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# Mutational Analysis of *Saccharomyces cerevisiae* U4 Small Nuclear RNA Identifies Functionally Important Domains

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**U4 small nuclear RNA (snRNA) is essential for pre-mRNA splicing, although its role is not yet clear. On the basis of a model structure (C. Guthrie and B. Patterson, Annu. Rev. Genet. 22:387–419, 1988), the molecule can be thought of as having six domains: stem II, 5′ stem-loop, stem I, central region, 3′ stem-loop, and 3′-terminal region. We have carried out extensive mutagenesis of the yeast U4 snRNA gene (*SNR14*) and have obtained information on the effect of mutations at 105 of its 160 nucleotides. Fifteen critical residues in the U4 snRNA have been identified in four domains: stem II, the 5′ stem-loop, stem I, and the 3′-terminal region. These domains have been shown previously to be insensitive to oligonucleotide-directed RNase H cleavage (Y. Xu, S. Petersen-Bjørn, and J. D. Friesen, Mol. Cell. Biol. 10:1217–1225, 1990), suggesting that they are involved in intra- or intermolecular interactions. Stem II, a region that base pairs with U6 snRNA, is the most sensitive to mutation of all U4 snRNA domains. In contrast, stem I is surprisingly insensitive to mutational change, which brings into question its role in base pairing with U6 snRNA. All mutations in the putative Sm site of U4 snRNA yield a lethal or conditional-lethal phenotype, indicating that this region is important functionally. Only two nucleotides in the 5′ stem-loop are sensitive to mutation; most of this domain can tolerate point mutations or small deletions. The 3′ stem-loop, while essential, is very tolerant of change. A large portion of the central domain can be removed or expanded with only minor effects on phenotype, suggesting that it has little function of its own. Analysis of conditional mutations in stem II and stem I indicates that although these single-base changes do not have a dramatic effect on U4 snRNA stability, they are defective in RNA splicing in vivo and in vitro, as well as in spliceosome assembly. These results are discussed in the context of current knowledge of the interactions involving U4 snRNA.**

Pre-mRNA processing occurs in a structurally dynamic RNA-protein complex, termed the spliceosome, which consists of four essential small nuclear ribonucleoprotein (snRNP) particles (U1, U2, U5, and U4/U6) and many auxiliary proteins (17, 18, 29, 39, 43, 50). The U1, U2, and U5 snRNPs, each containing one small nuclear RNA (snRNA), appear to be involved in recognition of the consensus signals in the intron and/or exon (8, 17, 32–34, 49, 51, 56, 57). The U4/U6 snRNP contains two different snRNA species (29, 43). While it has been proposed that U6 snRNA plays a catalytic role (27), the function of U4 snRNA in pre-mRNA splicing remains to be determined; a regulatory role has been suggested (28).

A model of U4, an essential (5, 9, 41) and well-conserved (31) snRNA, proposes six structural domains (6, 14, 18, 20, 38; see Fig. 4): stem II (nucleotides 1 to 19), 5′ stem-loop (20 to 53), stem I (54 to 65), central domain (66 to 90), 3′ stem-loop (91 to 142), and the 3′-terminal domain (143 to 160). U4 snRNA is associated with U6 snRNA through extensive base-pairing interactions involving the stem I and stem II regions (18). In a splicing extract, most domains of the yeast U4 snRNA are protected from RNase H digestion following annealing to appropriate complementary DNA oligonucleotides (53); this finding suggests that spliceosome components in addition to U6 snRNA may be associated with the U4 snRNA molecule. The yeast PRP4 protein is associated with the 5′ portion of U4 snRNA (4, 7, 13, 53); PRP3 (2), PRP6 (1), and PRP24 (38) are also part of the U4/U6 snRNP.

U4 snRNA enters the spliceosome as a tri-snRNP complex, U4/U6.U5. U4 snRNA either is absent from the spliceosome prior to or concomitant with the first step of the splicing reaction (15, 24, 36, 55) or becomes loosely attached during experimental manipulations (10). This finding suggests that the U4/U6 snRNP undergoes a conformational rearrangement(s) prior to the first step of the splicing reaction. Base-pairing interactions between U2 and U6 snRNAs (16, 21, 28, 52) indicate that U6 snRNA is involved in alternative RNA-RNA interactions, which is consistent with a U4/U6 snRNP conformational change. Currently little is known about how these conformational rearrangements occur and what triggers them.

In vivo assays of splicing complementation and snRNP assembly in *Xenopus* oocytes show that deletion of any of five U4 snRNA domains—stem II, 5′ stem-loop, stem I, central region, and Sm region—results in defective pre-mRNA splicing (46). The mechanism underlying the splicing defects is unclear except in the case of stem II deletion, which can be explained by the failure to form a U4/U6 snRNP complex. However, deletion of the 5′ stem-loop, stem I, or the central domain has no observable effect on U4/U6 snRNP formation in *Xenopus* oocytes (46). In vitro snRNP reconstitution studies with HeLa cell extracts indicate that deletion of stem II abolishes both the U4/U6 snRNP interaction and spliceosome assembly (48); the same study demonstrated that the 5′ stem-loop and stem I of human U4 snRNA are required for spliceosome assembly but not U4/U6 snRNA interaction. In yeast cells also, U4 snRNA stem II is important for the U4/U6 interaction (38). Mutations at positions 9, 10, 13, and 14 of U4 snRNA stem II cause a cold-sensitive (cs) phenotype, and mutations at positions 9 and

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14 can be suppressed by compensatory mutations of U6 snRNA (38). On the other hand, mutations in stem I of the yeast U4 snRNA (positions 58, 59, and 60) have no effect on cell growth (27). In addition, the 5′ stem-loop of yeast U4 snRNA is required for association with the PRP4 protein and with the U5 snRNP (13).

In *Xenopus* oocytes, deletion of the putative Sm site indirectly blocks U4/U6 di-snRNP formation because the mutant U4 snRNA cannot reenter the nucleus, where interaction with U6 snRNA occurs (46). The Sm region of human U4 snRNA is dispensable for spliceosome assembly but not for Sm binding (48).

In the past, two approaches have been used to assess the functional importance of snRNAs domains: phylogenetic comparison and mutational analysis. The former has limited utility, since phylogenetically conserved bases can tolerate substitutions and deletions (19, 23). We present here a detailed mutational analysis of the *Saccharomyces cerevisiae* U4 snRNA gene (*SNR14*). Using a pool of chemically synthesized single-stranded DNA molecules, we have obtained mutations at 105 of the 160 U4 snRNA residues. Mutations at 15 positions show lethal or conditional-lethal phenotypes. Two domains of U4 snRNA (stem II and 3′ terminal) were shown to be particularly susceptible to mutational change.

## MATERIALS AND METHODS

**Oligonucleotides.** Six oligonucleotides were made specifically for this mutational study. One of the oligonucleotides, BIG1 (240 bases in length), was synthesized by R. Barnett so that, on average, 1.5 changes were introduced randomly along its entire length. The sequences of the oligonucleotides are as follows: BGL, 5′-TCGTCCGAGATCTTTGTGTTTTT-3′; U4X, 5′-GGGCTCG GCGAATTCGAAAAATATGGTTGGGC-3′; U4W, 5′-GGGAGCTGGAAG CTTACTTACGTTTAATGCAT-3′; U4WP, 5′-GTCCAATTACTATTGTTAC TTACG-3′; BIG1, 5′ACTTACGTTTAATGCATTTTCTTTTCTTTCACTTCC TTCCTTAATACTCCATCCTTATGCACGGGAAATACGCAT ATCAGTGAGGATTCGTCCGAGATTGTGTTTTTGCTGGTTGAAAT TTAATTATAAACCAGACCGTCTCCTCATGGTCAATTCGGTGT TCGCTTTTGAATACTTCAAGACTATGTAGGGAATTTTTGGAATAC CTTTTTTCATTACCGATATTCATTCTTTCAAAGG-3′; and BIG2, 5′-GAA AAATATGGTTGGGCCCTCAATTGAATGTTTCAGTCCGGTATCCCTA ACCTATTTTTTGATATTACTTCCAAAGAAAAATAAACAGTCCCTTT GAAAGAATGAATATC-3′. The U3 snRNA primer was described previously (3).

**Yeast strains, plasmids and genetic methods.** Yeast strain W303-1a (*MAT***a** *can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*) was used for construction of the *SNR14* null strain. Plasmid pYX7A was constructed by inserting an *Eco*RI-*Eco*RV yeast genomic DNA fragment containing the *SNR14* gene between the *Eco*RI and *Sma*I sites of pUC18. The prp2-1 and *prp8-1* mutant strains were obtained from the Yeast Genetic Stock Center, Berkeley, Calif. The *prp18-1* mutant strain was given to us by J. Abelson. pYX8 is a derivative of pYX7A with the *Eco*RI site changed to *Bgl*II. pAPS2U4 was constructed by inserting a *Bam*HI-*Bgl*II fragment from pYX8 into the *Bam*HI site of pAPS2 (described as pINT2 in reference 35). pCU4BII is also a derivative of pYX7A in which a *Bgl*II site was introduced in the *SNR14* gene at the position corresponding to nucleotide 48 of the U4 snRNA. The *Bgl*II site was introduced by site-directed mutagenesis with single-stranded DNA template amplified by asymmetrical PCR (37) from the *SNR14* gene, using primers U4W and U4X at a ratio of 100:1. The second strand was synthesized by extension of the single-stranded DNA template annealed with primers BGL and U4X. The synthesized double-stranded DNA was digested with *Nsi*I and *Apa*I and was used to replace the 320-bp *Nsi*I-*Apa*I *SNR14*-containing fragment of pYX7A. pCU4H3 was constructed by inserting the *HIS3* gene as a *Bam*HI fragment (1.8 kb) into the *Bgl*II site of the pCU4BII. Plasmid pJHU4 was constructed by inserting an *Eco*RI-*Bam*HI fragment from pYX7A into pFL39 (12). The plasmid shuffle technique was described by Boeke et al. (11).

Yeast growth media were described previously (7). For growth of temperature-sensitive (ts) yeast strains, cells were cultured at 25°C (permissive temperature) or at 37°C (nonpermissive temperature). For cs yeast strains, cells were grown at 30°C (permissive temperature) or at 16°C (nonpermissive temperature). Plasmids isolated from *S. cerevisiae* were propagated in *Escherichia coli* JF1754 (42).

The *SNR14* null mutant strain JH1 (*MAT***a** *can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 SNR14*::*HIS3*) was constructed by introducing the wild-type *SNR14* gene carried on a *URA3-CEN-ARS* plasmid (pAPS2U4) into strain W303-1a; this was followed by replacement of the chromosomal *SNR14* gene with a 2.36-kb *Eco*RI-*Bam*HI DNA fragment from pCU4H3 that contains a null-mutant *SNR14* gene in which the *HIS3* gene had been inserted (*SNR14*::*HIS3*). Disruption of the chromosomal *SNR14* gene was confirmed by both genetic analysis and DNA blot hybridization (data not shown).

**RNA analysis.** Yeast RNA isolation and primer extension were performed as described by Hu et al. (22). Where appropriate, the primer extension products were quantified by a PhosphorImager (Molecular Dynamics model 400S). The stability of mutant U4 snRNAs was determined by primer extension, and a U2 snRNA primer was included as a loading control.

**Isolation and analysis of mutant plasmids.** Miniscale plasmid DNA preparation from *E. coli* was performed as described previously (37), with the exception that DNA from 1.5 ml of cells was dissolved in 40 μl of Tris-EDTA buffer and was purified further by precipitation of the residual RNA and proteins with addition of 20 μl of 7.5 M ammonium acetate on ice for 5 min. Following centrifugation for 2 min, DNA in the supernatant was precipitated with 2 volumes of ethanol. DNA prepared in this way was found to yield superior DNA sequencing results. Miniscale plasmid DNA isolation from yeast cells was carried out as described previously (44).

**In vitro analysis of splicing and spliceosome assembly.** Preparation of yeast extracts and in vitro splicing were carried out by the method of Lin et al. (26), with modifications described by Teigelkamp et al. (45); conditions for native gels and spliceosome assembly were as described by Cheng and Abelson (15).

## RESULTS

**Construction of an *SNR14* mutant library.** The strategy for construction of the mutant library is shown in Fig. 1A. A pool of oligonucleotides corresponding to the entire *SNR14* gene was synthesized chemically by substituting each nucleotide at random (37) with 0.63% of the other three. This pool of oligonucleotides, BIG1 (see Materials and Methods), was hybridized with oligonucleotide BIG2, and double-stranded DNA molecules were synthesized by extension from the annealed oligonucleotides with *E. coli* DNA polymerase I large fragment. The double-stranded DNA fragments, amplified by PCR (37), were digested with *Nsi*I and *Apa*I and then were ligated to the large fragment of pJHU4 (see Materials and Methods) following digestion with the same restriction enzymes. To determine whether the *SNR14* mutant library was random, DNA was isolated from 240 individual clones in the library and the DNA sequence of the resident *SNR14* gene was determined, (Table 1, Fig. 2, and Fig. 3). Among the 240 clones, 22.3% had no mutation, 33.5% had one mutation, 25% had two mutations, 12.5% had three mutations, and 6.7% had more than four mutations in the *SNR14* gene. This agrees well with the Poisson distribution, indicating that the *SNR14* mutant library is random.

**Genetic screening.** The library of *SNR14* mutations was introduced into *S. cerevisiae* JH1 (*SNR14*::*HIS3*) in order to test for the phenotypes conferred by the mutant alleles (see Materials and Methods). Plasmid shuffling and genetic screening are outlined in Fig. 1B. Following introduction of the mutant *SNR14* library into *S. cerevisiae* JH1, individual colonies were patched on solid selective medium and were incubated at 16, 25, or 37°C to determine which ones were conditional, dominant-negative mutants. Colonies grown at 25°C were then placed on 5-fluoro-orotic acid (5-FOA) solid medium in order to select against the wild-type *SNR14* gene carried on the *URA3-CEN-ARS* plasmid. Since the *SNR14* gene from the mutant library is the sole copy in the test strain, colonies that were unable to grow on 5-FOA medium were considered to harbor lethal mutations in the *SNR14* gene. Colonies grown on 5-FOA medium were tested for temperature sensitivity at 37°C and cold sensitivity at 16°C. Plasmids were rescued from the potential *SNR14* mutant strains, and restriction analysis of the plasmids was performed with *Eco*RI and *Bam*HI to ensure that these mutant *SNR14*-containing plasmids had no detectable gene rearrangement. The mutant plasmids were reintroduced into strain JH1. Four colonies of cells transformed with each mutant plasmid were retested for their growth phenotypes.

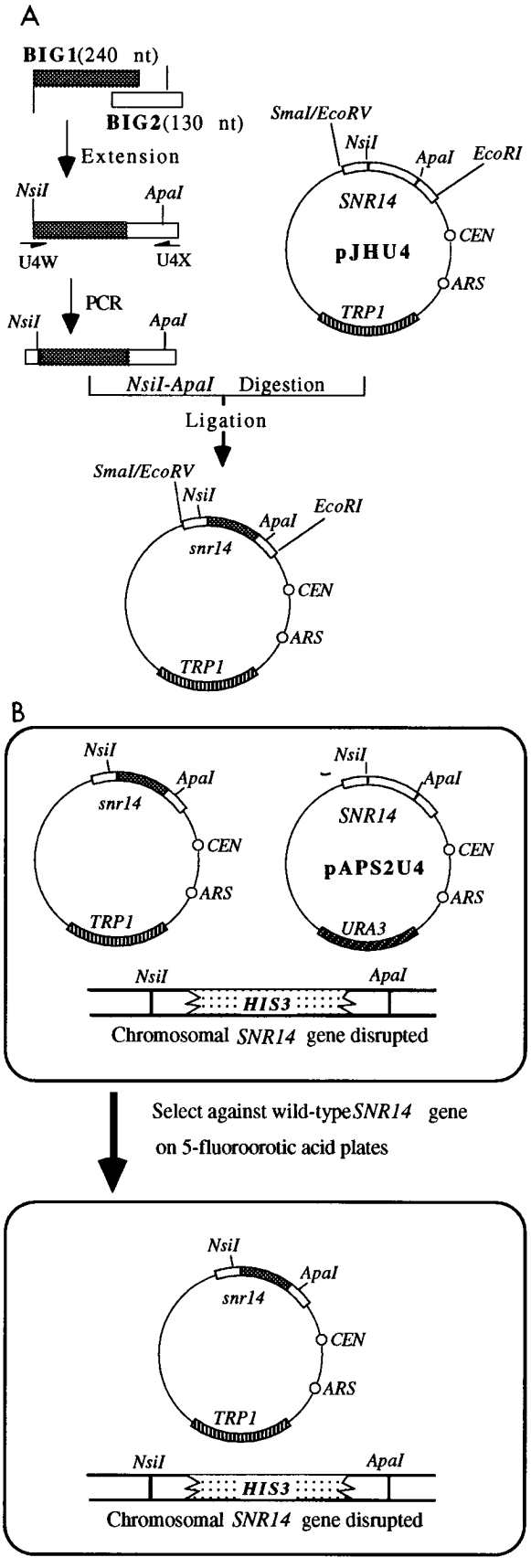



TABLE 1. Critical mutations in yeast U4 snRNA

| Mutated position[a] | Phenotype |
|---|---|
| Stem II (residues 1–19) | |
| Δ1–3 | cs |
| ΔC4 | cs |
| U6C | Lethal |
| ΔU8 | cs |
| G9C | cs |
| Δ9–10 | Lethal |
| C10A | cs |
| ΔC10 | Lethal |
| G13A | cs |
| G14A | cs |
| G14U | cs |
| G15A | cs |
| G15U | cs |
| ΔG15 | cs |
| Insertion of A at 18–19 | ts |
| 5′-stem-loop (residues 20-53) | |
| A45C | ts |
| G46C | Slow |
| Stem I (residues 54-65) | |
| G58A | ts |
| G58U | ts |
| ΔG58 | ts |
| G58C | ts |
| Central domain (residues 66-90) | |
| Δ62–88 | cs |
| 3′-stem-loop (residues 91-142) | |
| Δ131–133 | cs |
| 3′-terminal region (residues 143-160) | |
| U145G | Lethal |
| U146C | cs |
| U147G | Lethal |
| U149G | Lethal |

[a] Δ, nucleotide(s) that is deleted.

DNA sequence analysis was performed on those clones that showed lethal or conditional-lethal phenotypes.

We screened 3,500 independent colonies and obtained no dominant-negative mutants when the mutation-bearing plasmids were introduced into strain JH1 (*SNR14*::*HIS3*). Ten colonies showed a ts phenotype. Ninety-one colonies showed a cs phenotype; these correspond to 58 different mutations, since 33 of them occurred more than once during the screening. Thirty-six colonies showed a lethal phenotype, corresponding to 30 different mutations. In addition, from analysis of clones chosen at random, we also obtained 22 different *SNR14* alleles that conferred a lethal or conditional-lethal phenotype and 72

FIG. 1. (A) Construction of a mutant *SNR14* library. A pool of mutagenized oligonucleotides (BIG1; represented by a shaded box) of 240 nucleotides was hybridized with a pool of normal oligonucleotides (BIG2; represented by an open box) 130 nucleotides in length. Double-stranded DNA fragments were synthesized with *E. coli* DNA polymerase I large fragment by extension from the annealed oligonucleotides. The full-length extension products were amplified by PCR with primers U4X and U4W (see Materials and Methods). The PCR-amplified DNA fragments were digested with *Nsi*I and *Apa*I and were ligated to the large *Nsi*I-*Apa*I fragment of pJHU4. (B) Plasmid shuffling. Plasmid shuffling was performed as described previously (11). A mutant *SNR14* plasmid library was introduced into an *SNR14* null strain (JH1; see Materials and Methods), in which the chromosomal *SNR14* gene was disrupted by insertion of the *HIS3* gene. In this strain, a copy of the wild-type *SNR14* gene is carried on a *CEN-ARS* plasmid with a *URA3* marker. Transformants were replica plated on agar medium containing 5-FOA to select against the plasmid carrying the wild-type *SNR14* gene. Those cells that cannot survive on 5-FOA agar medium carry lethal mutations in the *SNR14* gene. Viable cells were tested at different temperatures for conditional-lethal phenotypes.

| Mutated positions | Mutated positions |
|---|---|
| **Stem II (residues 1-19)** | **Central domain (Residues 66-90)** |
| C4A | A66G |
| U5C | A67C |
| U6A | A67U |
| U8C | A68G |
| A11G | U69A |
| ΔC12 | U70G |
| | U71C |
| **5'-stem-loop (Residues 20-53)** | ΔU75 |
| C23A | U77G |
| C23G | A78G |
| U25C | ΔG84 |
| U25G | G84U |
| A29G | A85C |
| G40C | C87U |
| C42G | |
| C42U | **3'-stem-loop (Residues 91-142)** |
| +C @ 43-44 | C92G |
| A47G | C93G |
| A47U | C93A |
| G52A | A96G |
| | G98A |
| **Stem I (Residues 54-65)** | G99A |
| U54G | G108U |
| ΔU55 | +G @108-109 |
| C59G | U109G |
| ΔC59 | G114U |
| U60C | U127C |
| U60G | A129C |
| ΔG61 | ΔG131 |
| ΔU63 | A139C |
| G65A | G141U |
| | **3'-terminal region (Residues 143-160)** |
| | ΔG151 |

FIG. 2. Viable point mutations in yeast U4 snRNA. +X@, insertion of X at.

| Mutated positions | |
|---|---|
| U5G, A80G, U100G | G62C, T-18C |
| U8A, G52A, +G@62-63 | G62A, U105G, U127G |
| A16U, A-5G, T-22C | Δ63to64 |
| A17C, G40C | A67C, G131A |
| A18U, U25C, A36U | A68C, G84A |
| A18G, C101G | A68G, G107A |
| A20C, G52A | ΔA68, G151A, ΔC157 |
| A20U, G52A, C93U | Δ68to73 |
| ΔA20, G22A, +A @66-67, ΔC87 | U70A, U100G |
| G22A, C39G | U71A, +G@99-100, U109A |
| C23U, A73C, G107U | A73G, ΔC113 |
| ΔA24, T-22A, U31C | U77A, A80U |
| C28T, G137C | U77A, ΔA83 |
| A29G, U71G | A78G, C-16T, G141C |
| A29T, C39T, A188C | ΔC81, G84C |
| U31C, U-27C | C87U, ΔC157 |
| G35A, C87 | G88A, G99C |
| A36G, T-22G | G88A, C156U |
| A36U, C87G | G88A, ΔG110, U111A |
| U38G, C185T | C90G, +A@174-175 |
| C43U, A178G, A188C | U91G, A139C, ΔG142 |
| G46A, A76U | C92G, U100C |
| ΔG46, U80G | C93U, A139G |
| G46A, ΔA124 | C95A, Δ-22 to -29 |
| A47U, C87G | A96C, T162G |
| U48C, A122U | G98A, G99A |
| ΔU49, G62A, A129U | C101U, ΔA122 |
| Δ50to56 | C101U, G108U, A165G |
| U51A, U105A, A153C | C101U, Δ102to105 |
| G52A, A153U, C169U | A102G, U112G |
| G52A, A66G, G98A, G99A | A103G, ΔC-15 |
| U53C, C93U | U105G, G137A |
| U55G, G107A, C157A | U109C, C133G, ΔC220 |
| U56G, U77A | A122C, U154G |
| C59A, U64C | A153T, T-18G |

FIG. 3. Viable, informative multiple mutations in U4 snRNA. +X@, insertion X at; −, the position is upstream of the U4 snRNA coding region. Position numbers larger than 160 indicate 3′ to the U4 snRNA coding region; T instead of U indicates that the position is outside the U4 snRNA coding region.

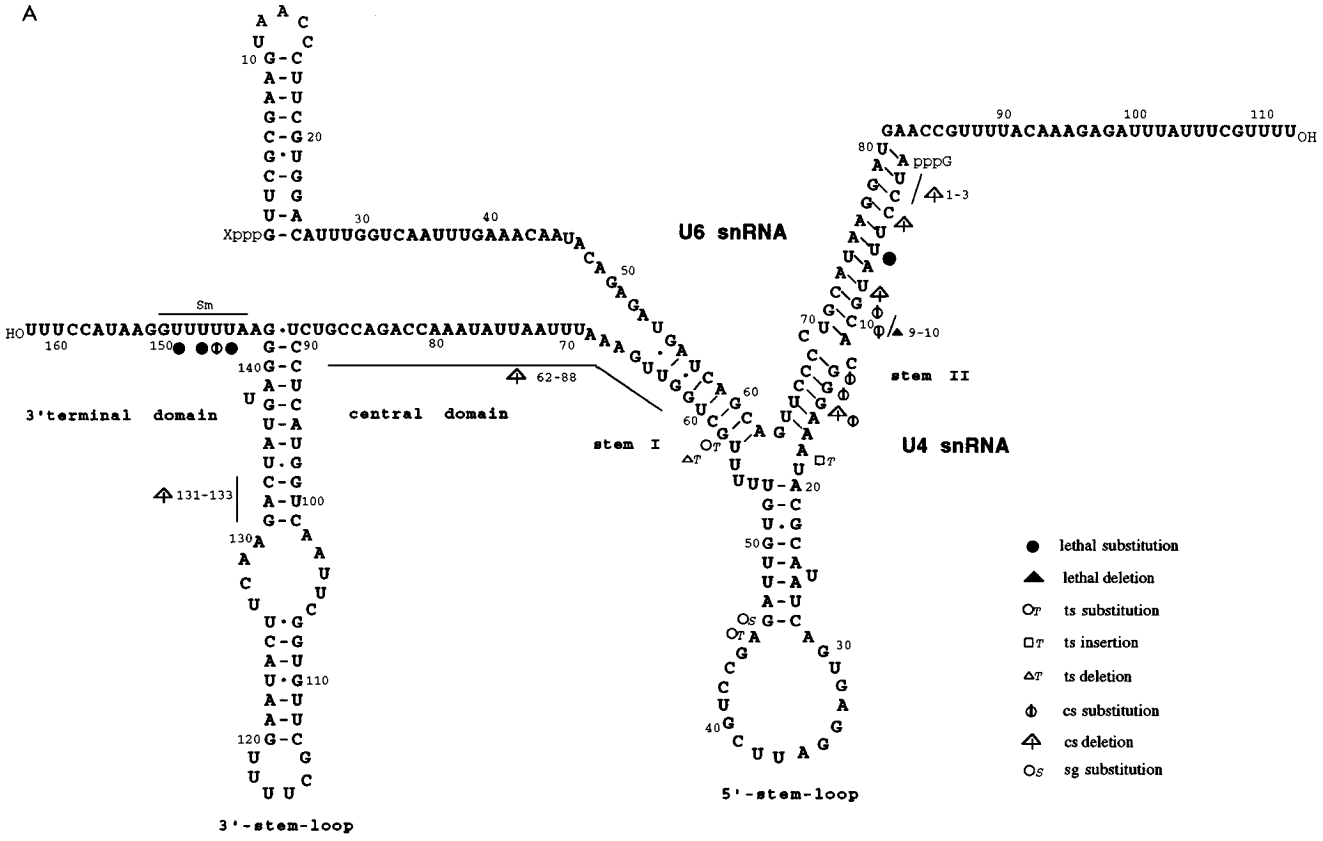
alleles that showed no growth defect under the conditions tested. Many of the lethal and conditional-lethal phenotypes were due to multiple mutations, as were an appreciable number of the pseudo-wild-type mutants. The former were not analyzed further, but the latter are included (Fig. 3) since they yield information about these nucleotide positions in U4 snRNA.

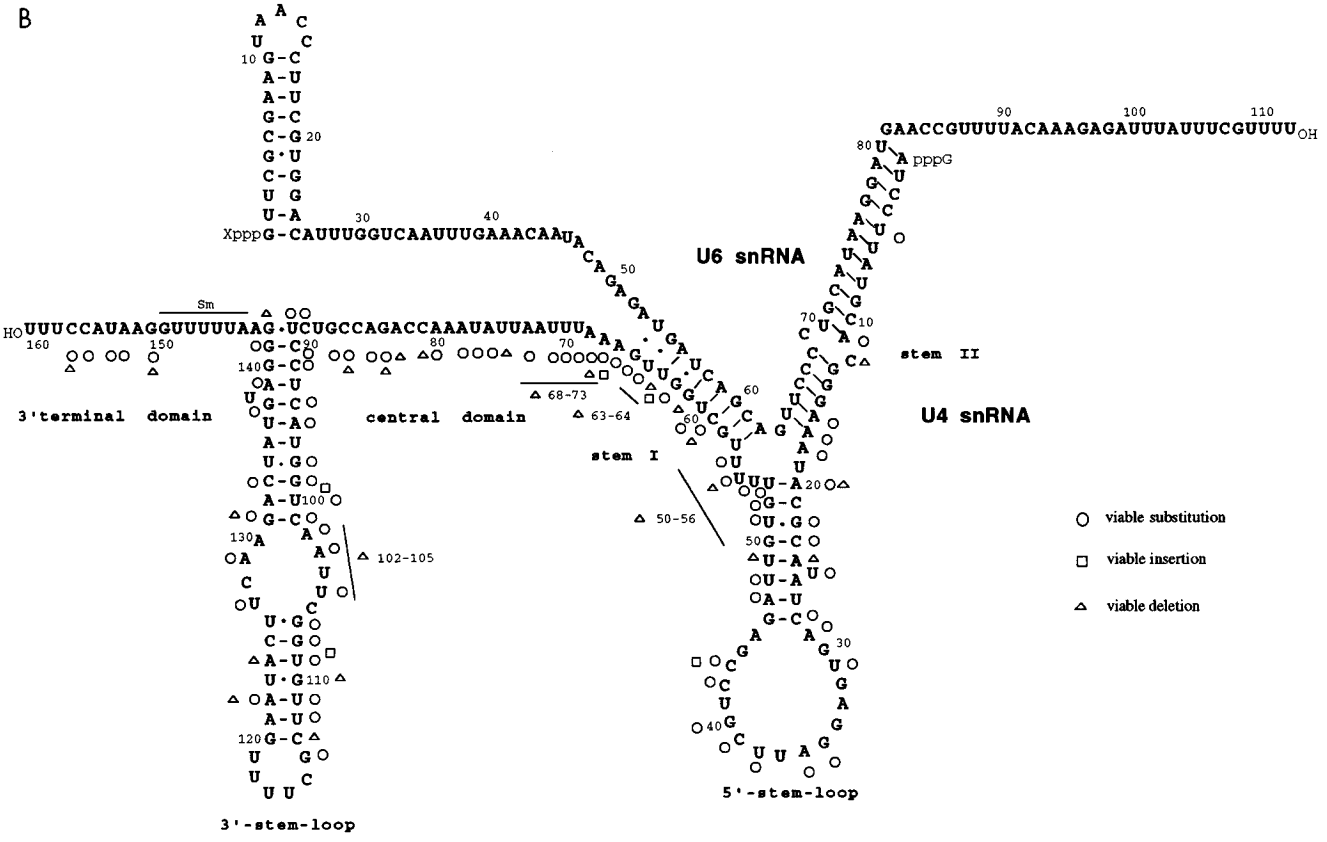
Results of the sequencing and genetic analyses of the informative mutations are summarized in Table 1, Fig. 2, and 3. The informative mutations (discussed below) are defined as base substitutions, insertions, and deletions with growth defects, as well as multiple mutations that do not show any growth detect. In the multiple mutations without phenotype, we assume that each mutation separately is innocuous and that the individual mutations do not compensate for the defects of one another.

**Critical mutations in U4 snRNA.** Table 1 and Fig. 4A summarize the U4 snRNA mutations that gave rise to a detectable lethal or conditional-growth phenotype. Fifteen nucleotide positions of the U4 snRNA were found to yield a phenotype: eight in stem II, two in the 5′ stem-loop region, one in stem I, and four in the 3′-terminal region. All of these are located in the regions that were found to be resistant to oligonucleotide-guided RNase H cleavage (53).

**(i) Stem II (nucleotides 1 to 19).** The stem II domain of U4 snRNA is more sensitive to mutational change than any other. Many mutations in this region showed either a lethal or cs phenotype (Fig. 4A and Table 1). Deletion of the three nucleotides at the extreme 5′ end of the U4 snRNA coding region resulted in a growth defect at 16°C. A deletion of residue C-4 conferred a cs phenotype, although substitution of an A at this position was without a growth defect. Similarly, different changes at positions U-6 and U-8 showed different growth phenotypes. All substitutions at nucleotides G-9, C-10, G-13, and G-14 showed a cs phenotype, in agreement with the results of Shannon and Guthrie (38). Position 15 is also critical since replacing it with A or U, or deleting it, caused a cs phenotype. Furthermore, addition of an A between positions A-18 and U-19 resulted in a ts phenotype. These results extend the critical region of stem II from the four positions that were previously shown (nucleotides G-9, C-10, G-13, and G-14 [38]) to nucleotides 1 through 15 (excepting U-5, A-11, and C-12; Fig. 4A and Table 1).

Five cs U4 snRNA mutants carrying changes in the stem II region were tested for their effects on in vivo splicing by quantitative measurement of pre-U3 snRNA accumulation at the nonpermissive temperature. Pre-U3 snRNA contains two species with differing intron sizes; these are distinguished readily from the single mature RNA species. Because the primer extension products from the mature U3 snRNA and the lariat intermediate are very similar in size, this assay is not used normally for assessing a splicing defect at step 2. Primer extension was performed with total yeast RNA isolated from the strains grown at the permissive temperature (30°C) or following a shift for 2 h to the nonpermissive temperature (16°C). The primer extension products were separated on a denaturing gel and were quantified by using a PhosphorImager. As shown in Fig. 5, mutant strains carrying a deletion of nucleotides 1 to 3 (Δ1-3), G13A, G14A, and G15A showed a significant increase in accumulation of pre-U3 snRNAs compared with the wild-type strain, while mutant G9C showed only a modest increase. Most mutant strains (Δ1-3, G9C, G13A, and G14A) accumulated substantial amount of pre-U3 snRNAs compared with the wild type, even at the permissive temperature. Three of the stem II mutations (Δ1-3, G13A, and G15A) were also tested in vivo for their effects on the splicing of actin pre-mRNA which can be used to assess splicing defects at both step 1 and step 2 (Fig. 6). As shown in Fig. 6, the wild-type strain


A
U6 snRNA
U4 snRNA
stem I
stem II
3'terminal domain
central domain
3'-stem-loop
5'-stem-loop
Sm
62-88
131-133
9-10
1-3
lethal substitution
lethal deletion
ts substitution
ts insertion
ts deletion
cs substitution
cs deletion
sg substitution


B
U6 snRNA
U4 snRNA
stem I
stem II
3'terminal domain
central domain
3'-stem-loop
5'-stem-loop
Sm
68-73
63-64
50-56
102-105
viable substitution
viable insertion
viable deletion

(lanes 1 and 2) did not accumulate either pre-mRNA or the lariat-intermediate species at the permissive or nonpermissive temperature. As a positive control, the *prp18-1* strain showed a significant amount of intermediate accumulation (lane 4), indicating a second-step splicing defect. (To our surprise, the *prp8-1* mutant strain showed accumulation of an intermediate RNA species at the permissive temperature and a pre-mRNA accumulation at the nonpermissive temperature.) The stem II mutations (Δ1-3, G13A, and G14A) showed an increase in pre-mRNA at the nonpermissive temperature (lanes 7 to 12), in qualitative agreement with the U3 snRNA data (Fig. 5). Significantly, there was no apparent accumulation of a lariat-intermediate species (Fig. 6), indicating that the defect conferred by these stem II mutations occurred at or before the first step of splicing. These results suggest that most stem II nucleotides are important or essential for U4 snRNA function and that U4 snRNA functions early in splicing, in agreement with previous observations (15, 24, 36, 55).

**(ii) 5′ stem-loop (nucleotides 20 to 53).** The loop portion of the 5′ stem-loop structure of U4 snRNA contains five conserved nucleotides (31). Oligonucleotide-directed RNase H cleavage experiments suggest that the 5′ portion of yeast U4 snRNA, including this domain, is associated with the PRP4 protein (53). In vivo deletion analysis has confirmed this and has indicated further that the 5′ stem-loop of U4 snRNA is required for U4/U6.U5 complex formation (13). The data presented here suggest that only two single-point mutations in the loop (A45C or at the first base pair of the stem, G46C) showed growth defects (Fig. 4A and Table 1). The ts defect conferred by mutation A45C was reflected in an accumulation of actin pre-mRNA (Fig. 6) as well as an inability to splice in vitro (see Fig. 9).

**(iii) Stem I (nucleotides 54 to 65).** Only one nucleotide, G-58, proved to yield a phenotype; mutations G58A, G58C, G58U, and ΔG-58 showed a strong temperature sensitivity (Fig. 7A and Table 1). This result differs from a previous observation with mutations at position G-58 (27). However, the phenotype was also seen when mutations were placed in strain YKS2 (26, 36), provided by H. D. Madhani and C. Guthrie, and thus is not strain specific. Mutations at positions G-58 that were assayed all showed accumulation of U3 snRNA pre-mRNA in vivo (Fig. 7B). These results suggest that G-58 is essential for U4 snRNA function.

**(iv) Central region (nucleotides 66 to 90).** The central region is very insensitive to mutation. A deletion of almost the entire region (nucleotides 62 to 88) yielded only a mild cold sensitivity (Fig. 4A and Table 1). An insertion of up to 76 nucleotides in this domain can also be tolerated with no detectable growth phenotype (unpublished results). Thus, the primary sequence of this domain may not be essential. Rather, it may serve as a spacer that separates the 5′ and 3′ domains of the U4 snRNA.

**(v) 3′ stem-loop (nucleotides 91 to 142).** Previous results (13) showed that replacement of the 3′ stem-loop of the yeast U4 snRNA with the comparable region from trypanosome U4 snRNA was lethal. In view of this, it was unexpected that only one mutation in our collection resulted in a phenotype; this was a small deletion of nucleotides 131 to 133 (Fig. 4A and Table 1). These data suggest that the overall structure of the 3′ stem-loop, rather than its particular nucleotide sequence, may be important for U4 snRNA function.

**(vi) 3′-terminal region (nucleotides 143 to 160).** The obvious region of functional importance in this domain is the supposed Sm-binding site (nucleotides 144 to 150). As shown in Fig. 4 and Table 1, all single-point mutations at this site (and no others in this domain) showed a growth defect; mutations U145G, U147G, and U149G conferred a lethal phenotype, and U146C conferred a cs phenotype. Mutant strain U146C showed approximately a threefold accumulation of pre-U3 snRNA at 16°C (Fig. 5) and no detectable change in actin mRNA splicing (Fig. 6).

**Mutant U4 snRNA stability.** To test the possibility that the phenotypes due to some of the U4 snRNA mutations could be attributed to RNA instability under nonpermissive conditions, we assayed the relative cellular amounts of U4 and U2 snRNAs (Fig. 8). Strains carrying mutations Δ1-3, G9C, and G58A did not show markedly reduced levels of U4 snRNA at the nonpermissive temperature compared with the permissive temperature and with U2 snRNA. The steady-state levels of U4 snRNA in G15A, A45C, and G46C mutants decreased at the nonpermissive temperature, although the total amounts present remained substantial. In contrast, mutant U146C in the putative Sm-binding site showed a significant decrease in U4 snRNA content, quite possibly due to its inability to interact with the Sm proteins.

**In vitro splicing and spliceosome assembly.** The data presented above indicate that critical U4 mutations in stem I and stem II inhibited in vivo splicing at or before the first step of the reaction. To determine whether U4 snRNA mutations also block splicing in vitro, we prepared splicing extracts from mutant strains G15A, A45C, and G58C as well as the wild-type strain and performed splicing assays in vitro. As shown in Fig. 9A, mutant extracts were defective in splicing (lanes 2 to 4) without accumulation of splicing intermediates, while the wild-type extract was fully active (lane 1). Furthermore, these mutant extracts could be complemented by a heat-inactivated *prp2-1* extract, indicating that there were no nonspecific inhibitory factors present in the mutant extracts. These in vitro results are in agreement with the in vivo findings (see above) that these U4 snRNA mutations are defective at or before the first step of splicing.

We have also determined spliceosome assembly in vitro with these extracts according to the methods described by Cheng and Abelson (15). As shown in Fig. 9B, the wild-type extract showed very efficient spliceosome assembly, while the extracts from the mutant G15A and G58C strains were much less active in assembly, accumulating primarily complex B, which contains pre-mRNA and U1 and U2 snRNPs (15). This finding is consistent with the results reported by Wersig and Bindereif (48) that stem II and stem I of the human U4 snRNA are involved in spliceosome assembly. It is not clear currently why mutation A45C inhibited splicing both in vivo and in vitro but did not show a major defect in assembly. It is possible that the defect caused by A45C is at a late stage of the spliceosome assembly pathway.

**Viable mutations in U4 snRNA.** Figures 2, 3, and 4B show nucleotide changes that do not yield a detectable phenotype. The pattern of viable mutations is, of course, the complement of the critical ones presented above and underlines two general conclusions.

First, stem II and stem I are very dissimilar in their sensi-

FIG. 4. (A) *S. cerevisiae* U4/U6 snRNA structure and locations of critical mutations. The U4/U6 snRNA structure is adapted from reference 18. Point mutations and informative deletions obtained from this study are presented. The phenotypes are designated as indicated (sg, slow growth). The numbers following the triangles indicate deleted nucleotide positions. (B) *S. cerevisiae* U4/U6 snRNA structure and location of viable mutations.

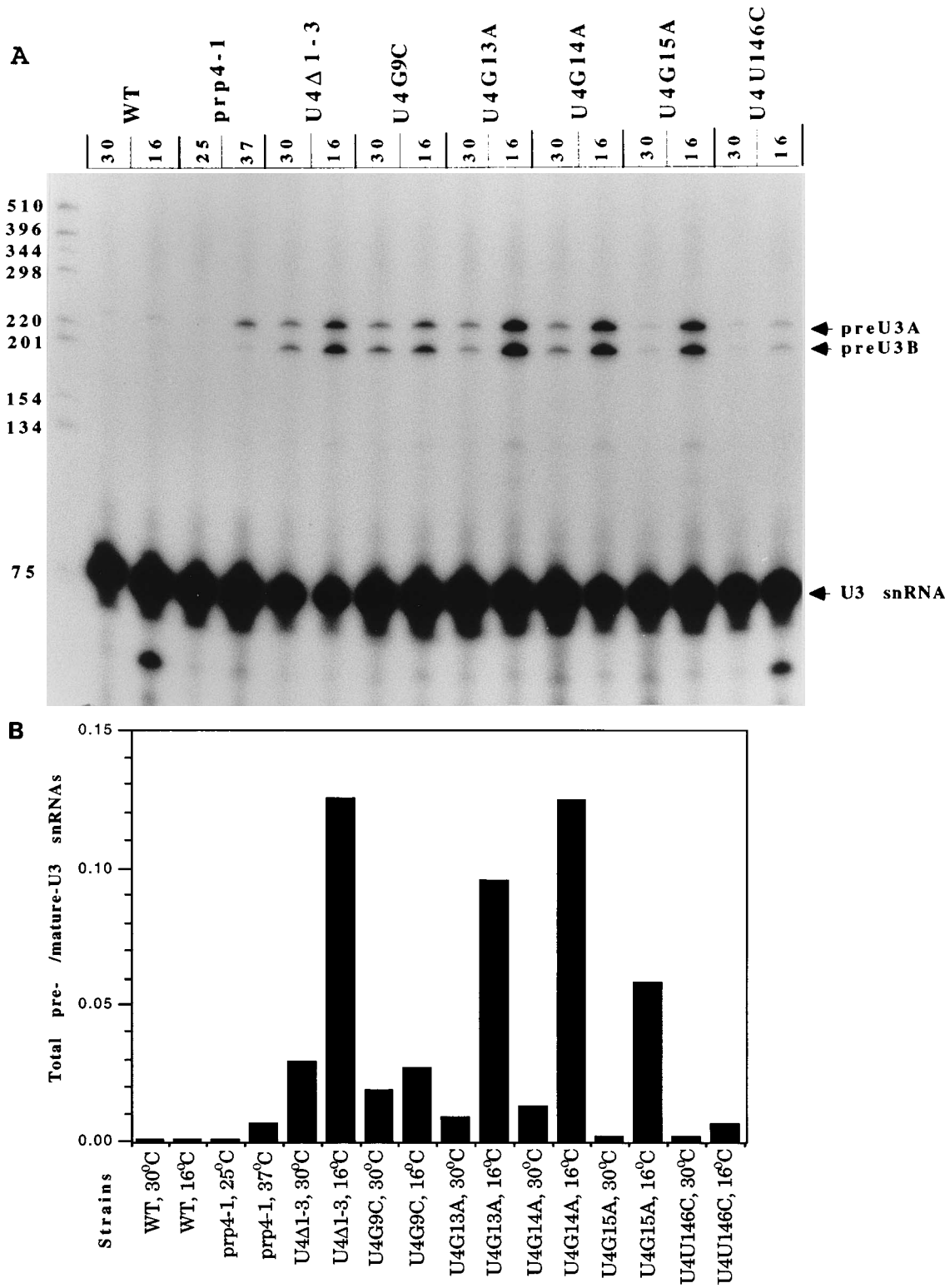



FIG. 5. Mutations in the stem II and 3′-terminal regions of the yeast U4 snRNA cause accumulation of pre-U3A and pre-U3B snRNAs in vivo. (A) Primer extension analysis. Total yeast RNA was isolated from wild-type (WT) and mutant strains following incubation for 2 h at a permissive (25°C for ts strains and 30°C for cs strains) or a nonpermissive temperature (37°C for ts strains and 16°C for cs strains). The RNA was subjected to primer extension analysis using an oligonucleotide primer complementary to a region that is common to both U3A and U3B (3). The strains used for RNA isolation and the temperatures for cell growth are indicated above the lanes. Mutant *prp4-1* was included for comparison. Positions DNA size markers are shown on the left in nucleotides, and bands corresponding to the pre-U3A, pre-U3B, and mature U3 snRNAs are designated with arrows on the right. (B) Quantification of the primer extension products with the aid of a PhosphorImager. The bar graph shows the ratios of total pre-U3 snRNAs (U3A plus U3B) to mature U3 snRNA.

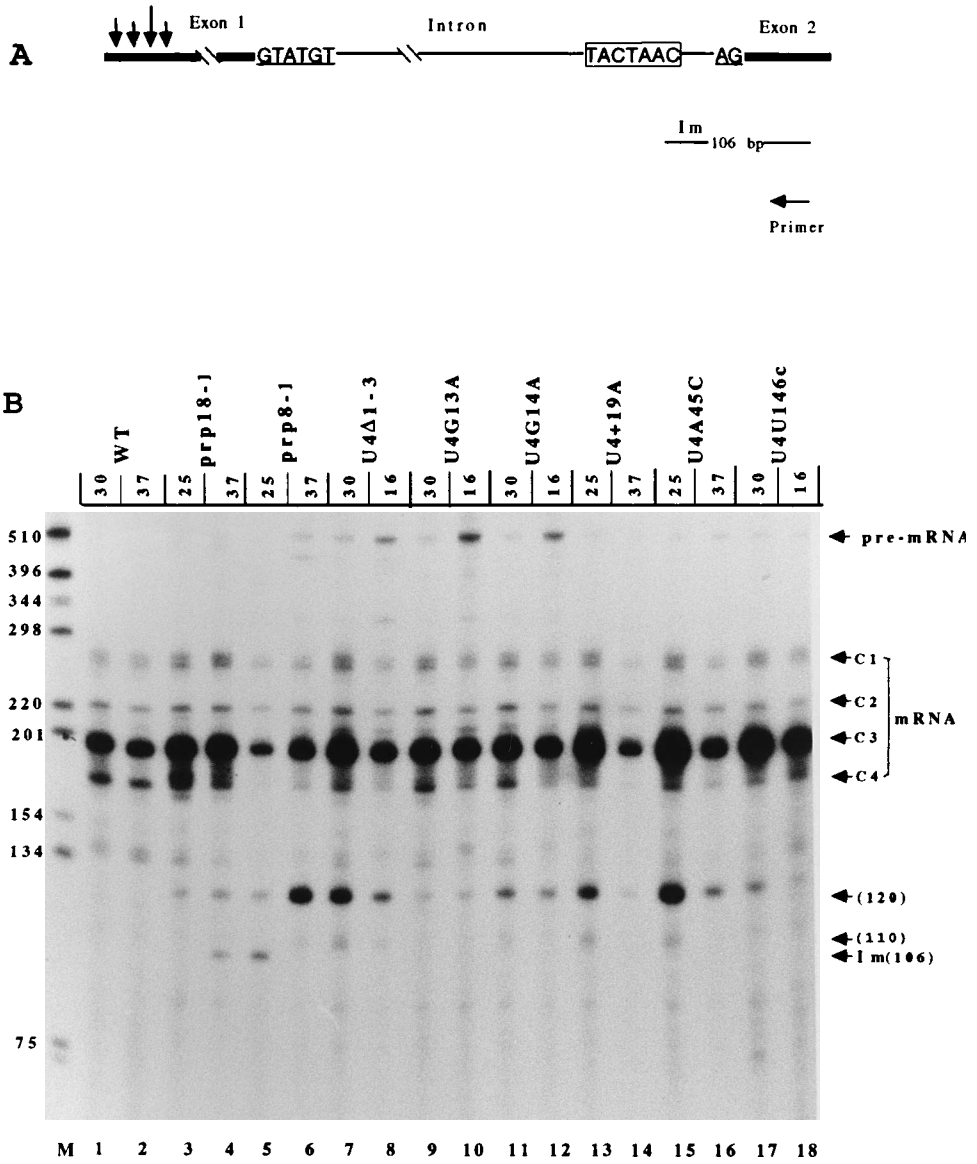



FIG. 6. In vivo splicing of actin pre-mRNA in mutant U4 snRNA strains. (A) Schematic diagram of actin pre-mRNAs. As indicated by the vertical arrows, the transcription of actin pre-mRNAs is initiated from four start sites, with the majority from start site 3 (30). The 5′ and 3′ splice sites are underlined, and the branch site is boxed. The location of the primer used in the experiment as well as the expected sizes of the primer extension products of the lariat intermediate (Im) are shown. (B) Primer extension analyses were done as described in Materials and Methods. Growth temperatures following the shift are indicated above the lanes. The *prp8-1* and *prp18-1* strains were included as positive controls for splicing defects at step 1 and step 2, respectively. Sizes of the molecular weight standards are shown on the left in nucleotides. The primer extension products from the four mature transcripts are designated C1, C2, C3, and C4. The location of the product (106 nucleotides) that is expected from the lariat intermediate is designated I m; this band is apparent only in the *prp18-1* strain at the nonpermissive temperature and the *prp8-1* strain at the permissive temperature (lanes 4 and 5). Two unidentified bands with sizes of 110 and 120 nucleotides are also visible in some lanes. WT, wild type.

tivity to mutation. Five nucleotides of stem II (U-5, A-11, C-12, A-16, and A-17) are insensitive to mutation, and 11 are sensitive (discussed above). In contrast, as is shown in Fig. 2, 3, and 4B, stem I, except for G-58, is almost impervious to mutational change. Single-base changes at many positions of stem I, including U54G, ΔU-54, C59G, U60C, U60G, ΔG-61, ΔU-63, and G65A, had no effect on cell growth, as was the case for a deletion of U63-U64. Furthermore, stem I mutations (except for G-58) do not show a phenotype even in the presence of mutations in other parts of the molecule (Fig. 3).

Second, the 5′ stem-loop is very tolerant of mutation. For example, single-point mutations in many positions of this domain (C-23, U-25, A-29, G-40, A-47, G-52; Fig. 2 and 4B) and even some double mutations (Fig. 3) showed no growth phenotypes. In addition, deletion of bases G-50 to U-56 had no detectable effect on cell growth (Fig. 3). The fully conserved A-45 (31) can be mutated to yield a ts phenotype, although at least one other fully conserved nucleotide, A-29, tolerates mutation.

It should be emphasized that some U4 snRNA mutations that show no effects on growth may nevertheless have a partial splicing defect. We have concentrated our current analysis of those mutations that showed a growth defect, while recognizing that other mutations with subtle effects might go undetected.

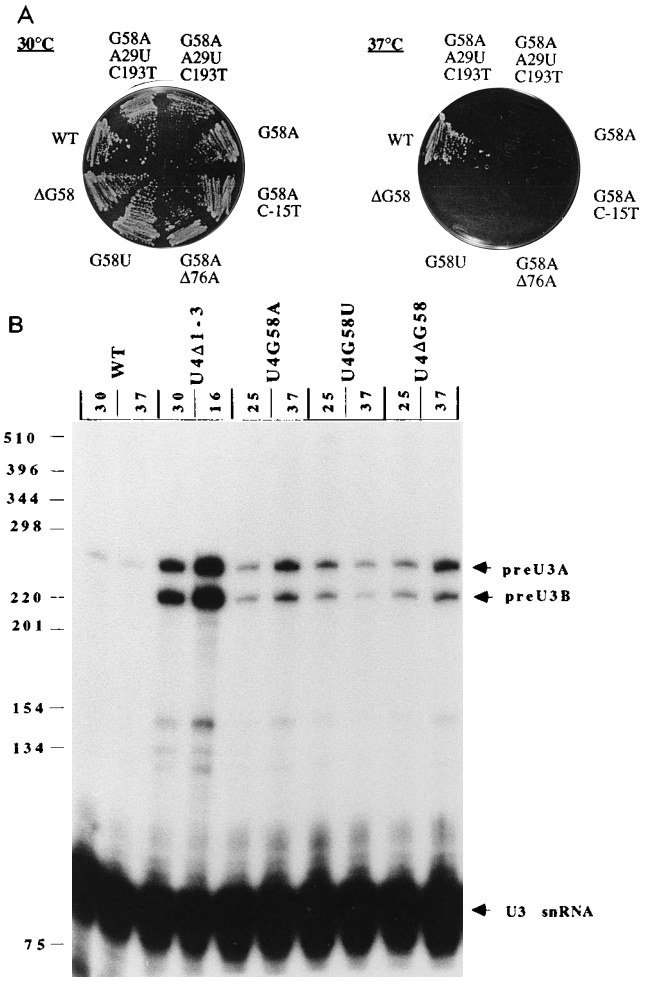



FIG. 7. U4 snRNA mutations at the G-58 position inhibit cell growth and cause accumulation of pre-U3A and pre-U3B snRNAs. (A) Cells harboring a wild-type (WT) or mutant *SNR14* gene on a plasmid (pFL39) were incubated on selective agar medium at 30 or 37°C for 2 days. (B) Total yeast RNA was isolated from wild-type and mutant strains following incubation for 2 h at a permissive (25°C for ts strains and 30°C for cs strains) or a nonpermissive temperature (37°C for ts strains and 16°C for cs strains). The samples were subjected to primer extension with an oligonucleotide that is complementary to a region common to both U3A and U3B (3). The strains used for RNA isolation and the temperatures for cell growth are indicated above the lanes. Negative (WT) and positive (U4Δ1-3) controls are included (lanes 1 to 4). DNA size markers are shown on the left, and bands corresponding to the pre-U3A, pre-U3B, and mature U3 snRNA are designated with arrows on the right.

## DISCUSSION

The studies reported here yield information on the effect of mutations in 105 of the 160 nucleotides of yeast U4 snRNA; 15 critical residues were found. Two regions of the molecule proved to be particularly sensitive to mutational change: stem II (nucleotides 1 to 19) and the putative Sm-binding region (nucleotides 144 to 150). In contrast, the 5′ stem-loop, stem I, the central region, and the 3′ stem-loop can tolerate changes with relatively little harm.

Stem II of U4 snRNA is the least tolerant to mutational change. We found that single-nucleotide substitutions and a three-nucleotide deletion at 11 of the 16 base-paired positions, including 4 positions found in an earlier study (38), confer lethal or conditional-lethal phenotypes. Mutational studies in HeLa cells (48) and *Xenopus* oocytes (46, 47) have indicated the importance of stem II for assembly of the U4/U6 snRNA. In addition, base pairing at two positions (U4 G-9/U6 C-72 and U4 G-14/U6 C-67) is important for in vitro RNA-RNA interaction of the deproteinized U4/U6 snRNAs (38). No evidence exists as to the importance of base pairing in other regions of stem II. However, the present studies show that mutational changes can be made in at least four positions of U4 snRNA stem II (nucleotides U-5, A-11, A-17, and A-18) without detectable effect on cell viability; it is also known that two positions on the U6 snRNA side of the stem II (nucleotides C-68 and C-72) can be deleted or otherwise altered without effect (27). Thus, while base pairing may be important to the function of stem II, this structure appears to be required for additional interactions with proteins or other RNAs.

This suggestion is supported by the following observations. (i) The *PRP24* gene has been identified as a suppressor of a U4 snRNA mutation, G14C, and its gene product is associated with the U6 snRNA (38), implying an association with U4/U6 snRNAs that is dependent on stem II of U4 snRNA. (ii) Stem II of both U4 and U6 snRNAs is not accessible to a complementary DNA oligonucleotide when in the snRNP form but becomes accessible when the snRNAs are deproteinized (53), suggesting protection by some associated protein(s).

Stem I, the other putatively base-paired region of U4/U6 snRNA, is a contrast to stem II. U4 snRNA nucleotides in stem I (nucleotides 59 to 65) are very tolerant of mutational change, but they are not accessible to a complementary DNA oligonucleotide, even in deproteinized form (53). Our results show that all U4 snRNA positions potentially involved in stem I, with the exception of G-58, can be changed with no discernible growth defects. Two of these nucleotides, C-59 and U-60, have already been noted in another study (27). In contrast to a previous finding (27), G-58 mutations in our hands led consistently to a ts phenotype. The mutations at this position also affected pre-mRNA splicing in vitro and in vivo (Fig. 7 and Fig. 9A). Furthermore, spliceosome assembly with mutant G58C extract is stalled at spliceosome complex B (Fig. 9), indicating that stem I of U4 snRNA is required for spliceosome assembly. While almost all nucleotides in stem I of U4 snRNA are tolerant to substitution or deletion, mutations in positions 59 and 60 the U6 snRNA stem I, which lie opposite nucleotides 60 and 59 of U4 snRNA, are deleterious (28). This is another example of asymmetric sensitivities to mutagenesis of the two strands that are potentially involved in helix formation (noted above for stem II).

Thus, current evidence indicates that (i) U4 snRNA stem I is very tolerant of mutational change, while U6 snRNA stem I is sensitive; and (ii) assembly of the U4/U6 snRNP does not appear to depend significantly on stem I base pairing. These conclusions once again suggest that sequences in these regions participate in other types of interactions. Base pairing between nucleotides in stem I of U6 snRNA and nucleotides near the 5′ end of yeast U2 snRNA (28) can explain partially the importance of the nucleotides in stem I of U6 snRNA and also suggests a dynamic interaction among U4, U6, and U2 snRNAs in which U4 snRNA must be dissociated from or loosely associated with U6 snRNA (28). However, the mutational flexibility of U4 stem I is difficult to reconcile with the role of nucleotides in this region as negative regulator of U6 snRNA (28). Perhaps the important interactions of stem I involve proteins.

Stem II, rather than stem I, may be important in the suggested role of U4 snRNA as a negative regulator of mRNA splicing (28), possibly mediated through RNA-protein interactions. Some of these proteins or RNAs may be required to recognize stem II and to facilitate the dissociation of U4 and U6 snRNAs at the late stages of spliceosome assembly. Ac-

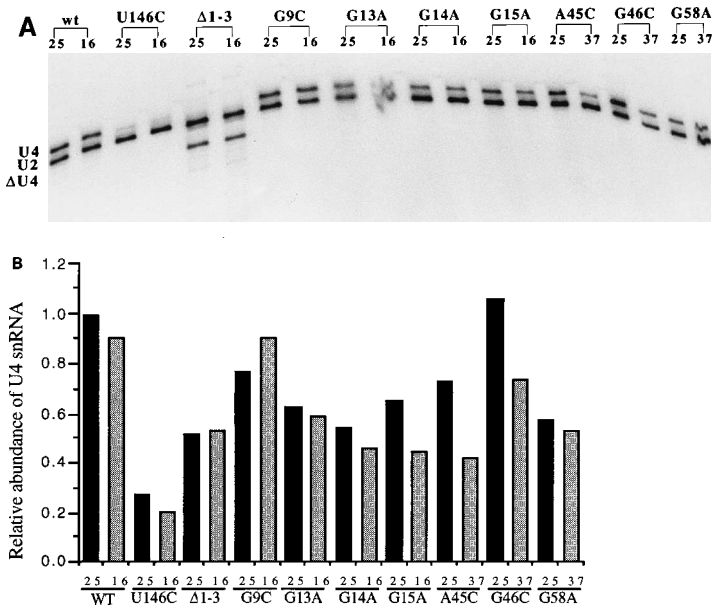



FIG. 8. Stability of mutant U4 snRNAs. (A) Primer extension analysis of mutant U4 snRNAs. Strains and the growth temperatures following the temperature shift (2 h) are shown above the lanes. Preparation of total yeast RNA and primer extension analysis were as described in Materials and Methods. Bands corresponding to the primer extension products of wild-type (wt) and deleted (in the case of Δ1-3) U4 snRNA are designated U4 and ΔU4, respectively. The yeast U2 snRNA was included as a loading control. (B) Quantification of primer extension products with the aid of a PhosphorImager. The bar graph shows the intracellular levels of U4 snRNA following normalization to U2 snRNA.

cording to this idea, single-point mutations in stem II would inhibit binding of the proteins; thus, dissociation of U4/U6 snRNAs as well as subsequent association of U2/U6 snRNAs would fail. This suggests three predictions: (i) in certain U4 snRNA stem II mutant strains (for example, single-base changes at positions 9, 10, 13, and 14), the U4/U6 snRNP stem II might be hyperstable, (ii) mutations that can strengthen the U2/U6 snRNA interactions would be expected to suppress hyperstable mutations in stem II of U4 snRNA, and (iii) the inability of mutant U4 snRNA to dissociate from U6 snRNA would block spliceosome assembly and yield a step-one defect in splicing. Some lines of evidence support this. First, in the G14C mutant strain, the U4/U6 snRNA complex becomes hyperstable so that both U4 and U6 snRNAs can be immunoprecipitated with antibodies against PRP24 protein, while only U6 snRNA can be immunoprecipitated in the wild-type strain (38). Second, a yeast U6 snRNA mutation (A91G), which could theoretically extend the proposed base pairing by one base pair between the 3′ region of U6 snRNA and the 5′ region of U2 snRNA (16, 52), has been identified as a suppressor of the U4 snRNA G14C mutation (38). Finally, we have shown in this study that U4 snRNA stem II mutation G15A blocks splicing in vitro at or before step 1, probably due to the inability to form a functional spliceosome (Fig. 7).

The structurally conserved 5′ stem-loop of U4 snRNA is required for association of the PRP4 protein and formation of the U4/U6.U5 snRNP complex (13). Changes at 20 of 33 positions of this domain, including a deletion (nucleotides 50 to 56) that might disrupt the potential stem, did not affect cell growth. However, single-base substitutions at positions A45C and G46C inhibit cell growth. Thus, these two positions may play an important role in interactions with PRP4 and/or U5 snRNP. In support of the former suggestion, we have observed that overexpression of *PRP4* from the *GAL1* promoter can suppress partially the ts phenotype due to the A45C substitution (unpublished observation). However, there is some difficulty in reconciling this with the result of spliceosome assembly analysis, since the A45C mutant extract was able to form complex A (Fig. 9B). Since the splicing is blocked in the same extract, it is not clear whether the complex A that forms in the mutant extract is normal.

The central parts of human U4 (nucleotides 57 to 72, corresponding to nucleotides 58 to 73 in *S. cerevisiae*) and U6 (nucleotides 36 to 55, corresponding to nucleotides 42 to 61 in *S. cerevisiae*) snRNAs have been shown in vitro to possess hammerhead catalytic activity (54). However, we have found that single-nucleotide changes in most of the central positions, including nucleotides corresponding to the invariable positions of the hammerhead, have no apparent effect on cell growth (Fig. 3). In fact, deletion of U4 snRNA nucleotides from 62 to 88, spanning all those corresponding to the invariable positions of the hammerhead, does not affect cell growth at or above 25°C. Therefore, it is unlikely that the catalytic or $Mg^{2+}$-binding activity of the U6-U4 hammerhead shown in vitro is essential for yeast in vivo splicing. The sequence in the central domain (nucleotides 69 to 88) of yeast U4 snRNA can be replaced with at least two directed repeats of a 38-nucleotide in length without any growth defect (unpublished results), supporting the notion that the primary role of the central domain is to keep the 5′ and 3′ domains of the snRNA at an optimal distance. This is in agreement with the previous finding that the central region is vulnerable to RNase H cleavage in the intact U4/U6 snRNP (53). This is also reminiscent of the central, nonessential domains of U1 (25) and U2 (40) snRNAs in *S. cerevisiae.*

Although the 3′ stem-loop (nucleotides 91 to 142) of U4 snRNA is essential (13), point mutations at 26 of 52 positions in this region showed no apparent growth defects, suggesting that the primary sequence of this domain is very flexible. In contrast, point mutations that confer a lethal or conditional-lethal phenotype have been identified at 4 of the 18 positions in the 3′-terminal domain of U4 snRNA. These mutations are

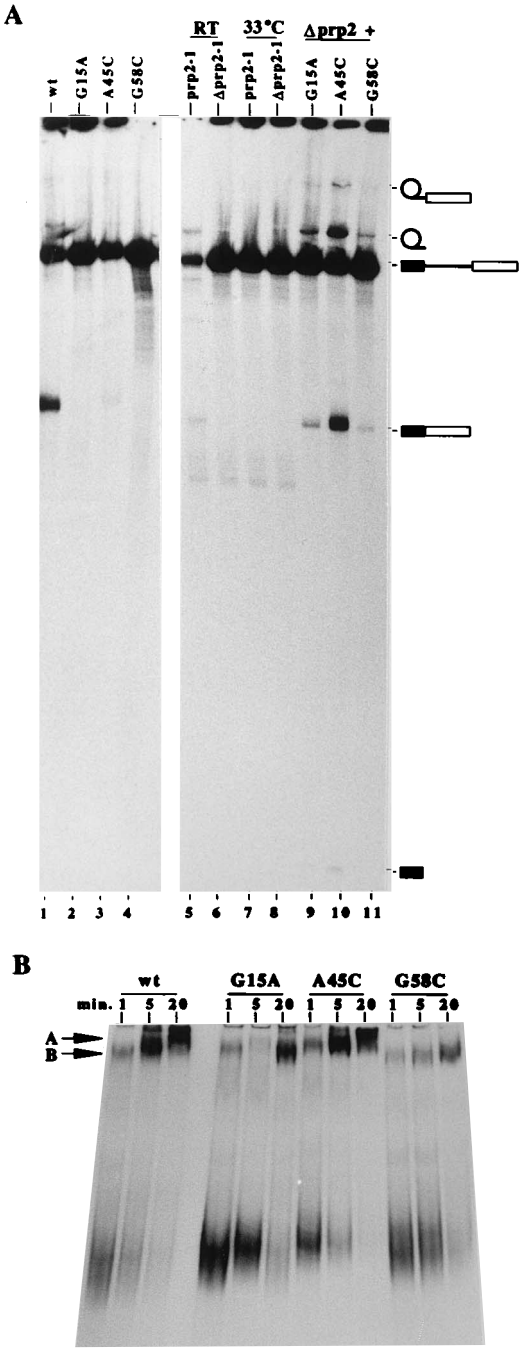



FIG. 9. In vitro RNA splicing and spliceosome assembly analyses. (A) Complementation of splicing activity of mutant U4 extracts by the heat-inactivated *prp2-1* extract. Extracts were prepared as described in Materials and Methods, and inactivation of *prp2-1* extract was achieved by incubating the extract at 37°C for 45 min. Splicing reactions were carried out at 33°C for 30 min except in lanes 5 and 6, in which cases the reactions were performed at room temperature (RT) to show the splicing activity difference between the heat-inactivated and untreated *prp2-1* extracts. The precursor, intermediates, and splicing product of actin RNA are represented at the right. wt, wild type. (B) In vitro spliceosome assembly. The spliceosome assembly analyses were performed as described by Cheng and Abelson (15). The reaction mixtures were incubated at room temperature for a length of time indicated at the top of each lane, and the complexes were resolved on 4% polyacrylamide gel in Tris-acetate-EDTA buffer. The spliceosome complexes are designated A and B (15).

clustered in the putative Sm-binding site, AAUUUUUGG. Although deletion of the corresponding region of the HeLa cell U4 snRNA did not have a great effect on in vitro splicing or spliceosome assembly, it abolished Sm binding (48). On the contrary, the putative Sm-binding site of the yeast U5 snRNA, UAUUUUUUGG, is remarkably tolerant to mutation despite its high degree of conservation (23). This could be due to the additional U residue in the putative Sm-binding site of U5 snRNA. Actually, many double mutations at the putative Sm site of U5 snRNA showed apparent growth defects, although most of the point mutations did not (23). These observations indicate strongly that the putative Sm-binding site of yeast snRNAs is important functionally.

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## REFERENCES

1. **Abovich, N., P. Legrain, and M. Rosbash.** 1990. The yeast *PRP6* gene encodes a U4/U6 small nuclear ribonucleoprotein particle (snRNP) protein, and the *PRP9* gene encodes a protein required for U2 snRNP binding. Mol. Cell. Biol. **10:**6417–6425.
2. **Anthony, J., and J. Woolford.** 1993. Personal communication.
3. **Ares, M. J., and A. H. Igel.** 1990. Lethal and temperature-sensitive mutations and their suppressors identify an essential structural element in U2 small nuclear RNA. Genes Dev. **4:**2132–2145.
4. **Banroques, J., and J. N. Abelson.** 1989. PRP4: a protein of the yeast U4/U6 small nuclear ribonucleoprotein particle. Mol. Cell. Biol. **9:**3710–3719.
5. **Berget, S. M., and B. L. Robberson.** 1986. U1, U2, and U4/U6 small nuclear ribonucleoproteins are required for in vitro splicing but not polyadenylation. Cell **46:**691–696.
6. **Bindereif, A., T. Wolff, and M. R. Green.** 1990. Discrete domains of human U6 snRNA required for the assembly of U4/U6 snRNP and splicing complexes. EMBO J. **9:**251–255.
7. **Bjørn, S. P., A. Soltyk, J. D. Beggs, and J. D. Friesen.** 1989. PRP4 (RNA4) from *Saccharomyces cerevisiae*: its gene product is associated with the U4/U6 small nuclear ribonucleoprotein particle. Mol. Cell. Biol. **9:**3698–3709.
8. **Black, D. L., and B. Chabot, and J. A. Steitz.** 1985. U2 as well as U1 small nuclear ribonucleoproteins are involved in premessenger RNA splicing. Cell **42:**737–750.
9. **Black, D. L., and J. A. Steitz.** 1986. Pre-mRNA splicing in vitro requires intact U4/U6 small nuclear ribonucleoprotein. Cell **46:**697–704.
10. **Blencowe, B. J., B. S. Sproat, U. Ryder, S. Barabino, and A. I. Lamond.** 1989. Antisense probing of the human U4/U6 snRNP with biotinylated 2′-OMe RNA oligonucleotides. Cell **59:**531–539.
11. **Boeke, J. D., J. Truehart, G. Natsoulis, and G. R. Fink.** 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. **154:**164–175.
12. **Bonneaud, N., O.-K. O., G. .Li, M. Labouesse, L. Minvielle-Sebastia, and F. Lacroute.** 1991. A family of low and high copy replicative, integrative and single-stranded *S. cerevisiae*/*E. coli* shuttle vectors. Yeast **7:**609–615.
13. **Bordonne, R., J. Banroques, J. Abelson, and C. Guthrie.** 1990. Domains of yeast U4 spliceosomal RNA required for PRP4 protein binding, snRNP-snRNP interactions and pre-mRNA splicing in vivo. Genes Dev. **4:**1185–1196.
14. **Bringmann, P., B. Appel, J. Rinke, R. Reuter, H. Theissen, and R. Luhrmann.** 1984. Evidence for the existence of snRNAs U4 and U6 in a single ribonucleoprotein complex and for their association by intermolecular base pairing. EMBO J. **3:**1357–1363.
15. **Cheng, S. C., and J. Abelson.** 1987. Spliceosome assembly in yeast. Genes Dev. **1:**1014–1027.
16. **Datta, B., and A. M. Weiner.** 1991. Genetic evidence for base pairing between U2 and U6 snRNA in mammalian mRNA splicing. Nature (London) **352:**821–824.
17. **Guthrie, C.** 1991. Messenger RNA splicing in yeast: clues to why the spliceosome is a ribonucleoprotein. Science **253:**157–163.
18. **Guthrie, C., and B. Patterson.** 1988. Spliceosomal snRNAs. Annu. Rev. Genet. **22:**387–419.
19. **Hamm, J., N. A. Dathan, and I. W. Mattaj.** 1989. Functional analysis of

mutant *Xenopus* U2 snRNAs. Cell **59:**159–169.
20. **Hashimoto, C., and J. A. Steitz.** 1984. U4 and U6 RNAs coexist in a single small nuclear ribonucleoprotein particle. Nucleic Acids Res. **12:**3283–3293.
21. **Hausner, T. P., L. M. Giglio, and A. M. Weiner.** 1990. Evidence for base-pairing between mammalian U2 and U6 small nuclear ribonucleoprotein particles. Genes Dev. **4:**2146–2156.
22. **Hu, J., Y. Xu, K. Schappert, T. Harrington, A. Wang, R. Braga, J. Mogridge, and J. D. Friesen.** 1994. Mutational analysis of the PRP4 protein of *Saccharomyces cerevisiae* suggests domain structure and snRNP interactions. Nucleic Acids Res. **22:**1724–1734.
23. **Jones, M. H., and C. Guthrie.** 1990. Unexpected flexibility in an evolutionarily conserved protein-RNA interaction: genetic analysis of the Sm binding site. EMBO J. **9:**2555–2561.
24. **Lamond, A. I., M. M. Konarska, P. J. Grabowski, and P. A. Sharp.** 1988. Spliceosome assembly involves the binding and release of U4 small nuclear ribonucleoproteins. Proc. Natl. Acad. Sci. USA **85:**411–415.
25. **Liao, X. L., L. Kretzner, B. Seraphin, and M. Rosbash.** 1990. Universally conserved and yeast-specific U1 snRNA sequences are important but not essential for U1 snRNP function. Genes Dev. **4:**1766–1774.
26. **Lin, R. J., A. J. Newman, S.-C. Cheng, and J. Abelson.** 1985. Yeast mRNA splicing in vitro. J. Biol. Chem. **260:**14780–14792.
27. **Madhani, H. D., R. Bordonne, and C. Guthrie.** 1990. Multiple roles for U6 snRNA in the splicing pathway. Genes Dev. **4:**2264–2277.
28. **Madhani, H. D., and C. Guthrie.** 1992. A novel base-pairing interaction between U2 and U6 snRNAs suggest a mechanism for the catalytic activation of the spliceosome. Cell **71:**803–817.
29. **Maniatis, T., and R. Reed.** 1987. The role of small nuclear ribonucleoprotein particles in pre-mRNA splicing. Nature (London) **325:**673–678.
30. **Munholland, J. M., J. K. Kelly, and A. G. Wildeman.** 1990. DNA sequences required for yeast actin gene transcription do not include conserved CCAAT motif. Nucleic Acids Res. **18:**6061–6068.
31. **Myslinski, E., and C. Branlant.** 1991. A study of U4 snRNA reveals the existence of an evolutionarily conserved secondary structure corresponding to 'free' U4 snRNA. **73:**17–28.
32. **Newman, A., and C. Norman.** 1991. Mutations in yeast U5 snRNA alter the specificity of 5′ splice-site cleavage. Cell **65:**115–123.
33. **Newman, A. J., and C. Norman.** 1992. U5 snRNA interacts with exon sequences at 5′ and 3′ splice sites. Cell **68:**743–754.
34. **Parker, R., P. G. Siliciano, and C. Guthrie.** 1987. Recognition of the TAC TAAC box during mRNA splicing in yeast involves base pairing to the U2-like snRNA. Cell **49:**229–239.
35. **Percival-Smith, A., and J. Segall.** 1986. Characterization and mutational analysis of a cluster of three genes expressed preferentially during sporulation of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6:**2443–2451.
36. **Pikielny, C. W., B. C. Rymond, and M. Rosbash.** 1986. Electrophoresis of ribonucleoproteins reveals an ordered assembly pathway of yeast splicing complexes. Nature (London) **324:**341–345.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Shannon, K. W., and C. Guthrie.** 1991. Suppressors of a U4 snRNA mutation define a novel U6 snRNP protein with RNA-binding motifs. Genes Dev. **5:**773–785.
39. **Sharp, P. A.** 1987. Splicing of messenger RNA precursors. Science **235:**766–771.
40. **Shuster, E. O., and C. Guthrie.** 1988. Two conserved domains of yeast U2 snRNA are separated by 945 nonessential nucleotides. Cell **55:**41–48.
41. **Siliciano, P. G., D. A. Brow, H. Roiha, and C. Guthrie.** 1987. An essential snRNA from *S. cerevisiae* has properties predicted for U4, including interaction with a U6-like snRNA. Cell **50:**585–592.
42. **Soltyk, A., M. Tropak, and J. D. Friesen.** 1984. Isolation and characterization of the *RNA2*$^+$ and *RNA11*$^+$ genes of *Saccharomyces cerevisiae*. J. Bacteriol. **160:**1093–1100.
43. **Steitz, J. A.** 1988. "Snurps." Sci. Am. **258:**56–60.
44. **Strathern, J. N., and D. R. Higgins.** 1991. Recovery of plasmids from yeast into *Escherichia coli*: shuttle vectors. Methods Enzymol. **194:**319–328.
45. **Teigelkamp, S., M. McMarvey, M. Plumpton, and J. Beggs.** 1994. The splicing factor PRP2, a putative RNA helicase, interacts directly with pre-mRNA. EMBO J. **13:**888–897.
46. **Vankan, P., C. McGuigan, and I. W. Mattaj.** 1990. Domains of U4 and U6 snRNAs required for snRNP assembly and splicing complementation in *Xenopus* oocytes. EMBO J. **9:**3397–3404.
47. **Vankan, P., C. McGuigan, and I. W. Mattaj.** 1992. Roles of U4 and U6 snRNAs in the assembly of splicing complexes. EMBO J. **11:**335–343.
48. **Wersig, C., and A. Bindereif.** 1990. Conserved domains of human U4 snRNA required for snRNP and spliceosome assembly. Nucleic Acids Res. **18:**6223–6229.
49. **Whittaker, E., and J. D. Beggs.** 1991. The yeast PRP8 protein interacts directly with pre-mRNA. Nucleic Acids Res. **19:**5483–5489.
50. **Woolford, J. L.** 1989. Nuclear pre-mRNA splicing in yeast. Yeast **5:**439–457.
51. **Wu, J., and J. L. Manley.** 1989. Mammalian pre-mRNA branch site selection by U2 snRNP involves base pairing. Genes Dev. **3:**1553–1561.
52. **Wu, J. A., and J. L. Manley.** 1991. Base pairing between U2 and U6 snRNAs is necessary for splicing of a mammalian pre-mRNA. Nature (London) **352:**818–821.
53. **Xu, Y., B. S. Petersen, and J. D. Friesen.** 1990. The PRP4 (RNA4) protein of *Saccharomyces cerevisiae* is associated with the 5′ portion of the U4 small nuclear RNA. Mol. Cell. Biol. **10:**1217–1225.
54. **Yang, J., R. Cedergren, and Nadal-Ginard.** 1994. Catalytic activity of an RNA domain derived from the U6-U4 RNA complex. Science **263:**77–81.
55. **Yean, S. L., and R. J. Lin.** 1991. U4 small nuclear RNA dissociates from a yeast spliceosome and does not participate in the subsequent splicing reaction. Mol. Cell. Biol. **11:**5571–5577.
56. **Zhuang, Y., H. Leung, and A. M. Weiner.** 1987. The natural 5′ splice site of simian virus 40 large T antigen can be improved by increasing the base complementarity to U1 RNA. Mol. Cell. Biol. **7:**3018–3020.
57. **Zhuang, Y., and A. M. Weiner.** 1989. A compensatory base change in human U2 snRNA can suppress a branch site mutation. Genes Dev. **3:**1545–1552.