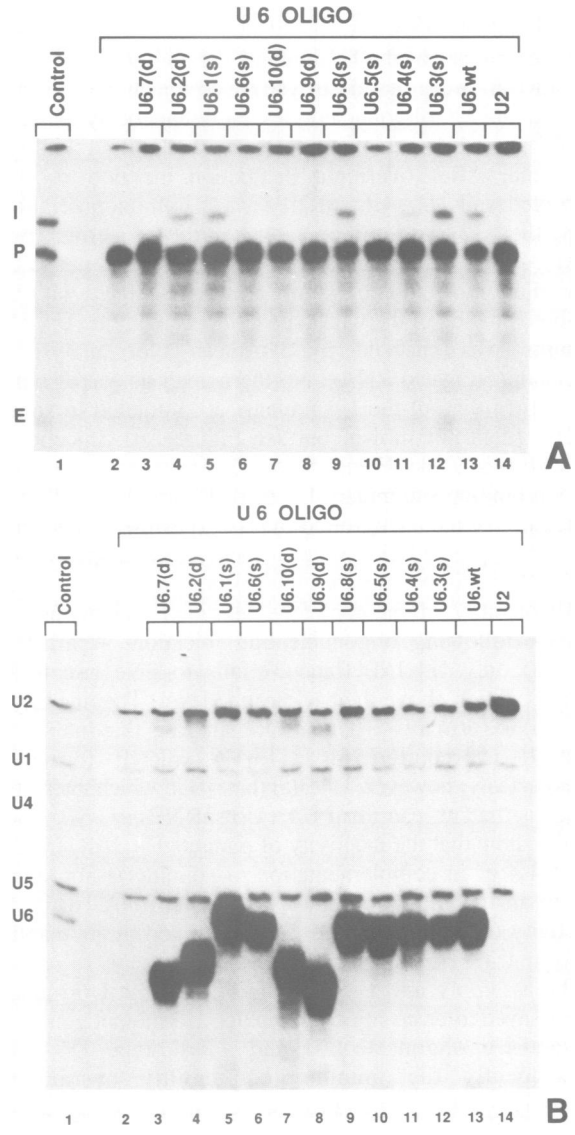

Fig. 3. Splicing complementation by U6 mutants. Analysis of splicing complementation (**A**) and transcript accumulation (**B**) of the U6 snRNA mutants. Treatments and lettering as for the U4 mutants (see Figure 2). A plasmid containing a wild-type U2 gene was injected as an additional negative control (lane 14).

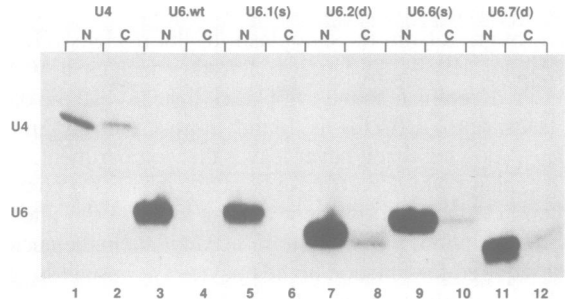

Fig. 4. Analysis of the intracellular distribution of U6 snRNAs. Wild-type U6, four different mutant U6 genes (see Figure 1B) and a wild-type U4 gene were injected into the nucleus of oocytes together with [α-^{32}P]GTP. After an overnight incubation cytoplasmic and nuclear fractions of the oocytes were separated into nuclear and cytoplasmic fractions and the U6 transcripts were analysed directly on a denaturing polyacrylamide gel. N, nuclear fraction; C, cytoplasmic fraction.

To differentiate between these possibilities we tested whether the U4 and U6 mutants were able to form U4–U6 snRNPs. For the analysis of the U4 mutants the endogenous U4 and U6 snRNAs were first destroyed by co-injection of the anti-U4 and anti-U6 oligonucleotides. Simultaneously genes for the different U4 mutants were co-injected with a wild-type U6 gene. After overnight incubation the oocytes were disrupted and U4–U6 snRNPs were immunoprecipitated using the Y12 anti-Sm monoclonal antibody (Lerner *et al.*, 1981). As the Sm proteins are bound to U4 and not to U6, precipitation of U6 occurs only if it is associated with U4. Co-precipitation of the wild-type U6 RNA with the U4 RNA mutants was checked by Northern blot analysis of immunoprecipitated RNAs. Northern analysis was also used to confirm that all the U4 mutants accumulated to similar extents (data not shown, but see Figure 2B). Injection of U6 wild-type alone (Figure 5, lane 2) or in combination with the Sm binding site mutant U4.6(d) (Figure 5, lane 8) gave a measure of the background in this experiment. The small amount of precipitation seen in these lanes is specific (cf. lane 11) and is presumably due to the presence of a very small quantity of incompletely degraded endogenous U4 snRNA following oligonucleotide injection. Apart from U4.6(d) only U4.1(d) (lane 3) fails to give rise to U6 coprecipitation above the background level. The inability of U4.1(d) to form a U4–U6 snRNP suggests that interaction stem II is required for U4–U6 snRNP formation. Unexpectedly, however, U4.3(s) (lane 4) in which interaction stem I is mutant, can form U4–U6 snRNPs *in vivo*. These results show that the inactivity of mutants U4.2(d), U4.3(s) and U4.4(d) in complementation of splicing is not simply due to an inability to interact with U6 snRNA, while the inactivity of U4.1(d) can be fully explained by its inability to form U4–U6 snRNPs.

The assembly into U4–U6 snRNPs of the U6 mutants was assayed after co-injection of each U6 mutant gene with the anti-U6 oligonucleotide and a wild-type U4 gene. Subsequently the immunoprecipitability by anti-Sm antibodies of the U6 mutants was tested. The U4 gene was co-injected because the partially degraded endogenous U6 remains bound to endogenous U4, as judged by Sm-immunoprecipitability (Figure 6, lanes 1–13), and thereby lowers the efficiency of assembly of the newly transcribed U6 into U4–U6 snRNPs (data not shown). To control that the efficiency of immunoprecipitation was similar in all lanes

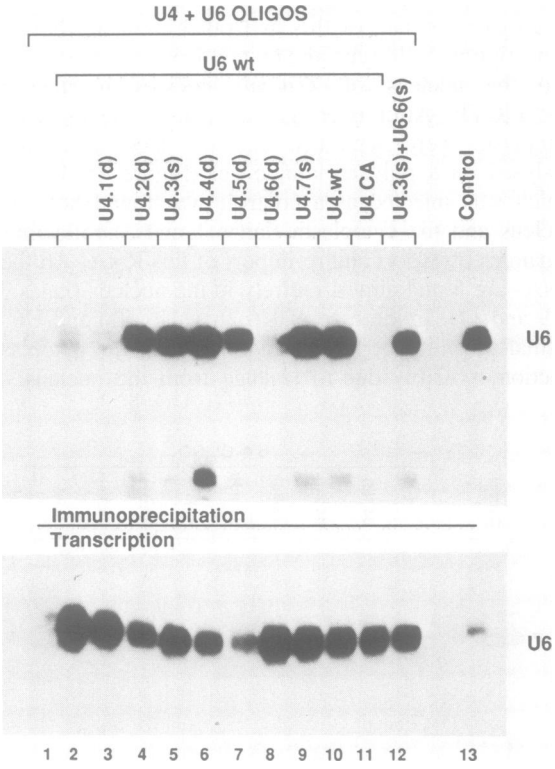

Fig. 5. Analysis of U4–U6 snRNP formation by U4 mutants. Fifteen *Xenopus* oocytes were simultaneously injected with two oligonucleotides complementary to U4 and U6 snRNAs (see Figure 1B) together with plasmids containing a wild-type U6 gene and a mutant U4 gene as indicated. After overnight incubation the oocytes were disrupted and analysed as follows. **Lower panel:** transcription. From an equivalent of five oocytes total RNA was prepared. The destruction of endogenous U6 RNA (lane 1) and transcription from the injected genes was analysed in a Northern blot of a fraction of this RNA by hybridization using an antisense RNA U6 probe. **Upper panel:** immunoprecipitation. From the remaining 10 oocyte equivalents snRNAs were immunoprecipitated using the Y12 anti-Sm monoclonal antibody. The immunoprecipitate was analysed by hybridization with a U6 probe. U4.CA, immunoprecipitation with a control antibody; U4.3(s)+U6.6(s), coinjection of the complementary mutants U4.3(s) and U6.6(s) (Figure 1B); control lane, immunoprecipitation and U6 accumulation in non-injected oocytes.

the filter was hybridized with a U4 probe (data not shown). The results are shown in Figure 6. The only mutant which had completely lost the ability to form detectable U4–U6 snRNPs was U6.7(d) (Figure 6, lane 3) underlining the importance of interaction stem II in snRNP assembly *in vivo*. In contrast to the results obtained with the U4 interaction stem I mutant, two mutants, U6.5(s) and U6.6(s) (Figure 6, lanes 10 and 6) which are mutant in regions adjacent to and within interaction stem I respectively, both showed strongly reduced immunoprecipitability (varying between 10 and 20% of wild-type in different experiments), indicating that they caused a reduction either in the formation or the stability of the U4–U6 snRNP. These results suggest that the failure of mutants U6.5(s) and U6.6(s) to complement is likely to be a result of their effects on the assembly, structure or stability of U4–U6 snRNPs, whereas U6.7(d) cannot complement because it is unable to enter the U4–U6 snRNP. The defective complementation of U6.9(d) and U6.10(d) is, on the other hand, not due to the inability to form stable U4–U6 snRNPs.

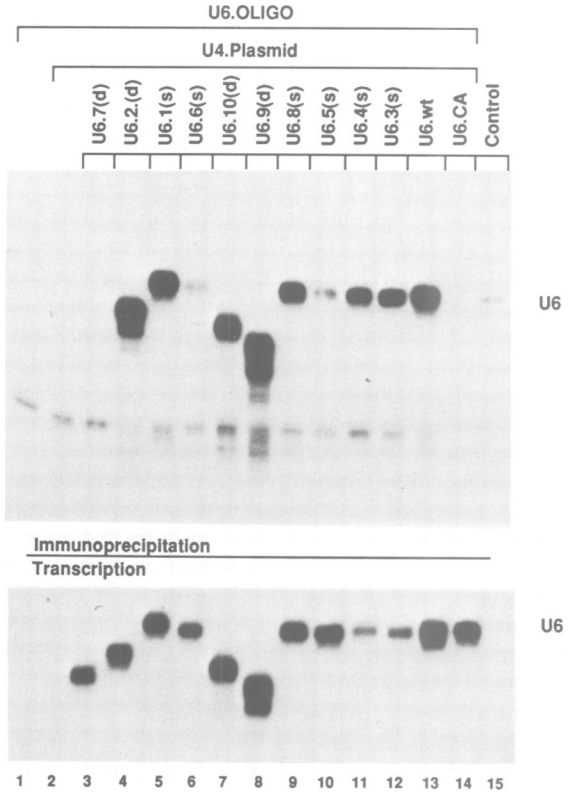

Fig. 6. Analysis of U4 – U6 snRNP formation by U6 mutants. Endogenous U6 was destroyed by injecting an oligodeoxynucleotide complementary to part of the U6 sequence (see Figure 1B). Simultaneously, a wild-type U4 gene in combination with each different U6 mutant gene was microinjected, as indicated above the lanes. After an overnight incubation 15 oocytes were disrupted and analysed as follows. **Lower panel:** transcription. From an equivalent of five oocytes total RNA was prepared. The destruction of endogenous U6 RNA and the transcription from the injected genes was analysed in a Northern blot of a fraction of this RNA by hybridization with a U6 probe. **Upper panel:** immunoprecipitation. From the remaining material snRNAs were precipitated using the Y12 anti-Sm monoclonal antibody. The immunoprecipitate was analysed by hybridization with a U6 probe. U6.CA, immunoprecipitation with a control antibody; control lane, immunoprecipitation and U6 accumulation in non-injected oocytes.

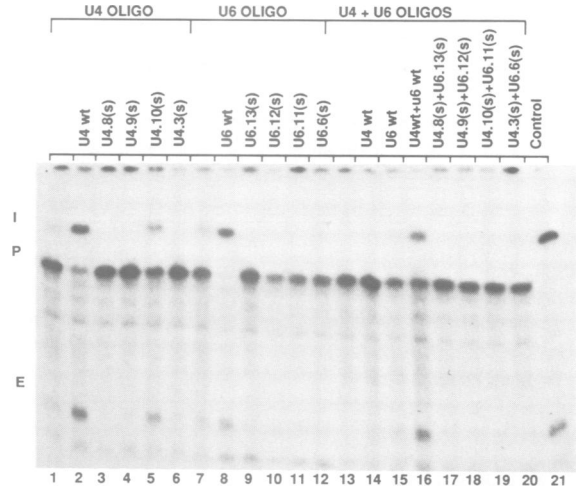

Fig. 7. Splicing complementation by complementary mutants. The mutants analysed were in interaction stems I and II (see Figure 1C). **Lanes 2–6:** splicing complementation by wild-type and mutant U4 genes, after destruction of endogenous U4. **Lanes 8–12:** splicing complementation by wild-type and mutant U6 genes, after destruction of endogenous U6. **Lanes 16–20**: splicing complementation by complementary wild-type and mutant combinations, after destruction of both endogenous U4 and U6. Control: splicing of pre-mRNA in non-treated oocytes.

Complementary mutations in the interaction domain restore U4 – U6 snRNP assembly but not splicing

We next investigated whether substitution mutations in interaction stem II would prevent splicing complementation and whether the defects in splicing caused by mutating the interaction stems could be overcome by co-injecting U4 and U6 genes with complementary mutations. In this way we hoped to determine whether the interaction domain was only required for snRNP assembly or whether function in splicing required the presence of particular sequences within the interaction stems. The mutants tested are shown in Figure 1C. The results of the splicing complementation assay are shown in Figure 7. As shown in lanes 1 – 12 none of the mutants, when injected alone, is capable of efficient complementation. Mutant U4.10(s) restored splicing to a reduced level (lane 5). In this particular experiment, the U6 oligonucleotide failed to inhibit splicing completely (Figure 7, lane 7). None of the U6 mutants (lanes 9 – 12) raised splicing efficiency above this background level. To demonstrate that both U4 and U6 snRNAs could be destroyed and subsequently complemented by transcripts of injected genes the experiment shown in lanes 13 – 16 was carried out. Following co-injection of anti-U4 and anti-U6 oligonucleotides no splicing was seen (Figure 7, lane 13). This inactivity cannot be complemented by injection of U4 or U6 DNA alone (lanes 14 and 15) but is complemented by injection of both DNAs together (lane 16). None of the combinations of complementary mutations is, however, capable of restoring splicing (lanes 16 – 20).

To determine whether the mutants were able to form U4 – U6 snRNPs, immunoprecipitation was tested in combination with either a wild-type counterpart or with the complementary mutant. The results are shown in Figure 8. Lanes 1 – 3 are the negative and positive controls for the analysis and show the effect of oligonucleotide injection either alone, together with U4 wild-type DNA, or together with both U4 wild-type and U6 wild-type DNA. None of the U6 mutants with alterations in interaction stem II were efficiently coprecipitated with wild-type U4 (lanes 4 – 6) although mutant U6.13(s) showed a very low level of U4 – U6 snRNP formation. U6.6(s), which is mutated in interaction stem I, showed ~20% of the wild-type level (Figure 8, lane 7, see also Figure 6, lane 6). When the U6 mutants were tested in combination with the complementary U4 mutants immunoprecipitability was restored (lanes 8 – 12), indicating that the potential to form basepairs in the interaction stems enhances U4 – U6 snRNP formation and/or stability. The particles formed are, however, non-functional, as shown by the results of splicing complementation (Figure 7). The coprecipitation of wild-type U6 with the U4 mutants is shown in lanes 13 – 16. There is a low background of Sm-precipitable U6 in the control (Figure 8, lane 12). The amount of coprecipitated U6 was slightly above this level for mutants U4.8(s) and U4.10(s) (lanes 13 and 15). This was expected in the case of U4.10(s), as this mutant was capable of complementing splicing at a low level (Figure 7, lane 5). Mutant U4.3(s), in which interaction stem I is altered, efficiently formed U4 – U6 snRNPs with wild-type

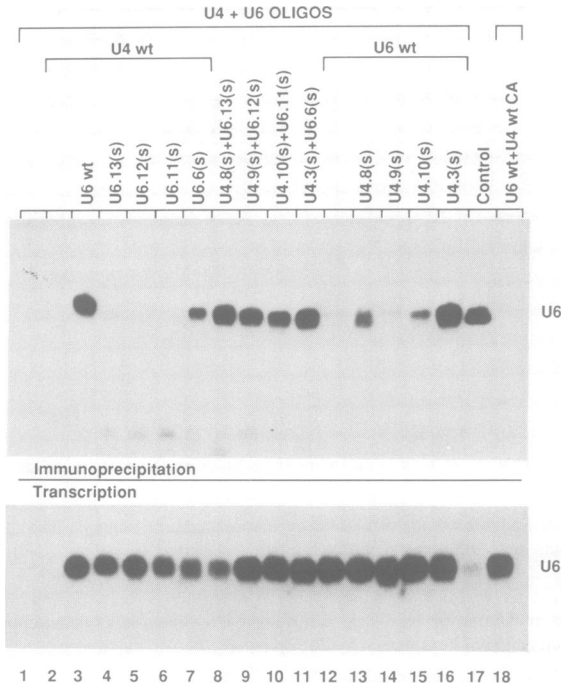

Fig. 8. U4–U6 snRNP immunoprecipitation with the complementary mutants. Transcription and immunoprecipitation by the Y12 anti-Sm monoclonal antibody were analysed as in Figures 5 and 6. **Lane 1:** RNA from oocytes injected with oligonucleotides alone. **Lane 2:** oocytes injected with wild-type U4 genes. **Lanes 3–7:** U4–U6 snRNP formation of U6 mutant genes co-injected with a wild-type U4 gene. **Lanes 8–11:** U4–U6 snRNP formation of U6 mutant genes co-injected with the complementary U4 mutants. **Lanes 13–16:** U4–U6 snRNP formation of mutant U4 genes co-injected with a wild-type U6 gene. Control: U6 RNA accumulation and immunoprecipitation in non-injected oocytes. U6 wt + U4 wt CA: transcription and immunoprecipitation with a control antibody from oocytes injected with wild-type U4 and U6 genes.

U6 (compare lanes 3 and 16) confirming the previous result (Figure 5, lane 5). Thus, mutations in both strands of interaction stem II have severe effects on U4–U6 immunoprecipitability which can be overcome by complementary mutation. Mutations in interaction stem I have an asymmetric effect, reducing immunoprecipitability when made in U6 but not in U4. In this case the deficiency of the U6 mutant can also be alleviated by the complementary mutation in U4. These results provide strong experimental support for the model of U4–U6 interaction proposed by Brow and Guthrie (1988). It is not possible to tell whether the mutation-induced defects in immunoprecipitability are due to reduced assembly of U4–U6 snRNPs, or to reduced stability of the snRNPs once formed. It is important to note that none of the complementary mutations are capable of restoring splicing, indicating that for activity in splicing not only snRNP assembly, but also particular sequences within the interaction stems, are necessary.

Discussion

It had previously been established that U4 and U6 are essential for splicing (Berget and Robberson, 1986; Black and Steitz, 1986; Brow and Guthrie, 1988). A first step on the way to answering the question of how they function is to define which domains of the U4–U6 snRNP are essential for splicing and then to determine which regions are required for U4–U6 snRNP assembly and what may be needed for interaction with other components of the spliceosome. We have begun this task by analysing a series of U4 and U6 mutants in *Xenopus* oocytes. The mutant phenotypes have been divided into three groups for the purpose of discussion.

Mutations which do not affect splicing

Neither of the 3′ hairpins flanking the Sm binding site in U4 snRNA are essential. The 3′-most hairpin can be replaced by a hairpin of unrelated sequence, whereas the second hairpin can be completely deleted without affecting either splicing or U4–U6 snRNP formation. These results are not totally unexpected, since these regions of U4 have not been conserved in evolution (Guthrie and Patterson, 1988). Bordonné *et al.* (1990) have reported that a yeast U4 mutant in which the 3′-most hairpin has been replaced by the corresponding hairpin from trypanosome U4 snRNA cannot support yeast growth. Similarly, mutation of the invariant G_{76} of U6 snRNA had no effect in oocytes, whereas this position has been shown to be important in a yeast *in vitro* splicing complementation system (Fabrizio *et al.*, 1990) and conversely, deletions in the single-stranded 3′ domain of U6 inactivated splicing in oocytes while having a less dramatic effect in the yeast *in vitro* system.

All of these results may reflect differences in the detailed mechanism of splicing between *Xenopus* and *Saccharomyces cerevisiae*. However, many other explanations of the differences are also possible. In both our experiments and those of Fabrizio *et al.*, the effect of snRNA mutation on single introns was tested. It is possible that different introns may turn out to have different requirements. In addition, differential effects of mutations which result in the substitution of analogous regions of yeast and vertebrate RNAs may be explicable by the effects of the two different substituted sequences on interactions with other components of the splicing machinery. Some substitutions may allow interactions to take place, when others do not. Further analysis will be required to elucidate the basis of the observed differences.

Mutations near the 5′ end of U6 do not interfere with splicing complementation activity. Interestingly, these mutants are affected in sequences which have been reported to be required for γ-methylation of the 5′ triphosphate of U6 RNA in HeLa cell extracts (Singh *et al.*, 1990). This might indicate that in *Xenopus* oocytes γ-methylation of U6 is not required for splicing. However, it will be necessary to demonstrate directly that these mutations affect U6 methylation in oocytes before accepting this conclusion. Mutant U6.2 transcripts are incapable of migration to the nucleus when microinjected into the cytoplasm of oocytes (Hamm and Mattaj, 1989) but are active in splicing complementation when the mutant gene is injected into the nucleus. This apparent contradiction was resolved by the demonstration that U6 transcripts do not leave the nucleus (Figure 4). This result has important implications for U4–U6 snRNP assembly. U4, like other polymerase II-transcribed snRNAs, leaves the nucleus after transcription and associates with snRNP proteins in the cytoplasm before re-entry into the nucleus (Mattaj, 1988). U6 assembly with the U4 snRNP must, however, take place in the nucleus. This means that if there is such a thing as a U4–U6 assembly/disassembly cycle during splicing (see Introduction), then the whole process can take place without requirement for cycling of the RNAs between the nucleus and cytoplasm. Although the

nuclear targeting sequences of U6 are non-essential in oocytes they may nevertheless play an important role in dividing cells, to allow re-accumulation of U6 in the nucleus after mitosis.

Mutants affected both in U4–U6 snRNP formation and in splicing

All of the U4 and U6 mutants in stem II of the interaction domain fell into this category. Both deletion and substitution mutations in this region had strong effects on both U4–U6 assembly and/or stability and on splicing complementation. When the complementary U4 and U6 substitution mutants were tested in pairs, the defect in U4–U6 assembly was apparently corrected, as judged by the efficiency of coprecipitation of U6 with U4 by anti-Sm antibodies. Interestingly, however, the inability of the mutants to complement splicing was not affected. These results show that the ability to form a base-paired helix in interaction stem II is not sufficient for function in splicing. There are sequence requirements that must also be fulfilled. This could reflect specific binding to these regions of U4–U6 snRNP proteins (e.g. the homologue of the yeast PRP4 protein, Bordonné *et al.*, 1990; Xu *et al.*, 1990). The proteins may in turn be required either to allow interactions with other components of the splicing machinery or for the apparent destabilization of the U4–U6 snRNP during splicing.

The mutants in interaction stem I produced unexpected results. It has previously been reported (Hamm *et al.*, 1989; Bindereif *et al.*, 1990) that the U6 strand of interaction stem I is required for exchange of U6 into U4–U6 snRNPs in HeLa cell extracts. However, our data show that only the U6 strand of the helix is required for normal U4–U6 assembly and/or stability, since simultaneous mutation in six of the eight bases in the U4 strand had no effect on U4–U6 snRNP immunoprecipitability, while mutation of the U6 strand reduced immunoprecipitability by ~80%. A similar assymetric effect was observed by Blencowe *et al.* (1989) in experiments in which U4–U6 snRNPs were probed with biotinylated oligoribonucleotides. Oligonucleotides hybridizing to stem I of U6 seemed to split the U4–U6 particle, whereas oligonucleotides hybridizing to the complementary U4 region did not. Second, the region of U6 required for U4–U6 snRNP immunoprecipitability does not correspond to the base-paired nucleotides since U6.5(s), which is mutated in the highly conserved ACACAG sequence upstream of the interaction stem, is as defective as the stem mutant in U4–U6 assembly/stability. These results imply the existence of a factor which interacts with the conserved ACACAG sequence, and possibly also the interaction stem, in whose absence U4–U6 snRNP assembly or stability does not attain wild-type levels. The existence of such a sequence-specific U6 binding protein could explain the conservation of these regions of U6 RNA. An alternative general explanation for the high conservation of U6, that it acts in the catalytic centre of the spliceosome (Brow and Guthrie, 1989) is extremely attractive. However, if applied to the sequences mutated in U6.5(s) and U6.6(s), that model would not predict, nor provide a ready explanation for, the observed reduction in U4–U6 snRNP assembly and/or stability.

Mutants affected in splicing but not in U4–U6 snRNP formation

This category, which includes the mutants in the interaction domains whose inability to function in splicing is not rescued by complementary changes, as discussed above, is potentially the most interesting, as it is likely to define regions of the U4 and U6 RNAs required for interaction with other components of the splicing machinery. It is very likely that some of these mutants, e.g. U4.3(s), will form snRNPs whose structures differ from that of the normal U4–U6 snRNP. Nevertheless, the positions of many of these mutations in the RNAs, and the evolutionary conservation of the sequences affected, argue in favour of the idea that the deletions or substitutions have affected regions required for *trans*-interactions. The region deleted in U6.9(d), for example, includes U6 sequences previously shown to affect the efficiency of U6 assembly into splicing complexes (Bindereif *et al.*, 1990). The conserved 5′ hairpin of U4, deleted in U4.2(d) is required in yeast for binding the PRP4 protein (Bordonné *et al.*, 1990). In *Xenopus*, or other vertebrate systems, no PRP4 homologue has so far been identified. Our results suggest that a search for this protein should be undertaken.

Two obvious steps which the mutants in this category might affect are the assembly of either the U4/5/6 particle or the spliceosome. We have thus far failed to detect a U4/5/6 particle in oocytes and, for technical reasons, experiments to assay the effects of the mutants on splicing complex formation are extremely difficult. Nevertheless establishing assays for these two processes will be the next step towards explaining and defining the defects in the mutants and thus the role of the wild-type U4 and U6 snRNAs in the splicing process.

Materials and methods

Oocyte microinjection

Microinjection was carried out as described previously (Hamm *et al.*, 1989) Oocytes were injected into the nucleus with oligonucleotides complementary to either U4 snRNA (at 800 μM) or U6 snRNA (at 300 μM) separately or in combination. The genes injected were wild-type or mutant versions of chicken U4 (Hoffmann *et al.*, 1986) and *Xenopus* U6 (Krol *et al.*, 1987). The concentration of each DNA injected was 250 μg/ml except where both U4 and U6 DNA were co-injected when the U6 concentration was reduced to 125 μg/ml. After overnight incubation, *in vitro*-generated T3 transcripts of pBSAd1 (Konarska and Sharp, 1987) were microinjected into the nucleus. 10–30 ng of RNA was injected per oocyte. After 90 min of further incubation oocytes were homogenized in homomedium (50 mM Tris–HCl pH 7.4, 5 mM EDTA, 1.5% SDS, 300 mM NaCl and 1.5 mg/ml Proteinase K). Protein was extracted with two phenol-chloroform extractions and RNA was precipitated with 3 vol of ethanol. To analyse splicing RNA equivalent to 0.5 oocyte was separated on a 10% denaturing polyacrylamide gel (acrylamide:bisacrylamide ratio of 40:1). Due to some variation in the efficiency of RNase H-mediated cleavage of the endogenous U snRNAs and of splicing efficiency, each mutant was tested at least three times in the splicing complementation assay. Consistent results were always obtained in repeat experiments. To analyse transcription 0.5 oocyte equivalents of RNA were separated on an 8% denaturing gel, transferred to a genescreen membrane (electrotransfer in 27 mM citric acid, 34 mM Na_2HPO_4; 300 mA for 1 h at 4°C) and hybridized with a mixture of radioactively labelled antisense RNA probes against U1, U2, U4, U5 and U6. Prehybridization was carried out for 1 h at 42°C (50% formamide, 5 × SSC, 1% SDS, 1 × Denhardt's solution, 50 mM Na_2HPO_4) Hybridization was carried out for 12–16 h at 42°C (50% formamide, 5 × SSC, 1% SDS, 1 × Denhardt's solution, 50 mM Na_2HPO_4). Washing was done twice in 2 × SSC, 0.1% SDS for 30 min at 65°C. Subsequently the membranes were autoradiographed at −80°C.

In vitro RNA synthesis

RNA synthesis was carried out as described previously (Hamm *et al.*, 1989).

Immunoprecipitation of U4–U6 snRNPs with Y12 antibody

Oligonucleotides and plasmids were injected into the nucleus of oocytes as described above (oocyte microinjection). After overnight incubation 15 oocytes were homogenized in 1.5 ml of oocyte extraction buffer (10 mM

Tris–HCl pH 8.0, 150 mM NaCl and 40 U RNasin/ml). One third of this mixture was extracted twice with phenol-chloroform. The RNA was precipitated with 3 vol of ethanol. RNA from 0.5 oocyte was separated on an 8% denaturing acrylamide gel, transferred to genescreen membrane and hybridized as described above. The remaining homogenate of 10 oocytes was spun in an Eppendorf centrifuge for 10 min to pellet the pigment and yolk from the oocytes. To 1 ml of the cleared supernatant 10 μl of 10% NP-40 was added. This solution was mixed with 40 μl protein A–SepharoseCL-4B beads to which 20 μl of Y12 anti-Sm monoclonal antibody (Lerner *et al.*, 1981) had been bound during an overnight incubation at 4°C in IPP 500 (10 mM Tris–HCl pH 8.0, 500 mM NaCl, 0.1% NP-40, 0.1% sodium azide). After revolving for 2 h, the beads were washed four times for 10 min with 1 ml of IPP500. The RNA was released from the beads by digestion in 400 μl homomedium (see above) at 37°C for 30 min. The beads were pelleted, the supernatant was extracted with phenol-chloroform and the RNA was precipitated with 3 vol of ethanol. The total precipitate from 10 oocytes was separated on an 8% denaturing polyacrylamide gel and transferred to a genescreen membrane. The filter was probed with a U6 and subsequently, to control the efficiency of precipitation, with a U4 probe.

Mutagenesis

The U4 and U6 genes were cloned into pBS+ for generating single-stranded plasmid DNA. Mutations were introduced using an Amersham Mutagenesis kit.

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