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Research Article

# Two Domains of Yeast U6 Small Nuclear RNA Required for Both Steps of Nuclear Precursor Messenger RNA Splicing

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**U6 is one of the five small nuclear RNA's (snRNA's) that are required for splicing of nuclear precursor messenger RNA (pre-mRNA). The size and sequence of U6 RNA are conserved among organisms as diverse as yeast and man, and so it has been proposed that U6 RNA functions as a catalytic element in splicing. A procedure for in vitro reconstitution of functional yeast U6 small nuclear ribonucleoproteins (snRNP's) with synthetic U6 RNA was applied in an attempt to elucidate the function of yeast U6 RNA. Two domains in U6 RNA were identified, each of which is required for in vitro splicing. Single nucleotide substitutions in these two domains block splicing either at the first or the second step. Invariably, U6 RNA mutants that block the first step of splicing do not enter the spliceosome. On the other hand, those that block the second step of splicing form a spliceosome but block cleavage at the 3′ splice site of the intron. In both domains, the positions of base changes that block the second step of splicing correspond exactly to the site of insertion of pre-mRNA–type introns into the U6 gene of two yeast species, providing a possible explanation for the mechanism of how these introns originated and adding further evidence for the proposed catalytic role of U6 RNA.**

PRECURSOR MESSENGER RNA (PRE-MRNA) SPLICING IN the nucleus takes place on a large multicomponent particle, termed the spliceosome (*1*). The function of the various components or trans-acting factors in the spliceosome is to fold the intron into a splicing substrate and to catalyze the reaction. The trans-acting factors include a large number of different proteins (perhaps more than 30) and five small nuclear RNA's (snRNA's)—U1, U2, U4, U5, and U6. The snRNA's associate with a set of seven common core proteins, in addition to other proteins occurring only with certain of the snRNA's, to form the small nuclear ribonucleoproteins (snRNP's) (*1, 2*).

Pre-mRNA splicing is a two-step process. In the first step, the 5′ splice site is cleaved and, in a coordinated reaction, the 5′ G residue of the intron is linked in a 2′,5′-phosphodiester linkage to an A residue near the 3′ splice junction. In the second step, the 3′ splice site is cleaved, the exons are joined, and the intron is released as a "lariat." Hydrolysis of adenosine triphosphate (ATP) is required for this process in vitro (*3*).

The group I and group II self-splicing introns are found in mitochondria and chloroplast genes, and group I introns have been extensively studied in a *Tetrahymena* ribosomal RNA gene (*4*). The splicing mechanism for these introns also consists of two phosphotransfer reactions. The group II self-splicing reaction mechanism is, in fact, identical to that for pre-mRNA's, which suggests a common evolutionary origin for the two processes (*5*). Although pre-mRNA splicing and group II self-splicing proceed by the same reaction pathway, the two reactions have distinct biochemical requirements. Group II self-splicing proceeds with no added factors, whereas a myriad of trans-acting factors, as well as hydrolysis of ATP, are required for pre-mRNA splicing. The group II intron itself is the catalyst of the self-splicing reaction and, as such, has a conserved and complex secondary and tertiary structure. By contrast, introns in the nucleus have the bare minimum of information at 5′ and 3′ splice sites required for identification of these sites by the trans-acting factors, and intron sequences appear to have no conserved secondary or tertiary structure (*3*). If these two processes have a common evolutionary origin, then we are led to the hypothesis that pre-mRNA splicing, despite its requirements for protein and ATP, is an RNA-catalyzed reaction. If this is so, then the catalyst for pre-mRNA splicing must be one or more of the snRNA's. In this view, each of the snRNA's can be thought of as a group II intron in pieces, and the spliceosome as a way of reassembling the intron from its parts (*6*).

Unequivocal evidence establishing a catalytic role in splicing for any of the snRNA's is lacking. Functional roles have been established for the U1 and U2 snRNA's. Namely, a sequence at the 5′ end of U1 RNA forms base pairs with the 5′ splice site and a sequence near the 5′ end of U2 RNA forms base pairs with the branchpoint sequence near the 3′ splice site (*1*). These interactions

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serve to identify two important regions of the intron. It is possible, however, that U1 or U2 RNA could have a further role as the catalyst. Base-pairing interactions of U4, U5, or U6 RNA with the intron have not been identified. U4 and U6 RNA's, however, form base pairs with each other and are found in the same snRNP (*1*). Disruption of the extensive U4-U6 base-pairing interaction destabilizes the association of U4 with U6 and precedes the catalytic activation of the spliceosome (*7, 8*).

U6 RNA has properties that suggest it may have a catalytic function in pre-mRNA splicing. Its size and sequence are the most conserved of any of the snRNA's (*9*). The presence of an intron in a highly conserved region of the *Schizosaccharomyces pombe* U6 RNA gene (*10*) led to the hypothesis that U6 RNA functions as a catalytic element with U4 RNA being its negative antisense regulator (*11*).

We have developed a procedure for in vitro reconstitution of functional yeast U6 snRNP's (*12*). In this procedure, U6 RNA in an active splicing extract is destroyed by incubation of the extract with a deoxyoligonucleotide complementary to U6 RNA. The DNA-RNA hybrid is cleaved by endogenous ribonuclease H (RNase H) in the extract. During further incubation, the deoxyoligonucleotide itself is degraded by endogenous deoxyribonuclease (DNase) in the extract. This makes it possible to test the activity of synthetic U6 RNA added back to the extract. We showed that synthetic U6 RNA is incorporated into the U4-U6 snRNP and, in the presence of pre-mRNA, into spliceosomes (*12*). The exogenous U6 RNA efficiently restores splicing activity to the extract. This procedure allowed us to undertake a detailed investigation of the structural basis of U6 RNA function. We have now constructed a set of point mutations in highly conserved sequences in the synthetic U6 RNA and have identified two domains at which single nucleotide substitutions or deletions affect splicing. Certain of these point mutants block splicing completely. Others allow the first step of splicing to occur but block the second step.

**Mutagenesis of the central domain of U6 RNA.** We initially focused on the central domain of U6 RNA [nucleotides (nt) 28 to 54] (Fig. 1). This region does not appear to have any secondary structure (it can pair with exogenous oligonucleotides and is the target for inactivation by RNase H in our procedure). The central domain is not paired with U4, thus the nucleotides in this region do not have the possible dual roles during assembly and splicing that those in the U4-U6 stems might have (*9*). Of the 27 nucleotides in this region, 13 are conserved in those organisms from yeast to man in which the U6 sequence is known. In particular, the sequence ACAGAGA (nt 47 to 53) is conserved (*9*).

In order to synthesize U6 RNA in vitro with bacteriophage T7 RNA polymerase, we had constructed a synthetic U6 RNA gene abutted to a T7 promoter (*12, 13*). To construct sequence variants, we resynthesized the gene with a degenerate synthetic oligonucleotide. Each synthetic step corresponding to positions 34 to 53 in U6 RNA was performed in the presence of mutagenic concentrations of the other three phosphoramidites (*14*). Random clones (100) arising from this synthesis were sequenced. A total of 37 point mutations was found, 23 of which occurred only once. Three single and two multiple deletions were also isolated. RNA from each of the mutant genes was synthesized and tested for activity in the complementation assay (*15*). The results are summarized in Fig. 1. Many of the mutations in conserved sequences (A34, U37, G39, A41, and A42) had no effect on splicing. However, all but one of the changes in the sequence ACAGA (nt 47 to 51) affected splicing. We therefore focused on this region and prepared synthetic oligonucleotides that allowed us to isolate all possible single variants in this region (*16*). All of these were assayed (Figs. 1 and 2A). Changes in

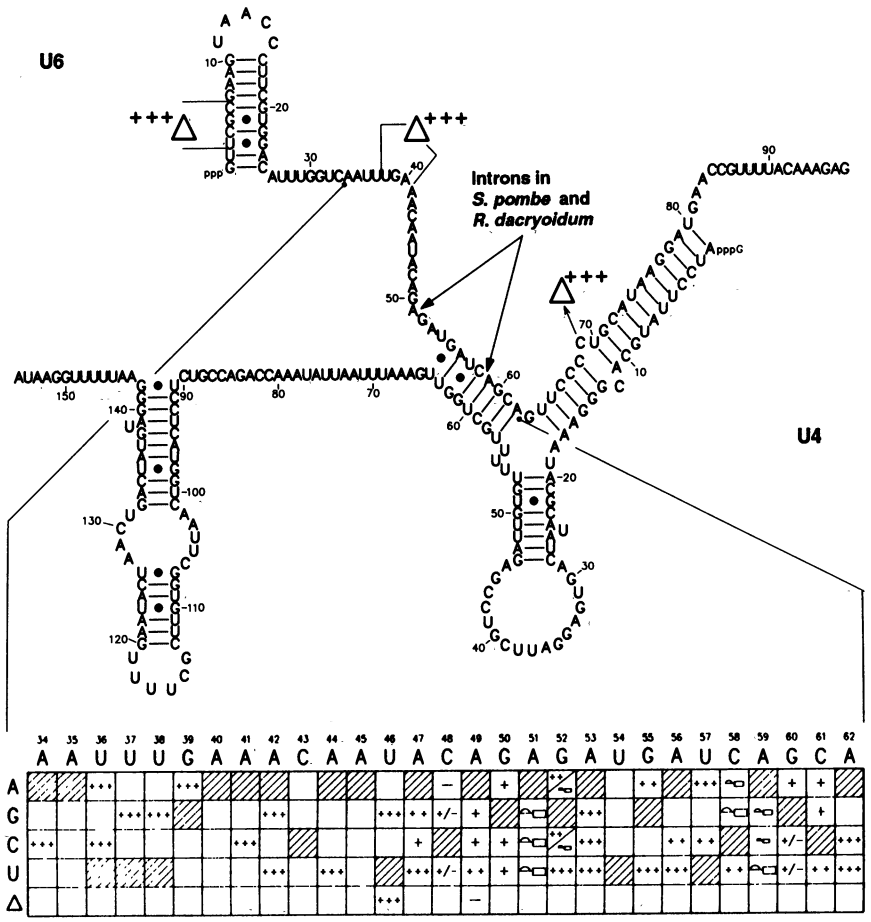


**Fig. 1.** Mutations in U6 RNA block pre-mRNA splicing in vitro. The proposed secondary structure of the yeast U4-U6 snRNA's is shown (*9*). The table summarizes the effects of point mutations at nucleotides 34 to 62 of U6 RNA on pre-mRNA splicing in vitro. +++, As reactive as wild type (80 to 100 percent splicing efficiency); ++, somewhat less reactive than wild type (50 to 80 percent splicing efficiency); +, significantly inhibited (20 to 50 percent splicing efficiency); +/−, only faint signal of products detected (less than 10 percent splicing efficiency); −, no products detected; ++, lariat exon 2, 50 to 80 percent splicing efficiency in combination with a barely detectable block in the second step of splicing. The lariat symbols signify various amounts of block in the second step of splicing (the greater the symbol, the greater the block); Δ, deletions of nucleotides. For each mutant, the data were derived from one to six independent experiments.

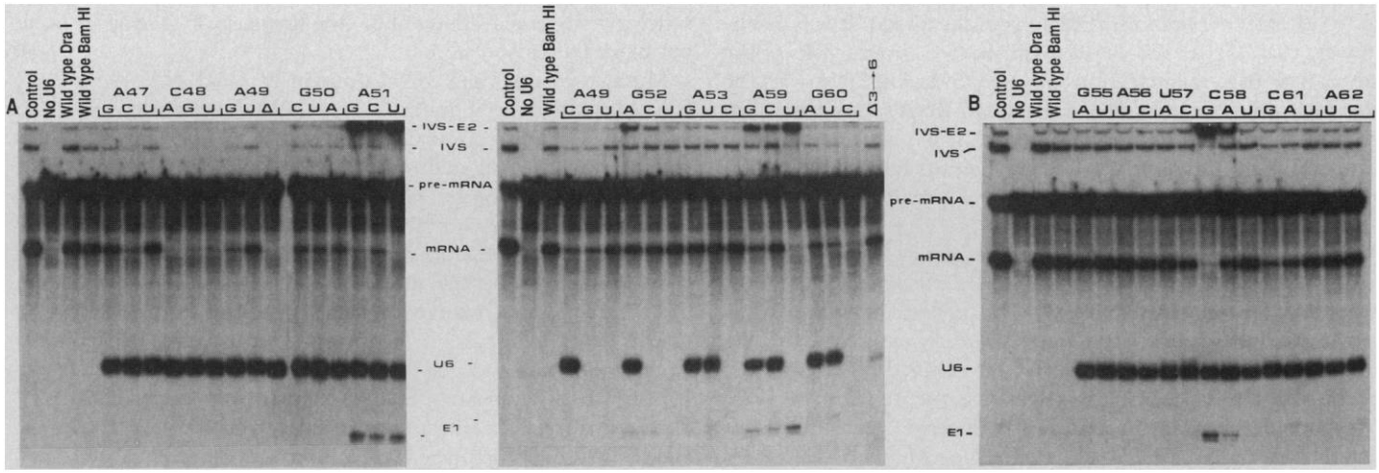


**Fig. 2.** (**A** and **B**) Reconstitution of splicing activity by U6 RNA mutants. Yeast splicing extract was first treated with 300 nM oligonucleotide d1 in the presence of ATP to cleave the endogenous U6 RNA. Reconstitution was then performed as described (*12*) with 40 nM of each synthetic U6 RNA clone (10 nM Δ3 → 6) that had been 3′-truncated with Bam HI and labeled with $^{32}P$ at a specific activity of 7.7 cpm/fmol (except in some of the cases where U6 transcripts were unlabeled) (*37*). Splicing activity was assayed with $^{32}P$-labeled actin pre-mRNA (0.2 nM) at a specific activity of 10,000 cpm/fmol. Splicing efficiency was quantified by Cerenkov counting of the appropriate bands excised from the dried gels as described (*26*). The value for the Bam HI–truncated wild-type U6 was taken as 100 percent. Reconstitution with the wild-type U6 RNA 3′-truncated with Dra I is also shown. The positions of the intermediates—lariat–exon 2 (IVS-E2), lariat intron (IVS), and exon 1 (E1)—are indicated. Control, mock-treated extract, without oligonucleotide d1.

the U6 sequence ACAGA blocked splicing either at the first or the second step.

All changes to C48 drastically affected splicing activity: C48 to A abolished splicing, and with C48 to G or U, only a small amount of spliced product was seen.

Differential effects were seen from the alteration of A47, with A47 to C being the most drastic of these changes. The same was true for A49, where changes to G or C were more drastic than the change to U. All changes to G50 inhibited splicing significantly.

All three substitutions at position A51 allowed the first step of splicing to occur but blocked the second step. The second step was completely blocked when A51 was changed to U. Substitution with G or C allowed small amounts of product to be formed. In order to carry out the first step of splicing, U6 RNA must function correctly in all stages of spliceosome assembly. The failure of the A51 (and to a lesser extent of the G52) mutants to carry out the second step of splicing indicates that this base, or a structure including it, is required for the second step of splicing.

**U6 RNA mutants that block the second step of splicing and allow 5′ splice site cleavage and branchpoint formation.** It is possible that the failure of the A51 mutants to carry out the second step of splicing is due to errors made during the first step. It is known, for example, that mutations in the 5′ splice site, although allowing the first step of splicing to take place, block the second step (*17*). Possibly there is an editing function that examines the nature of the branch before committing the complex to the next step.

To map precisely the point of branch formations and 5′ cleavage, we conducted primer extension analysis. Synthetic oligonucleotides labeled at their 5′ end with $^{32}P$ were used to prime reverse transcription of actin pre-mRNA isolated from reaction mixtures (Fig. 3). Primer 1 hybridizes near the 3′ splice site, 3′ to the normal branchpoint. Extension of primer 1 is blocked by the branched nucleotide resulting in a 43-nt product. All A51 mutants gave rise to lariats, which, in turn, gave rise to a product of identical size in this experiment. Primer 2 is complementary to sequences in the intron just 3′ to the 5′ splice site. In this case, reverse transcriptase is blocked by the branchpoint giving rise to a 69-nt product. Again,

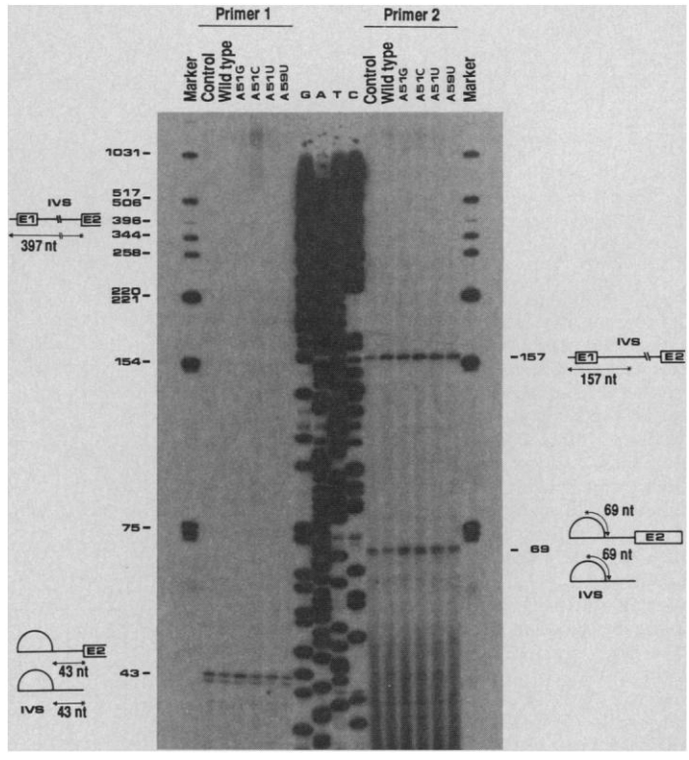


**Fig. 3.** Primer extension analysis of pre-mRNA spliced in the presence of U6 RNA mutants. Primer extension was essentially performed as described (*38*). Yeast splicing extract was first treated with 300 nM oligonucleotide d1 in the presence of ATP to cleave the endogenous U6 RNA. Reconstitution was then performed with unlabeled U6 RNA mutants as indicated, and splicing activity was assayed with unlabeled actin pre-mRNA (0.4 nM). Total RNA was extracted with phenol, precipitated with ethanol, and used in each primer extension reaction with 50,000 cpm of primer labeled at the 5′ end with [γ-$^{32}P$]ATP as described (*29*). Lanes labeled G, A, T, and C are reference analyses of DNA sequence of an actin clone in M13, with primer 1 as primer. Size markers are 5′ end-labeled, Hpa I–digested pBR322, and sizes are given as the number of nucleotides. In the diagrams beside the gel results, action exons (E1, E2) are shown as open boxes and intron sequences as a solid line. Control, mock-treated extract, without oligonucleotide d1.

substitution of A51 with G, C, or U did not give rise to any change in the length of this product, an indication that the site of 5′ cleavage is unchanged. Thus, 5′ cleavage and branchpoint formation are not affected by mutations in A51. The defect is only in the second step of splicing.

**U6 RNA mutants that block the first step of splicing and prevent the formation of the spliceosome.** In spliceosome assembly, U6 snRNP, together with U4 and U5 snRNP's, joins an early complex that includes U1 and U2 snRNP's (*7, 18*). Destruction of U6 RNA by RNase H results in accumulation of the U1-U2 complex (Fig. 4, complex B). In our reconstitution experiment, the association of $^{32}$P-labeled U6 RNA with the spliceosome can be demonstrated by electrophoresis in a non-denaturing polyacrylamide gel (*12*). It was important to know whether mutations that block the first step of splicing still allow correct spliceosome assembly. In the control experiment (Fig. 4, wild type), $^{32}$P-labeled U6 RNA was in two complexes, A1 and A2. A1 is the penultimate complex before formation of the active complex, and A2 is the active complex and contains splicing intermediates. Mutation of C48 to A completely blocks the association of U6 with the spliceosome. (All other mutations that block the first step of splicing that have been tested have the same effect.) We have investigated the effect of many of the point mutants on the assembly of snRNP's, and the results so far indicate that most mutant U6 RNA molecules associate with U4 and form complexes with a normal electrophoretic mobility. Thus the failure of mutant U6 molecules to enter the spliceosome must occur at a later step.

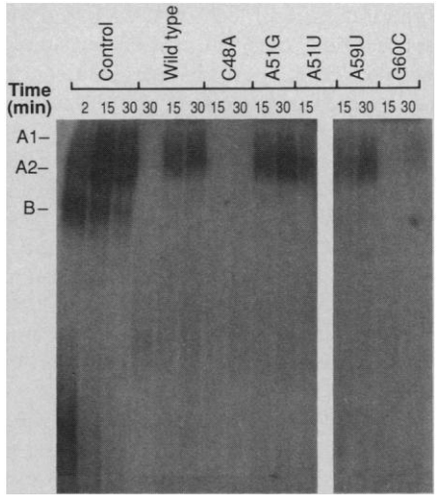


**Fig. 4.** Spliceosome formation by U6 RNA mutants. Yeast splicing extract was first treated with 300 nM oligonucleotide d1 in the presence of ATP to cleave the endogenous U6 RNA. Reconstitution was performed with 15 nM of various U6 RNA transcripts that were 3′-truncated with Bam HI and labeled with $^{32}$P at a specific activity of 800 cpm/fmol. Spliceosome formation was assayed by incubating unlabeled actin pre-mRNA (4 nM; more substrate was used in this experiment to build up detectable levels of each complex) for the times indicated, except in the first lane of the wild-type reaction where precursor was not added. After adding heparin, complexes were isolated in a nondenaturing gel as described (*7, 12*). Control, mock-treated extract, without oligonucleotide d1, using labeled actin pre-mRNA. The nomenclature of the complexes is that proposed in (*7*).

**Mutations near the *S. pombe* U6 intron insertion site: Analysis of the stem I region.** A large number of snRNA genes have been sequenced, but until recently none had been shown to have introns. The discovery that the *S. pombe* U6 RNA gene has an intron was therefore a surprise; even more surprising was the fact that this intron, as judged by its sequence, is very likely equivalent to those in pre-mRNA and is therefore removed by the same mechanism (*10*). The intron is located between nucleotides analogous to C58 and A59 of *S. pombe* U6 RNA in the U4-U6 pairing stem I region of *Saccharomyces cerevisiae* U6 RNA (Fig. 1).

It has thus been proposed (*11*) that this region of U6 RNA might be in the catalytic site of the spliceosome. During splicing of a pre-mRNA, an aberrant event could have occurred that resulted in the insertion of an intron into the U6 RNA. Through reverse transcription and recombination, the normal U6 RNA gene could thus have acquired an intron. The finding that the *S. cerevisiae* U3 gene also has an intron shows that this is not an obligatory route for the acquisition of introns by snRNA genes (*19*) (U3 is not a component of the spliceosome). Nonetheless, this phenomenon clearly focuses interest on the stem I region of U6 RNA. We have now constructed variants of this region. Changes were made in nucleotides 55 to 62 (*20*). Only changes in nucleotides CAGC (nt 58 to 61) had any significant effect on splicing (Fig. 2B). In all U6 RNA's, C58 is conserved as a pyrimidine (*9*). Thus, changing C58 to U had little effect. However, changing C58 to A or G blocked the second step of the reaction and, as with changes in A51, resulted in the accumulation of intermediates.

Changing A59 to U also resulted in the accumulation of intermediates, whereas substitution with C or G had a less dramatic effect (Fig. 2A). As with A51 mutants, the accuracy of 5′ splice site cutting and lariat formation were not altered when U6 RNA containing an A59 to U mutation was used to complement inactivated extracts (Fig. 3). Changing G60 to C or U blocked splicing almost completely, whereas U6 RNA with G60 altered to A had low complementing activity. As with other mutations blocking the first step, U6 RNA containing a G60 to C mutation failed to enter the spliceosome (Fig. 4). All changes to C61 also blocked the first step of splicing.

Nucleotides 55 to 62 of U6 RNA are thought to form base pairs with U4 in stem I (*9*). The failure of U6 mutants with changes in G60 and C61 to complement could therefore be explained by the resulting mispairing. Interestingly, changes in U6 that would result in mispairing at positions 55 to 57 and 62 have little or no effect. Changes in C58 and A59, however, do not prevent formation of the spliceosome but rather block the second step of splicing. These results lend some support to the idea that the *S. pombe* U6 RNA gene may have acquired its intron at this position precisely because of the close proximity of U6 RNA to the intron in the spliceosome. U6 RNA genes in a wide variety of organisms have been examined for the presence of introns (*21*). One other independent example was found in the yeast *Rhodosporidium dacryoidum*. In this case the site of the intron insertion was adjacent to nucleotides corresponding to A51 and G52. Thus, in both cases, the intron is located next to nucleotides that when changed block the second step of splicing.

**Effect of deletion of four nucleotides in the 5′ stem of U6 on RNA stability.** During our search for random mutants in the

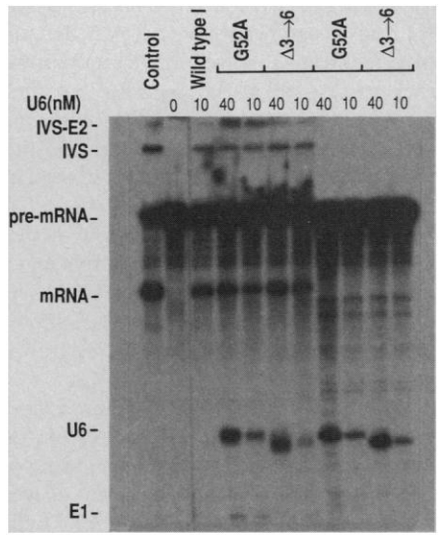


**Fig. 5.** U6 Δ3 → 6 RNA is unstable. Yeast splicing extract was first treated with 300 nM oligonucleotide d1 in the presence of ATP to cleave the endogenous U6 RNA. Reconstitution was performed with unlabeled wild-type U6 RNA and with $^{32}$P-labeled G52 to A and Δ3 → 6 U6 mutant RNA's as indicated. Splicing activity was monitored with $^{32}$P-labeled actin pre-mRNA (0.2 nM). The last four samples were incubated with buffer only. Control, mock-treated extract, without oligonucleotide d1.

central domain of U6 RNA, we found some unexpected mutations and deletions outside of the central domain (Fig. 1). One of these was the deletion of nucleotides U3 to C6 in the 5′ stem of U6 RNA (Δ3 → 6). This truncated U6 RNA could effectively restore splicing activity, yet its stability in the extract was reduced (Figs. 2 and 5). U6 RNA containing a G52 to A mutation (which is as stable as wild-type U6 RNA) and Δ3 → 6 U6 RNA were compared (Fig. 5). Both RNA's were incubated with yeast extract or with buffer only. Δ3 → 6 was clearly unstable in the extract. The fact that the residual U6 RNA that remained in the reaction after degradation was still able to restore splicing of actin pre-mRNA is not surprising. Even 0.5 nM U6 RNA (which is one-twentieth to one-eightieth the concentration normally used for reconstitution) restored splicing efficiently (*12*).

The first 26 nucleotides of human U6 snRNA contain information necessary and sufficient for capping (*22*). The cap structure of this U6 snRNA consists of a γ-monomethyl phosphate structure (*22*), in contrast to the trimethyl G cap in the other snRNA's. The cap structure of mRNA's has been shown to enhance the stability of mRNA by protecting it against 5′ exonucleolytic degradation (*22, 23*). Thus, the lack of four nucleotides in the 5′ stem of U6 RNA could be sufficient to impede the formation of the γ-monomethyl phosphate cap structure, leaving the RNA unprotected against degradation.

**Possible catalytic function for U6 RNA in pre-mRNA splicing**. The suggestion that U6 RNA functions as the catalyst in splicing was first made because of the extraordinary conservation of the U6 sequence in evolution (*9*). U6 RNA stands out most clearly when the *S. cerevisiae* snRNA sequences are considered. *Saccharomyces cerevisiae* U1 and U2 RNA's are much longer than their mammalian counterparts and sequences are conserved only in limited regions. The U4 and U5 RNA sequences in *S. cerevisiae* are almost totally divergent from the mammalian sequences (*9*).

Our results show that changes in the conserved U6 RNA bases A51, C58, A59, and, to a lesser extent, G52, allow spliceosome formation but block the second step of splicing. To our knowledge, these are the only snRNA mutants so far found that block the second step of splicing. Interestingly, it is rare to find mutations in snRNA's that have any effect on splicing. Mutations in those parts of U1 and U2 RNA's that are known to form base pairs with the intron have an effect on the first step of splicing (*24*). But many changes in U1 and U2 RNA's outside these regions have no effect (*25, 26*). Only a few mutations (of many tested) in U5 RNA affect splicing (*27*). And, as our results have shown, changes in some conserved nucleotides in U6 RNA also do not affect splicing. Thus, the finding of two contiguous blocks of sequences that are crucial to splicing is significant. We suggest that these two sequence blocks may be directly involved in the catalytic mechanism in the sense that they participate in the chemistry of the second phosphotransfer reaction. The accumulation of lariat–exon 2 intermediates caused by mutations in C58, A59, A51, and G52 provides a possible explanation for the origin of the introns in the U6 RNA genes of the yeast species *S. pombe* and *R. dacryoidum* (*10, 21*). If these nucleotides are very close to the 3′ splice site at the moment of intron cleavage and exon ligation, and an aberrant splicing reaction takes place, the intron might reintegrate into the U6 RNA. In vivo analysis of single mutations in *S. cerevisiae* U6 RNA shows that most of the single lethal nucleotide substitutions cluster in the same two blocks of sequences (*28*).

We are aware that there are other explanations that can explain the crucial role of these two sequence blocks, however. At least two proteins are required for the second step of splicing. These proteins have been identified in a screen of yeast temperature-sensitive mutants for splicing defects (*29*). ATP hydrolysis is also required for the second step of splicing (*30*). It is possible that a large conformational change, mediated by ATP hydrolysis, is required after the first step of splicing. The mutant changes that we have identified could block this structural transformation through failure to interact with one of these proteins or because a necessary RNA structure involved in this transformation could not form.

We have observed that altered U6 RNA variants that block the first step of splicing invariably fail to enter the spliceosome. This is a puzzling result, especially because changes in adjacent, conserved nucleotides that block the second step of splicing allow spliceosome formation. An interesting explanation for this finding is that these nucleotides participate in base-pairing interactions, and that these interactions must occur early in spliceosome assembly. It appears that ATP hydrolysis is required at virtually every step of assembly, except for the first step (U1 RNA binding to the pre-mRNA) (*31*). The sequences of four yeast proteins implicated in splicing offer an explanation for the role of ATP hydrolysis. Two of the proteins, PRP5 and CA8 (*32*), are closely related in sequence to the growing family of RNA helicase–like proteins, originally defined by the eukaryotic translation initiation factor eIF-4A (*33*). The other two proteins, PRP16 and PRP22 (*34*), are closely related in sequence to each other and have ATP binding sites, but are not closely related to the eIF-4A family. These may be members of a second class of RNA helicases. These RNA helicases may inspect RNA structures that form at various stages in assembly. If the structures are correct they may mediate, via ATP hydrolysis, changes that move the spliceosome along the assembly pathway. If incorrect structures are formed, assembly would be aborted. This proofreading mechanism could explain the extraordinary accuracy of pre-mRNA splicing. In this context, U6 RNA variants that fail to enter the spliceosome could prevent the formation, early in assembly, of a structure that is closely monitored and that is required for a subsequent irreversible step. It will be interesting to find mutations in U6 that block the first step of splicing, but which allow complete assembly of the spliceosome to occur.

In this regard we have noted that the conserved sequence ACAGA, strongly implicated as having a crucial role in the splicing mechanism, could potentially form base pairs with a conserved sequence in U2 RNA, adjacent to the UACUAAC interaction site (*26*). This pairing would allow a near continuous helix to form, one strand of which is U2 RNA and the other strand the intron and U6 RNA. However, mutations of those bases in U2 that are predicted to interact with U6 have only a small effect on splicing in an in vitro complementation assay similar to that of our study (*35*). Further experiments are required to rule out the occurrence of this base-pairing interaction, however. Also, the active site of the group I intron, which binds a guanosine cofactor, has been shown to include a base-paired stem containing an ACAGA sequence (*36*). It may be that the active site of the spliceosome is a similarly complex structure with interactions between several of the snRNA's and the intron.

A proof that RNA is the catalyst in pre-mRNA splicing would ideally include a demonstration that RNA alone can catalyze the reaction. This may prove impossible to do, however, because snRNA's have evolved to function with proteins.

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13. The U6 RNA gene attached to the T7 promoter was cloned with the use of six synthetic oligonucleotides as described (*12*). Oligonucleotide d, 5′-CATCCTTATGCAGGGGAACTGCTGATCAtctctgtattgtttcaaattG-3′, was doped for generating 20 random changes within a region of DNA (signified by small letters) corresponding to the central domain of U6 RNA.
14. The concentration of contaminating nucleotides was calculated from the binomial distribution, such that the region chosen for mutagenesis contained on the average one change per molecule [J. B. McNeil and M. Smith, *Mol. Cell. Biol.* **5**, 3545 (1985)]. For generating 20 random changes, we used 14 mol of wild-type base solution and 1 mol of equimolar random mix containing all four bases. To obtain a high mutagenesis efficiency, with low yield of wild-type clones, we used oligonucleotide c, 5′-CATTTGGTCAAUUUGAAACAAUACAGAGAUGAUCAGCAGUUCCCCUGCA-3′, which is complementary to oligonucleotide d. Oligonucleotide c contains U instead of T. Transformation of this construct in *Escherichia coli* strain JM101, which contains an active *N*-glycosylase, results in excision of the uracils and creation of apyrimidinic sites. This reduces the biological activity of the parental strand, and the majority of the progeny contains the desired mutation [T. A. Kunkel, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488 (1985)].
15. DNA was isolated from each clone by a small-scale alkaline lysis procedure, which gives clean DNA [R. Kraft, J. Tardiff, K. S. Krauter, L. A. Leinwand, *Biotechniques* **6**, 544 (1988)] usable for both sequencing with Sequenase (USB) and in vitro transcription with T7 RNA polymerase.
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20. For the construction of the stem I mutants, the oligonucleotide d2, 5′-CATCCTTATGCAGGGGAACtgctgatcA-3′, was doped at each individual position from nucleotides 55 to 62 (doped positions are indicated by small letters) by the use of an equal proportion of the other three bases. Oligonucleotide d2 was used for total U6 RNA gene synthesis together with the uracil-containing oligonucleotide c and oligonucleotide d3, 5′-TCTCTGTATTGTTTCAAATTG-3′.
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32. PRP5 and CA8 are putative helicase proteins involved in pre-mRNA splicing in *S. cerevisiae*. PRP5 (pre-RNA processing mutant) directly participates in nuclear splicing and spliceosome assembly and closely resembles members of a family of RNA helicases that is characterized by several invariant sequence motifs [G. Dalbadie-McFarland and J. Abelson, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4236 (1990)]. CA8 is the product of an RNA helicase gene, which was isolated in a search for additional members of the RNA helicase family [T.-H. Chang, J. Arenas, J. Abelson, *ibid.*, p. 1571]. The CA8 gene is identical to the PRP28 gene, the product of which is involved in nuclear pre-mRNA splicing. A single nucleotide mutation in the conserved region of the PRP28 gene, which shares similarly with the RNA helicase genes, blocks the first step of splicing and prevents cell survival at 18°C (E. Strauss and C. Guthrie, personal communication).
33. Mammalian eukaryotic translation initiation factor eIF-4A was the first of the RNA helicases to be characterized [B. K. Ray *et al.*, *J. Biol. Chem.* **260**, 7651 (1985)].
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37. $^{32}$P-Labeled or unlabeled U6 RNA transcripts gave the same results in the in vitro reconstitution assay. U6 RNA (mutant or wild type) was transcribed in a 20-μl reaction volume in the presence of 2 μCi of [α-$^{32}$P]UTP (uridine 5′-triphosphate) and 1 mM each of unlabeled ATP, CTP (cytidine triphosphate), GTP (guanosine triphosphate), and UTP. RNA transcribed under these conditions has a specific activity of 7.7 cpm/fmol. After several half-lives and loss of the $^{32}$P label, this RNA can be used like unlabeled RNA without any differences in reconstitution efficiency when compared to $^{32}$P-labeled or unlabeled RNA transcripts.
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