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Efficient association of U2 snRNPs with pre-mRNA requires an essential U2 RNA structural element

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To understand the role of U2 RNA structure in pre-mRNA splicing we have characterized several cold-sensitive mutations in an essential stem–loop of yeast U2. Although mutant U2 is stable in vivo after a shift to restrictive temperature, splicing is rapidly inhibited, suggesting a direct effect on U2 function rather than U2 synthesis or snRNP assembly. Splicing complexes form at 23°C in both mutant and wild-type extracts; however, stable association of mutant U2 snRNPs with pre-mRNA in vitro is inefficient at 15°C, a temperature permissive for spliceosome assembly in wild-type extracts, indicating that the cold-sensitive defect is in U2 snRNP association with the assembling spliceosome. In vivo RNA structure probing reveals that the bulk of U2 RNA is misfolded in the mutants, even at permissive temperature. We propose that U2 stem–loop IIa is recognized by an assembly factor that assists U2 snRNP binding to pre-mRNA and that the cold sensitivity is due to a critical deficiency of correctly folded U2 for spliceosome assembly at low temperatures. Evolutionary conservation of the potential to form an interfering alternative RNA structure suggests the possibility that splicing could be regulated negatively at an early step by control of U2 snRNA conformation.

[*Key Words*: Splicing; spliceosome; cold-sensitive mutations; yeast]

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Removal of introns from nuclear pre-mRNA occurs in a large RNA–protein complex called the spliceosome (for reviews, see Steitz et al. 1988; Woolford 1989; Guthrie 1991; Ruby and Abelson 1991). Four small nuclear ribonucleoprotein particles (snRNPs), U1, U2, U4/U6, and U5, combine with pre-mRNA and each other in an ordered pathway to form the active complex. In mammals, the U2 snRNP associates with pre-mRNA early during complex formation at a site in the intron that includes the branchpoint (for review, see Steitz et al. 1988). The association of U2 with the intron requires U2AF (Ruskin et al. 1988; Zamore and Green 1989) and is influenced by other proteins (Kramer 1988; Garcia-Blanco et al. 1989) that recognize the polypyrimidine tract in the intron to mediate U2 binding. Binding of mammalian U2 snRNPs can occur on transcripts lacking a 5′-splice site and in extracts where the 5′ end of U1 snRNA has been removed (Steitz et al. 1988), but some feature of the U1 snRNP may be required for U2 binding in mammalian extracts (Barabino et al. 1990). In both yeast and mammals, U2 RNA is required to base-pair near the intron branchpoint for efficient splicing to occur (Parker et al. 1987; Wu and Manley 1989; Zhuang and Weiner 1989).

The spliceosome assembly pathway in yeast extracts differs from that observed in mammalian cell extracts. In yeast, binding of the U2 snRNP requires a conserved intron branchpoint sequence UACUAAC (Legrain et al. 1988; Ruby and Abelson 1988; Seraphin and Rosbash 1989), as well as prior binding of the U1 snRNP (Ruby and Abelson 1988; Seraphin and Rosbash 1989). There is no strong dependence on a polypyrimidine tract or the 3′-splice site for in vitro spliceosome assembly in yeast (Woolford 1989), although many yeast introns are U-rich in the region between the branchpoint and the 3′-splice site (Parker and Patterson 1987), and changes in the pyrimidine content of this region can influence efficiency of 3′-splice site use in vivo (Patterson and Guthrie 1991). Of the yeast pre-RNA processing (*prp*) mutants (for review, see Guthrie 1991; Ruby and Abelson 1991), the *PRP9* gene product has been shown to be required for stable association of the U2 snRNP with the pre-mRNA in vitro (Abovich et al. 1990). A fraction of U2 snRNPs in yeast splicing extracts can be immunoprecipitated by antibodies against an epitope-tagged Prp9 protein, suggesting it may mediate U2 addition to the assembling spliceosome as part of a complex that interacts directly with the U2 snRNP (Abovich et al. 1990). The *PRP5* gene product, a putative RNA helicase (McFarland and Abelson 1989), is also reported to be required for this step (see McFarland and Abelson 1990; Ruby and Abelson 1991). In addition, there is evidence for a factor that recognizes the UACUAAC element before U2 snRNP binding (Seraphin and Rosbash 1989, 1991). The relationships between these yeast factors and proteins, such as mamma-

lian U2AF, are unclear. Despite these differences, U2 RNA sequences are highly conserved, and mammalian U2 sequences can replace those of yeast for splicing in vitro (McPheeters et al. 1989) and in vivo (Shuster and Guthrie 1990; Miraglia et al. 1991).

What special features of the U2 snRNP are recognized by spliceosome assembly factors? The known U2-specific intrinsic snRNP proteins A′ and B′′ (for review, see Luhrmann 1988) would seem obvious candidates, but U2 snRNPs lacking the A′ and B′′ proteins function in splicing (Hamm et al. 1989; Pan and Prives 1989). Direct recognition of the RNA moiety of the snRNP is also possible; and thus far two RNA elements unique to U2 are known to be essential for U2 snRNP function in yeast: the branchpoint interaction region (nucleotides 32–39, Parker et al. 1987; Miraglia et al. 1991) and stem–loop IIa (nucleotides 48–67, Ares and Igel 1990). Both of these regions of U2 snRNA are highly conserved, and either of them could interact with an assembly factor during U2 binding to pre-mRNA. The branchpoint interaction sequence can be altered, and the snRNA will still function in splicing as a suppressor U2 (Parker et al. 1987; Wu and Manley 1989; Zhuang and Weiner 1989), suggesting that the primary sequence in this region has no conserved function besides base-pairing with the intron (Wu and Manley 1989). Experiments with oligodeoxynucleotide-targeted RNase H digestion of U2 snRNPs (Steitz et al. 1988; Zillman et al. 1988) or the masking of parts of the snRNP with 2′-modified oligoribonucleotides (Barabino et al. 1989; Lamond et al. 1989) produce different answers to the question of the role in spliceosome assembly of either the branchpoint interaction element or the conserved sequences at the very 5′ end of U2 (which base-pairs with U6 snRNA; Hausner et al. 1990; Datta and Weiner 1991; Wu and Manley 1991). A possibility suggested by genetic analysis of U2 RNA is that stem–loop IIa is required for binding of U2 snRNPs to pre-mRNA early in spliceosome assembly (Ares and Igel 1990; Miraglia et al. 1991).

To study the role of U2 stem–loop IIa in splicing we have analyzed the effects of temperature on splicing in cold-sensitive yeast U2 mutations in vivo and in vitro. We find that mutants carrying lesions in stem–loop IIa are cold sensitive for splicing in vivo. In splicing extracts made from cold-sensitive mutants, the formation of U2 snRNP-containing splicing complexes is also cold sensitive. The mutations disrupt the folded structure of U2 in vivo, but temperature-dependent changes cannot be detected. Taken together, the results indicate that efficient formation of an appropriate U2 RNA secondary structure is important for assembly of U2 snRNPs into splicing complexes and suggest that stem–loop IIa of U2 RNA is required for stable association of the U2 snRNP with pre-mRNA, perhaps through interaction with an assembly factor.

Results

Growth phenotypes of point mutations in U2 stem–loop IIa

Previous work indicated that non-Watson–Crick appositions in the 53–62 bp within stem–loop IIa of yeast U2 produce a recessive cold-sensitive or heat- and cold-sensitive growth phenotype on agar plates containing rich medium (Ares and Igel 1990). The positions of these mutations in the secondary structure model for yeast U2 are shown in Figure 1. The single-base changes have the following phenotypes on plates (Ares and Igel 1990): C62G, lethal; G53C, heat and cold sensitive, slow growing at 30°C; G53A, cold sensitive; and C62U, cold sensitive. Compensatory double mutations G53C : C62G and G53A : C62U grow like wild type.

We examined the growth phenotypes of the G53A and C62U mutants in more detail. In rich liquid medium and on plates, strains carrying the cold-sensitive mutations continue to grow slowly at 18°C, the restrictive temperature, but never achieve the cell density or colony size of wild type (not shown). Cells are not killed by long-term exposure to restrictive temperature because when cultures are shifted to restrictive temperatures or diluted and plated at permissive temperature, viable cell numbers are consistent with estimates of cell number with optical density measurements (not shown). Taken to-

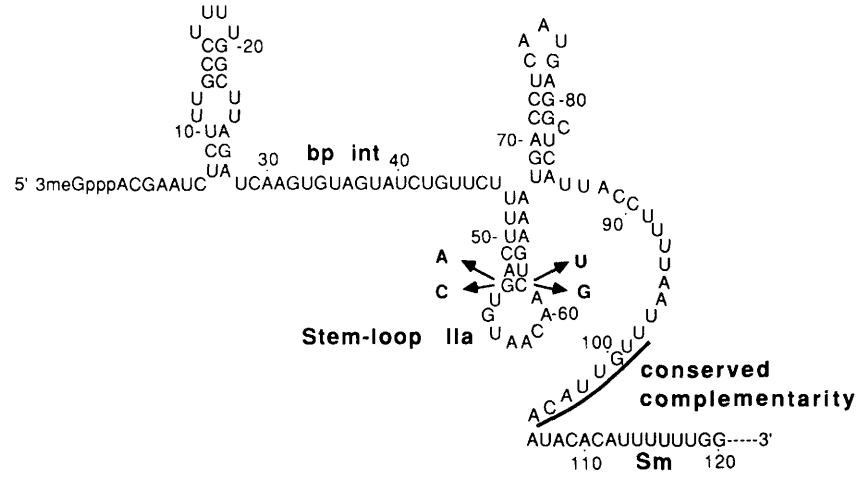

Figure 1. Location of U2 stem–loop IIa mutations in the U2 secondary structure. Alterations of the G53–C62 base pair are indicated. The heavy line indicates the phylogenetically conserved complementarity to the loop of stem–loop IIa. (bp int) Branchpoint interaction region; (Sm) core snRNP protein-binding site.

gether, the data indicate that the defect in the U2 mutants is reversibly inhibitory to growth rather than lethal.

Splicing is cold sensitive in the U2 stem–loop IIa mutants

To determine whether the cold-sensitive U2 RNA mutations have a direct effect on splicing, we isolated RNA from wild-type and mutant strains immediately after a shift from 30°C to restrictive temperature (18°C) and examined the splicing of intron-containing transcripts. The U3A and U3B genes contain introns that differ in size by 27 nucleotides (Myslinski et al. 1990), allowing us to measure the levels of both pre-U3 transcripts in the same sample. An oligonucleotide complementary to the second exon of U3A and U3B was annealed to total RNA and extended with reverse transcriptase. Strains with wild-type U2 showed no change in the ratio of pre-U3 to mature U3 after the temperature shift (Fig. 2). The G53A and C62U single mutants contain slightly elevated levels of unspliced U3 transcripts at permissive temperature (Ares and Igel 1990), and upon a shift to restrictive temperature, the levels increase dramatically (Fig. 2). In the G53A : C62U compensatory double mutant, wild-type growth is restored (Ares and Igel 1990), and little or no accumulation of unspliced U3 is observed after temperature shift, indicating that the cold-sensitive splicing inhibition found in both single mutants is suppressed.

To compare the time course of splicing inhibition by the U2 RNA mutations with that of a known temperature-sensitive mutation affecting splicing, we shifted a *prp4* strain carrying a heat-sensitive U4 snRNP protein (Banroques and Abelson 1990; Xu et al. 1990) from 23°C to 36°C. Unspliced U3A accumulated to a similar extent in the *prp4* strain as in the U2 mutants (Fig. 2, cf. lanes 7). The extent of pre-U3B accumulation on *prp4* inactivation is less, most likely as a result of strain background differences in levels of U3B expression. We conclude that splicing of pre-U3 transcripts is inhibited in the U2 mutants soon after a shift to restrictive temperature.

U3 RNA is relatively stable; hence, we did not expect to see substantial immediate loss of spliced U3 as a result of inhibition of splicing. To determine whether splicing continued at low levels in the U2 mutants at restrictive temperature we assessed splicing of the first intron of the unstable *MATa1* mRNA (Miller 1984). There is a rapid loss of mature *MATa1* mRNA and the accumulation of pre-mRNA in a *prp2* mutant after temperature shift (Miller 1984). We performed primer extension analysis in the presence of ddCTP in place of dCTP using an appropriate primer (Fig. 3). The *MATa1* pre-mRNA has no G residues between the 3′ end of the primer and the G at the 3′-splice site of the first intron so that primer extension on *MATa1* pre-mRNA gives a 38-nucleotide product. *MATa1* transcripts lacking the first intron should direct a 70-nucleotide termination product at the first G upstream of the 5′-splice site (Astell et al. 1981). RNA isolated from our strain background yielded a 57-nucleotide product (Fig. 3) as a result of a polymorphism in the *MATa1* first exon (M. Zavanelli, M. Ares, and R. Samaha, unpubl.; see also Miller 1984). The two cold-sensitive mutants G53A and C62U showed a rapid increase in the ratio of unspliced to mature transcript

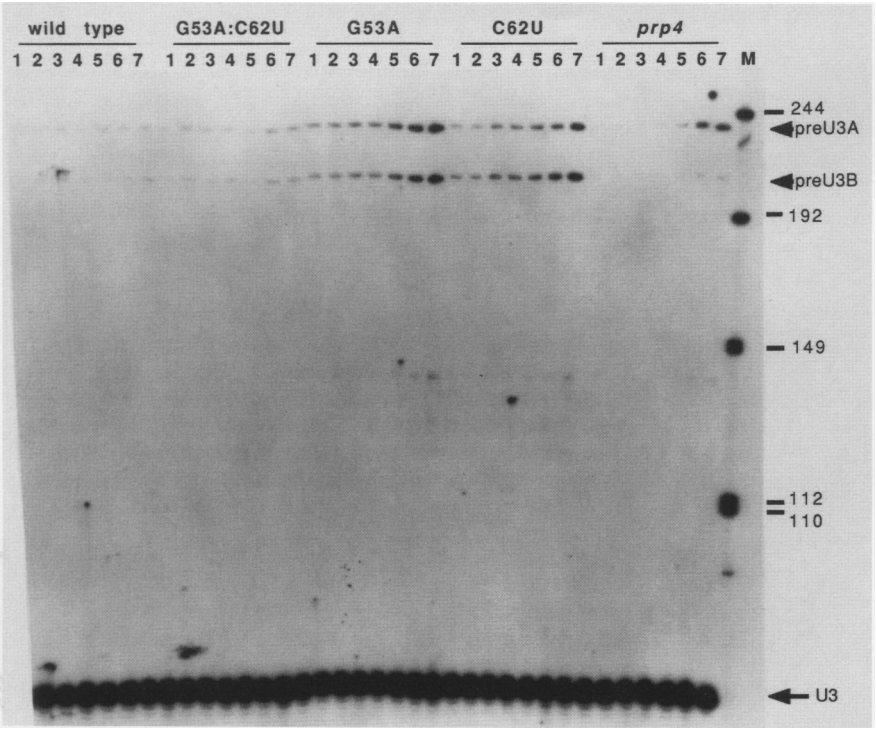

Figure 2. Inhibition of pre-U3 splicing with temperature shift in cold-sensitive U2 mutant strains. Cells were grown at 30°C and shifted to 18°C (wild type, G53A : C62U, G53A, and C62U) or grown at 23°C and shifted to 36°C (*prp4*) for 0 (lanes *1*), 5 (lanes *2*), 10 (lanes *3*), 20 (lanes *4*), 45 (lanes *5*), 60 (lanes *6*), or 120 (lanes *7*) min. RNA was extracted and used in a primer extension assay with a labeled oligonucleotide complementary to the second exon of U3A and U3B genes. The arrow indicates the position of migration of U3; arrowheads indicate those of pre-U3 cDNAs. The product at ~140 does not correspond to any known U3 precursor or splicing intermediate and represents either a strong stop in one of the introns or a degradation product of pre-U3. Markers (lane *M*) are filled-in *Hpa*II fragments of pUC13.

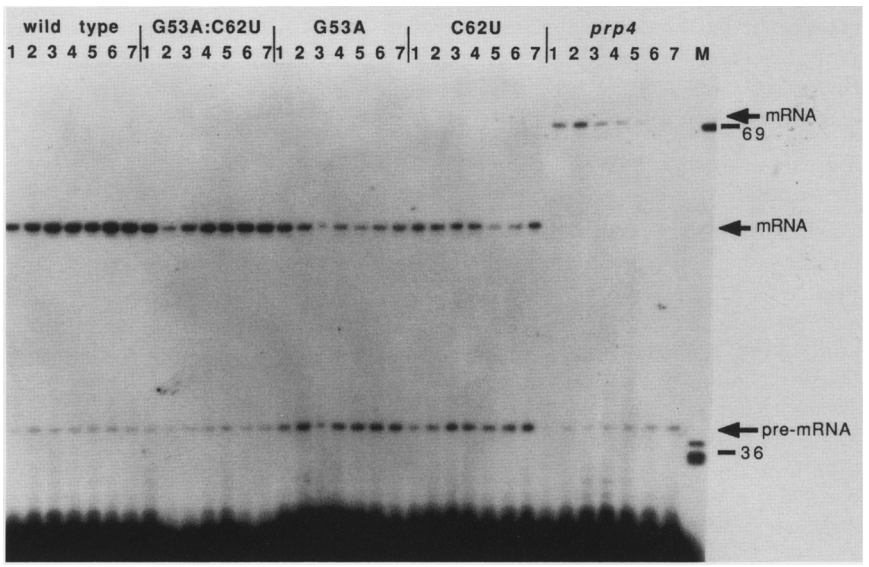

Figure 3. Inhibition of splicing of the *MATa1* gene first intron with temperature shift in cold-sensitive U2 mutant strains. Cells were grown at 30°C and shifted to 18°C (wild type, G53A : C62U, G53A, and C62U) or grown at 23°C and shifted to 36°C (*prp4*) for 0 (lane *1*), 5 (lane *2*), 10 (lane *3*), 20 (lane *4*), 45 (lane *5*), 60 (lane *6*), or 120 (lane *7*) min. RNA was extracted and used in a primer extension assay with a labeled oligonucleotide complementary to the second exon of the *MATa1* gene. The primer is positioned such that the first G in the template encountered by the enzyme during primer extension is either the G at the 3′-splice site (arrow, pre-mRNA) or either one of two Gs (depending on the *MATa1* allele; see text) found in the first exon (arrows, mRNA). The reaction contains ddCTP in place of dCTP. Markers (lane *M*) are filled-in *Hpa*II fragments of pUC13.

within 5–10 min after the shift to restrictive temperature (Fig. 3, cf. G53A lane 1 with G53A lanes 2 and 7). Complete loss of spliced *MATa1* mRNA was not observed (G53A and C62U, lanes 7), even after much longer times (not shown). In contrast, splicing inhibition in the *prp4* mutant was established more slowly, with kinetics similar to that observed by Miller (1984) for *prp2*; therefore, by 120 min spliced transcripts were not detectable (cf. G53A lane 7 and C62U lane 7 with *prp4* lane 7). This is not the result of greatly different stabilities of *MATa1* transcripts at 18°C versus 36°C because unspliced *MATa1* transcripts do not accumulate in wild type after a shift to 18°C (Fig. 3, wild type, lane 7), and the heat-sensitive U2 mutant G53C behaves like the cold-sensitive U2 mutants with respect to spliced and unspliced *MATa1* RNAs after a shift to 36°C (not shown). Splicing of the *MATa1* first intron in the G53A : C62U double mutant is unaffected by a shift to restrictive temperature, indicating that the splicing defects seen in the single mutants are suppressed by restoration of Watson–Crick base-pairing between the nucleotides at these positions (Fig. 3).

Although splicing is greatly inhibited in the U2 mutants, it is not completely blocked, suggesting that a U2 function before the first step of splicing is slowed in the mutants and has become growth rate limiting in the cold. To accurately determine the effectiveness of the cold-sensitive block is difficult owing to uncertainties about the rates of synthesis, splicing, or degradation of splicing precursors and products, as well as the activity of the critical components at permissive temperature and rapidity and extent of inactivation of these components after the temperature shift. Because the steady-state ratio of *MATa1* pre-mRNA to mRNA in the mutants is greater than wild type at permissive temperature and increases to a maximum within 5–10 min after the temperature shift, we conclude that the inhibition of splicing is most likely the result of a direct effect of cold on the function of mutant U2. Primer extension experiments designed to detect accumulation of lariat–exon 2 splicing intermediates after a shift to restrictive temperature for a number of spliced transcripts (*MATa1*, *RPL32*, *CRY1*, actin) showed no accumulation of such intermediates (data not shown), indicating that the U2 function rendered cold-sensitive in these mutants is required for the first step of splicing. We conclude that a U2 function before the first step of splicing is slowed in the mutants and has become growth rate limiting in the cold.

Stability of mutant U2 is unaffected by shift to restrictive temperature

U2 stem–loop IIa mutations do not affect U2 RNA stability at permissive temperature (Ares and Igel 1990). To exclude the possibility that the temperature-sensitive growth and splicing-inhibition phenotypes are the result of a rapid destabilization of the mutant U2 RNAs at the restrictive temperatures, we compared the steady-state levels of the mutant RNA to pseudo-wild-type U2 RNA at 30, 18, and 36°C (Fig. 4). The pseudo-wild-type U2 has a C → U change at position 121 that does not significantly affect U2 RNA accumulation or function and allows discrimination of two different U2 RNAs in a mixture (Ares and Igel 1990). The cold-sensitive mutant U2 RNAs accumulate to equivalent levels with respect to the pseudo-wild-type U2 at all temperatures (Fig. 4). Therefore, a decrease in the level of mutant U2 RNA at the restrictive temperatures is not responsible for the temperature-sensitive growth or splicing-inhibition phenotype.

Association of U2 snRNPs with pre-mRNA is cold-sensitive in splicing extracts from the mutants

To identify the U2 function compromised by disruption of stem–loop IIa, we prepared splicing extracts from iso-

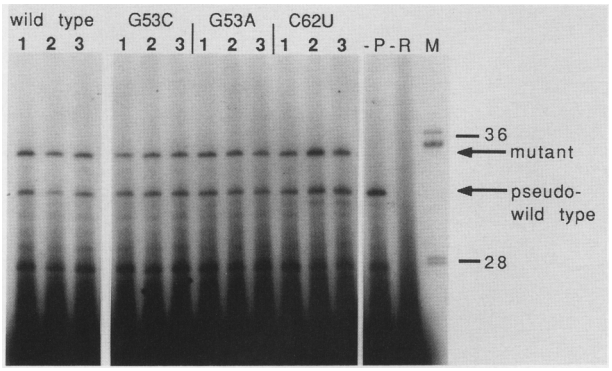

Figure 4. Steady-state levels of cold-sensitive mutant U2 RNA at different temperatures. Plasmids containing wild-type, G53C, G53A, or C62U U2 genes were introduced into a haploid yeast strain containing a pseudo-wild-type U2 gene marked by the C121U mutation. Cultures were grown at either 30°C (lanes *1*), 18°C (lanes *2*), or 36°C (lanes *3*). A control strain with only the U2 C121U plasmid was grown at 30°C (lane *−P*). RNA was extracted and used in a primer extension assay with a U2 primer (23T) placed so that the first U in the template encountered by the enzyme is either U121 (arrow, pseudo-wild type) or U118 (arrow, mutant). The reaction contains ddATP in place of dATP. A reaction without template is shown (lane *−R*). Markers (lane *M*) are end-filled *Hpa*II fragments of pUC13. All lanes are from the same gel; intervening lanes were cut out.

genic strains containing mutant U2. Strain BJ81 contains a wild-type U2-coding region fused to a *GAL*-regulated promoter in the chromosome (Miraglia et al. 1991) and is galactose dependent unless a functional, constituitively expressed U2 gene is present on a plasmid. After the desired U2 mutation is introduced, the strain is grown on glucose at permissive temperatures, and only the mutant U2 accumulates in the cell. Extracts are prepared as usual at 4°C (Lin et al. 1985) and are active when assayed at 23°C, indicating that the cold treatment necessary for the extraction procedure does not irreversibly inactivate the mutant U2 (Fig. 5A,B). The experiments presented in Figure 5 were performed using the same wild-type and mutant extracts and have been repeated with multiple C62U and G53A extracts. We have not observed cold-sensitive splicing or splicing complex formation in any wild-type extract.

Using a labeled actin pre-mRNA similar to that described by others, we assayed splicing (Lin et al. 1985). RNA was extracted at times after initiation of the splicing reactions and separated on denaturing gels to evaluate reaction of the pre-mRNA. Splicing intermediates and products are detected within 5 min in wild-type extracts at 23°C (lane 4) but are not apparent until 10 min in either wild-type extracts at 15°C (lane 5) or mutant extracts at 23°C (lane 5) nor in mutant extracts at 15°C until between 10 and 20 min of incubation (lane 6). The delayed appearance of splicing intermediates and products suggested that an early step in the process, perhaps spliceosome assembly, might be cold sensitive.

We monitored spliceosome assembly by native gel electrophoresis and could detect splicing complexes identified and characterized by others (Pikielny and Rosbash 1986; Pikielny et al. 1986; Cheng and Abelson 1987; Seraphin and Rosbash 1989, 1991). The collection of complexes in bracket 1 (Fig. 5B) form late in the reaction (including a species containing the splicing intermediates and products; data not shown) and are equivalent to the A complexes of Cheng and Abelson (1987) and complexes I and II of Pikielny et al. (1986), and contain the U2, U4/U6 or U6, and U5 snRNPs (Pikielny et al. 1986; Cheng and Abelson 1987). The complex in bracket 2 is equivalent to the B complex (Cheng and Abelson 1987) or complex III (Pikielny et al. 1986), which contains U2, but not U4/U6 or U5 (Pikielny et al. 1986; Cheng and Abelson 1987). This complex contains unreacted precursor and requires ATP for its formation (Pikielny et al. 1986; Cheng and Abelson 1987; data not shown). The complex in bracket 3 also contains unreacted precursor, does not require ATP for formation (not shown), and may represent commitment complexes (Seraphin and Rosbash 1989, 1991).

Splicing reactions were carried out at two temperatures (23°C and 15°C) and sampled at times after the addition of the splicing extract to the reaction (Fig. 5B). Complexes in bracket 3 form early and efficiently in both mutant and wild-type extracts, suggesting that this step in splicing complex formation is unaffected by the U2 mutations. This is consistent with the representing commitment complexes that form in U2-depleted extracts as described by Seraphin and Rosbash (1989, 1991). The first U2-containing complex (bracket 2) can be detected after 1 min in wild-type extracts at 23°C (Fig. 5B, wild type at 23°C, lane 2) and within 2 min in either mutant extracts at 23°C (lane 3) or wild-type extracts at 15°C (lane 3). In mutant extracts at 15°C, however, substantial amounts of this complex never form (lanes 1–7). These observations indicate that the stable addition of U2 snRNPs to the assembling spliceosome is cold sensitive in mutant extracts. Formation of bracket 1 complexes is also delayed and inefficient in mutant extracts at 15°C (lanes 5–7). These complexes are visible by 5 min in mutant and wild-type extracts at 23°C (lane 4), form more slowly in wild-type extracts at 15°C (lane 5), and are detected at low levels in mutant extracts at 15°C (lanes 5–7). Given the proposed dependence of the formation of bracket 1 complexes on the assembly of the bracket 2 complex (Pikielny et al. 1986; Cheng and Abelson 1987), it is likely that the inhibition of formation of these complexes is the result of the impact of temperature on the formation of the first U2-containing complex.

Estimates of initial rates of complex formation in these extracts using densitometry suggest that C62U mutant extracts assemble U2-containing complexes only twofold more slowly than wild type at 23°C, but more than fivefold more slowly at 15°C. That is, wild-type extracts assemble U2-containing complexes at a rate almost 6-fold lower at 15°C than at 23°C, whereas the mutant extracts assemble U2-containing complexes nearly 15-fold more slowly at 15°C than at 23°C. Because

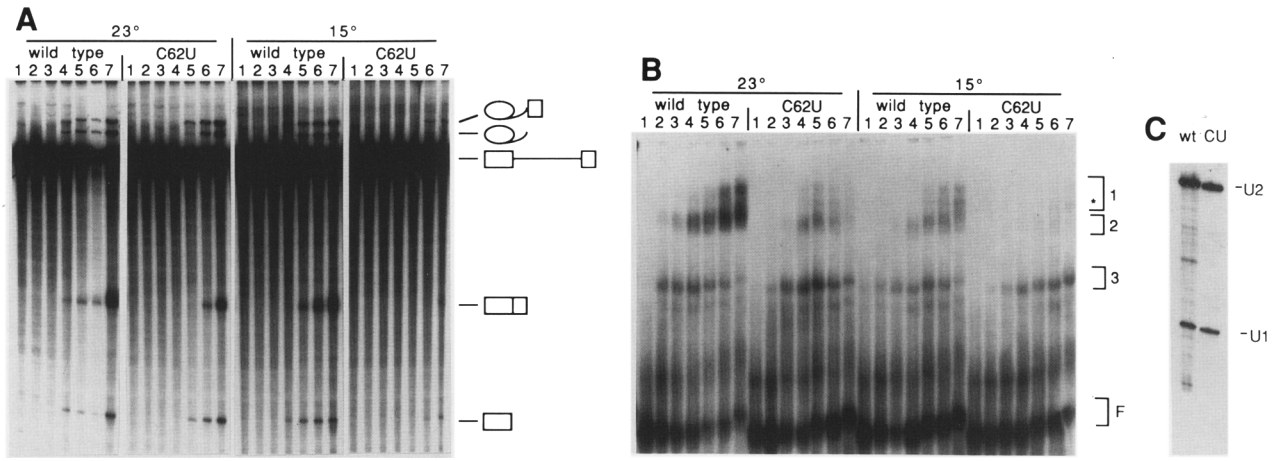

Figure 5. Splicing is cold sensitive in extracts from U2 stem–loop IIa mutants. (*A*) Time course of substrate reaction in mutant and wild-type splicing extracts at different temperatures. Splicing reactions were assembled using a labeled actin pre-mRNA and extracts from the indicated strains, incubated at the indicated temperatures and sampled at 0 (lanes *1*), 1 (lanes *2*), 2 (lanes 3), 5 (lanes *4*), 10 (lanes 5), 20 (lanes 6), and 40 (lanes 7) min. RNA was extracted and separated on a denaturing gel by electrophoresis. Positions of migration of the precursor, intermediates, and products are indicated at *right*. All samples are from the same experiment: A photograph of the autoradiogram was cut and rearranged to present positions as in *B*. (*B*) Time course of splicing complex formation in mutant and wild-type splicing extracts at different temperatures. Splicing reactions were assembled using a labeled actin pre-mRNA and extracts from the indicated strains, incubated at the indicated temperatures, and sampled at 0 (lanes *1*), 1 (lanes *2*), 2 (lanes 3), 5 (lanes *4*), 10 (lanes 5), 20 (lanes 6), and 40 (lanes 7) min. Samples were taken, quenched, and loaded on a native acrylamide–agarose gel to separate splicing complexes. Complexes labeled in brackets *1*, *2*, and *3* are discussed in the text. Bracket *F* indicates the position of migration of naked substrate RNA. (*C*) Mutant U2 is abundant in splicing extracts. RNA was extracted from the indicated splicing extracts and used in a primer extension reaction with two labeled primers: one complementary to U1 and the other complementary to U2. Positions of migration of full-length primer extension products derived from U1 and U2 RNAs are indicated.

multiple components and multiple steps are required to assemble a U2-containing complex (for review, see Ruby and Abelson 1991), a lack of understanding of which, if indeed the same, step is rate limiting in the two extracts, and the relationship of splicing complex formation rate in vitro to splicing rate in vivo complicates interpretation. Nonetheless, the results indicate that extracts made from the cold-sensitive U2 mutant are differentially impaired for in vitro splicing complex formation in the cold and suggest that U2 snRNP addition to the assembling spliceosome is the cold-sensitive step.

After 40 min of incubation, the amount of splicing intermediates and products generated in the mutant and wild-type extracts at 15°C was similar, in spite of the dramatic inhibition of complex formation. This is because the amount of pre-mRNA participating in complex formation in our wild-type extracts greatly exceeds the amount that eventually becomes spliced, probably because a factor required for the splicing reactions, but not for splicing complex formation, is limiting. The small amounts of U2-containing complexes that form inefficiently in the mutant extracts are sufficient to produce amounts of splicing intermediates and products similar to that observed in wild-type extracts where splicing complex formation is efficient. This indicates that although the capacity of the extract for the substrate cleavage–ligation reactions is only modestly affected, the efficiency of stable association of U2 snRNPs with pre-mRNA in vitro is reduced dramatically by the stem–loop IIa mutations at low temperatures.

To exclude the possibility that mutant U2 RNA is poorly represented or unstable in mutant splicing extracts, we measured amounts of U2 RNA present in wild-type and mutant splicing extracts (Fig. 5C). As an internal control, we monitored the amounts of U1 RNA. There are equivalent concentrations of both U2 and U1 RNAs in both the wild-type and the C62U splicing extracts used for the experiments in Figure 5, A and B, as well as others (not shown), indicating that the in vitro splicing defect is not due to poor extractability or representation of U2 snRNA in mutant extracts.

The secondary structure of the bulk of mutant U2 RNA is perturbed in a temperature-independent fashion

To determine whether conditional U2 function might be correlated with changes in the conformation of U2 during a shift to restrictive temperatures, we probed the structure of U2 in vivo using dimethylsulfate (DMS). DMS reacts with N1 of adenosines and N3 of cytosines, which are not involved in base-pairing (Inoue and Cech 1985; Moazed et al. 1986) and can be applied to live yeast cells for RNA structure-probing purposes (Ares and Igel 1990).

We analyzed the 5′ domain of U2 RNA in the wild-type and mutant strains grown at the permissive temperature of 30°C or shifted to 18°C or 36°C (Fig. 6A). Nucleotides in the complementary sequences required to form stem–loop IIa are relatively protected from reac-

tion with DMS, whereas the loop nucleotides are reactive, consistent with the existence of this stem–loop in a large fraction of wild-type U2 molecules in vivo, at least at 30°C (Ares and Igel 1990; Fig. 6A, wild-type, lane 2). The nucleotides in the phylogenetically conserved complementarity to the loop (Fig. 1) are also reactive (Fig. 6A and B), in agreement with genetic experiments showing that this complementarity is dispensable, and, hence, cannot be absolutely required to pair with the loop during the function of U2 in splicing (Ares and Igel 1990). The wild-type pattern of nucleotide accessibility observed at 30°C is also observed at 18 and 36°C (Fig. 6A, wild type, lanes 2–4).

Changes in the reactivities of residues in the single mutants at 30°C are very apparent and do not change detectably with temperature (Fig. 6A). For both the G53C and G53A mutations, there is a loss of reactivity of residues A57, A58, and C59. Increased reactivity of C51 and A52 is also observed, as well as at position 53 where an unreactive G residue has been replaced with A or C, in both cases introducing unpaired, DMS-reactive nucleotides. In addition, there is decreased reactivity of A103 and C104 in both of these mutants (Fig. 6A). These data are consistent with disruption of at least part of the stem of stem–loop IIa and pairing of the loop with residues in the conserved complementarity, as depicted in Figure 6C, causing the bulk of the U2 to be folded into a structure similar to that proposed by Keller and Noon (1985; Fig. 6C).

In the C62U mutant, a decrease in the reactivity of A57, A58, and C59 is also detected; however, there is also a loss of reactivity of A60 and A61 and a less signif-

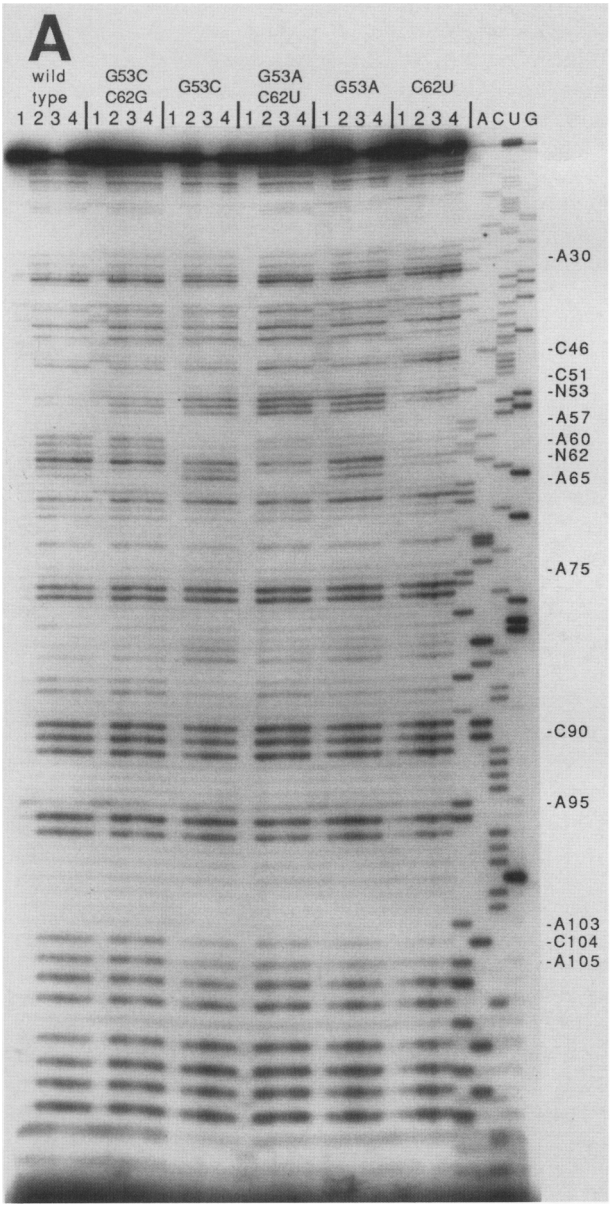

Figure 6. Altered secondary structure in U2 mutants. (*A*) DMS reactivity of individual nucleotides in wild-type and mutant U2. Cells with the indicated U2 genes were incubated at 30°C (lanes *1*,*2*), 18°C (lanes *3*), or 36°C (lanes *4*) for 4 hr and treated with DMS for 2 min, and the reaction was stopped (lanes *2–4*); or they were incubated without DMS and then treated with stop solutions before the addition of DMS (stop control, lanes *1*). RNA was extracted and sites of modification were mapped relative to an RNA sequence ladder generated by primer extension using unmodified wild-type U2 RNA and dideoxynucleoside triphosphates. (Lane *A*) Reaction contained ddTTP; (lane *C*) ddGTP; (lane *U*) ddATP; (lane *G*) ddCTP. Position of migration of the dideoxynucleotide-generated termination products for selected nucleotides are indicated at *right*. Note that DMS modification-induced termination products are 1 nucleotide shorter than the corresponding dideoxynucleotide termination product. (*B*) DMS reactivities of nucleotides in stem–loop IIa and the conserved complementarity. Sequence from position 45–113 is shown. Relative reactivity is indicated as follows: (Solid minus) Strongly protected; (open minus) protected; (open plus) reactive; (solid plus) strongly reactive. (*C*) Changes in reactivity and alternative folding in the G53A mutant. Sequence from position 45–113 is shown, with the mutant position circled. Changes in reactivity are indicated as follows: (+) Increased reactivity; (−) decreased reactivity; (o) little or no change in reactivity. A65, A66, and A67 are not shown pairing but could be paired, as discussed in the text.

icant gain of reactivity in C51 and A52 (Fig. 6A). Because the residue at position 62 was changed from a C to an unreactive U, we are unable to determine how this residue is affected. The C62U mutation extends the potential for base-pairing an additional 2 bp and, hence, may stabilize a protecting interaction between A60 and U99, and between A61 and U98 (Fig. 6C). Because the reactivity of 6 nucleotides decreases (A57, A58, C59, A60, A103, and C104) and only 2 increase slightly (C51 and A52) in the C62U mutant (Fig. 6A), it is possible that both helices form simultaneously in a pseudoknot (Pleij et al. 1985; Puglisi et al. 1988; Ares and Igel 1989).

There is little change in the reactivities of A66 or A67 in any of the mutants, suggesting that either the mutations do not disrupt the potential interactions with U49 and U48 (and also produce a pseudoknot) or that they pair elsewhere, perhaps with U87 and U86 (see Fig. 6C). For unknown reasons A65 is more reactive than might be expected of a paired A residue even in wild type, making the significance of its high reactivity in the mutants difficult to interpret.

The wild-type pattern of reactivity is only incompletely restored in both the compensatory double mutants G53C : C62G and G53A : C62G (Fig. 6A). Reactivities of residues 57–61 in the loop of stem–loop IIa, as well as A103 and C104 in the conserved complementarity are restored to near wild-type levels in the double mutants, indicating that the alternative folding depicted in Figure 6C is suppressed. In contrast, the increased reactivity of C51 and A52 observed in the single mutants is not suppressed, suggesting that the stem may form less efficiently in these double mutants than in wild type, although neither strain is temperature sensitive.

It is important to note that in instances where two or more distinct folded forms are present in roughly equal amounts, the patterns of reactivity will be superimposed, making structure description difficult. The predominant misfolded forms consistent with the structure-probing data (Fig. 6A) are similar to those proposed previously for U2 structure based on phylogenetic considerations (Keller and Noon 1985; Ares and Igel 1989). The mutations could affect protein binding, and this might account for some of the alterations in DMS reactivity, further complicating the interpretation of the nucleotide reactivities. In spite of these difficulties, none of the effects on U2 RNA structure in the single mutants is influenced in a dramatic way by temperature, suggesting that cold sensitivity is not due to the conversion of the bulk of U2 from a correctly folded form at permissive temperature to a misfolded, nonfunctional form at restrictive temperature. We conclude that the main impact of these mutations on U2 RNA structure is the temperature-independent disruption of stem–loop IIa.

Discussion

We have characterized several point mutations in U2 snRNA that are cold sensitive for growth and splicing. We have shown that unspliced transcripts accumulate soon after a shift to restrictive temperature (Figs. 2 and 3), but that in contrast to the heat-sensitive U4 snRNP mutant *prp4*, the cold-sensitive U2 mutants continue splicing at reduced rates at restrictive temperature (Fig. 3). Association of U2 snRNPs with actin pre-mRNA transcripts is impaired in mutant splicing extracts in the cold (Fig. 5), indicating that the stem–loop IIa structure perturbed by the mutations is essential for this association. Surprisingly, the secondary structure perturbations caused by the mutations are largely temperature independent, suggesting that either a small fraction of U2 need be correctly folded for function or that there is sufficient interconversion of folded forms to supply spliceosome assembly reactions (Fig. 6).

A U2 RNA secondary structure is required for association of U2 snRNPs with pre-mRNA

Mutations in stem–loop IIa cause several phenotypes that allow an important RNA structure–function correlation to be made. Alteration of the Watson–Crick base pair between U2 nucleotides 53 and 62 causes the bulk of U2 snRNA in the cell to be folded differently from wild type and in a fashion that disturbs the integrity of stem–loop IIa (Fig. 6), an essential structure (Ares and Igel 1990). Disruption of stem–loop IIa is accompanied by reduced rates of splicing in vivo (Ares and Igel 1990), especially at restrictive temperatures (Figs. 2 and 3), as well as inefficient association of U2 snRNPs with pre-mRNA at restrictive temperatures in vitro (Fig. 5). These data argue that stem–loop IIa is required for U2 snRNP association with pre-mRNA during spliceosome assembly.

The stem–loop may function by interacting with one or more splicing factors reported to be required for U2 snRNP binding to the assembling spliceosome (Abovich et al. 1990; Ruby and Abelson 1991). The *PRP9* gene product is required for U2 snRNP-containing complexes to form in vitro, and a fraction of U2 snRNPs are precipitated by an antibody against an epitope-tagged Prp9 fusion protein (Abovich et al. 1990). It has been argued that Prp9 does not fit the definition of a U2 snRNP protein; hence, a reasonable hypothesis is that Prp9 protein is a spliceosome factor that associates with the fraction of U2 snRNPs participating in splicing (Abovich et al. 1990). The *PRP5* gene product is reported to be required for U2 snRNP participation in splicing complexes in vitro (Ruby and Abelson 1991), and either of these could also be involved in a direct physical interaction with stem–loop IIa or as part of a complex that must interact with stem–loop IIa. Prp9 has a zinc finger-like motif possibly associated with RNA binding (Legrain and Choulika 1990), whereas Prp5 has the DEAD signature of RNA helicases (for reviews, see Ruby and Abelson 1991; Guthrie 1991). Additional experiments will be required to determine which, if any, of these components may interact directly with stem–loop IIa. Because of the broad phylogenetic conservation of the stem–loop IIa sequence (Guthrie and Patterson 1988; Ares and Igel 1989, 1990),

structural elements of components interacting with it may also be conserved.

Mutations in stem–loop IIa could conceivably block function in one or both of two ways. First, a mutation could cause a structural distortion of the stem owing to the presence of a mismatched base pair. This distortion could reduce binding by spliceosome assembly factors that might interact with the stem–loop. Alternatively, and consistent with the structure-probing data (Fig. 6), a mutation could block efficient formation of stem–loop IIa such that only a small fraction of U2 molecules in the cell were folded to contain it (albeit in distorted form), and this population might be too small to support efficient splicing. In the second model, the absence of the stem–loop IIa, rather than a subtle alteration of its structure, causes the defect. Additional experiments will be necessary to determine whether one or both of these mechanisms are at work.

RNA misfolding and cold sensitivity

How do mutations in stem–loop IIa render U2 snRNP function in spliceosome assembly cold-sensitive (Figs. 2, 3, and 5) and yet cause temperature-independent misfolding (Fig. 6)? A heat-sensitive phenotype might have been expected of a mutation predicted to decrease the thermal stability of an essential RNA helix. Why do mutant strains grow almost as well as wild-type strains at permissive temperature even though most of their U2 is misfolded? This apparent conflict can be reconciled by a kinetic hypothesis (Fig. 7). In this model, U2 RNA is in a state of kinetic interconversion or flux between alternatively folded forms in vivo (and in splicing extracts), only one of which can function in assembly. This interconversion might or might not require an enzyme and would produce a characteristic steady-state population distribution, which could be sampled in a structure-probing experiment. Near equilibrium, the most stable forms will predominate, and the distribution will be altered by mutations that change the relative stabilities of alternatively folded forms. If the rate of interconversion is high, a functional conformation may occur with sufficient frequency to supply spliceosome assembly reactions with correctly folded U2 at permissive temperature. In the cold, the rate of interconversion could be expected to drop, without appreciably affecting the steady-state distribution. The spliceosome assembly reactions would rapidly deplete the small pool of correctly folded U2 snRNPs, interconversion would be too slow to replenish it, and U2 refolding would become the growth rate-limiting step.

Kinetic data on rates of simple interconversion between alternative RNA secondary structures with equivalent or similar stabilities are not extensive. Neither are data on the effects of temperature on such rates, although practically speaking, low temperatures are used to stabilize nucleic acid structure in the laboratory (e.g., see Williamson et al. 1989), whereas heat treatment can be used to renature and "activate" RNA molecules that have adopted nonfunctional structures (e.g., see Walstrum and Uhlenbeck 1990 and references therein). It is possible that there is little or no interconversion and that the cold-sensitive step is the addition of the U2 snRNP to the prespliceosome. In this case, the natural cold sensitivity of this step would be masked by an excess of correctly folded U2 in wild-type cells and become apparent only when a limited amount of correctly folded U2 is available, as in the mutants.

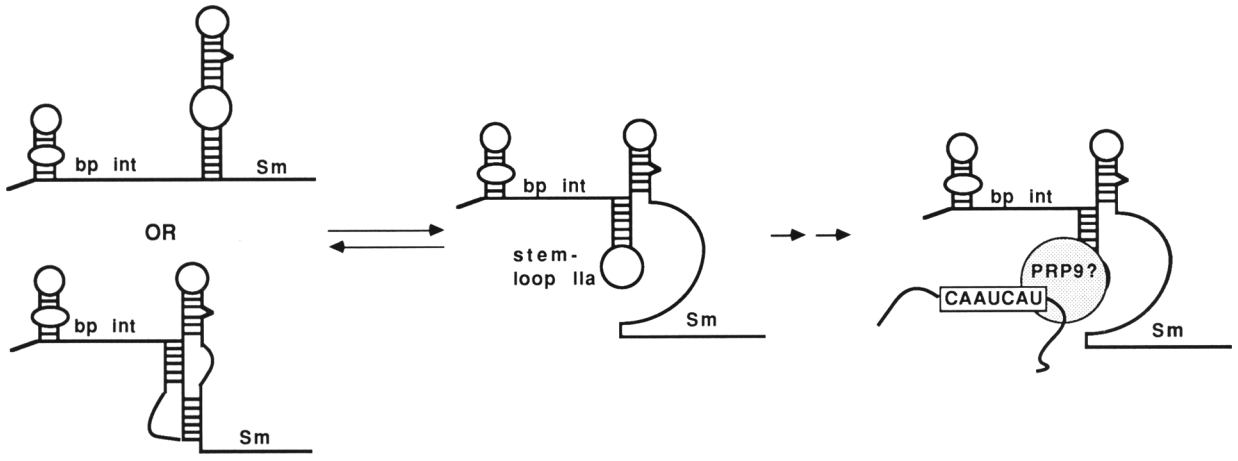

Figure 7. Model for early steps in U2 snRNP assembly into splicing complexes. In wild type, most U2 is folded to contain stem–loop IIa (*middle*). Spliceosome assembly factors, perhaps including Prp9 protein, interact with stem–loop IIa to bind U2 to the assembling spliceosome near the pre-mRNA branchpoint (*right*). U2 folded to contain stem–loop IIa (*middle*) is in flux with alternatively folded forms (*left*), owing to interconversion. Mutations that reduce the stability of stem–loop IIa relative to the competing helix that could form between the loop and the conserved complementarity will drive the distribution to the left, and most of the U2 in the cell will not contain stem–loop IIa. At high temperatures, interconversion is efficient, and although present in small steady-state amounts, sufficient U2 containing stem–loop IIa is continuously generated by interconversion to feed the spliceosome assembly reactions. At low temperature in the mutants, the rate of interconversion drops and splicing is inhibited.

What is the role of the competing helix?

The unusual presence of a potentially interfering, dispensable, yet highly conserved complementarity within the U2 structure is enigmatic. Phylogenetic analysis reveals multiple compensating base changes that preserve complementarity with the loop of stem–loop IIa (Guthrie and Patterson 1988; Ares and Igel 1989), and yet this complementarity is dispensable (Ares and Igel 1990). Even in U2 RNA from divergent eukaryotes, such as trypanosomes and their relatives (Hartshorne and Agabian 1990; Tschudi et al. 1990), an analogous, conserved complementarity persists (M. Ares, unpubl.). This argues that some feature of U2 RNA function is enhanced by conservation of an alternative RNA structure that disrupts stem–loop IIa. Previously, we suggested that positive U2 function could be augmented by this structure but that its contribution could not be essential for splicing (Ares and Igel 1990). It is also possible to conceive a general negative regulatory role for this sequence, given its conservation and the structure-probing data suggesting that it participates in a helix at the expense of stem–loop IIa in the mutants (Fig. 6). A factor sensitive to a signal indicating that splicing is to be turned off might direct the interconversion of the U2 snRNPs in the cell to an alternatively folded, nonfunctional conformation, and splicing would be shut off rapidly at an early step. Regulation of RNA function by alternative secondary structures has been implicated in translation of λcIII mRNA, which influences the lysis–lysogeny decision after infection (Altuvia et al. 1989), as well as in the replication control of ColE1 plasmids (Masukada and Tomizawa 1986). If such a system operates on U2, the need for general negative regulation of splicing (and *trans*-splicing that requires U2; Tschudi and Ullu 1990) must also be conserved. So far, the only environmental stimulus reported to invoke a general inhibition of splicing is heat shock (Bond 1988; Muhich and Boothroyd 1988; Shukla et al. 1990; Delannoy and Caruthers 1991; Yost and Lindquist 1991). In any event, the availability of yeast U2 mutants lacking this complementarity provides experimental approaches to these questions.

Materials and methods

Yeast strains

All strains were grown according to standard procedures (Sherman et al. 1986). For the in vivo analysis of splicing inhibition and structure probing we used an isogenic set of strains generated by plasmid shuffling and varying only in the U2 allele they carry on a centromeric *LEU2* plasmid (Ares and Igel 1990). They are *MAT***a**, *leu2-3,112*, *ura3-52*, *his4-619*, *lys2*, and *snr20*::*URA3* carrying YCpU2 with the indicated allele. For the RNA stablity experiments the strains also carry a *LYS2* centromeric plasmid with a pseudo-wild-type U2 allele marked by the C121U mutation as an internal U2 RNA stability reference (Ares and Igel 1990). As indicated, this strain background has a polymorphic **a** allele at the *MAT* locus. The temperature-sensitive splicing mutant containing the *prp4* allele was obtained from the Yeast Genetic Stock Center (Berkeley, CA) and expresses a *MAT***a** allele consistent with the published sequence (Astell et al. 1981; see also Miller 1984).

For splicing extracts, an isogenic set of strains based on BJ81 was used. BJ81 is *MAT***a**, *GAL2*, *leu2-3,112*, *ura3-52*, *trp1*, *pep4-3*, *prb1*, *prc1*, and contains a glucose-repressible wild-type U2 allele integrated into the chromosome (Miraglia et al. 1991). When transformed with the appropriate U2 allele on a centromeric *LEU2* plasmid and grown on glucose, only the plasmid-borne U2 gene is expressed.

Yeast strains were grown in 50 ml of YEPD or synthetic complete media lacking the appropriate amino acids (Sherman et al. 1986) to mid-log phase of growth (A_{600} = 0.5–1.0), diluted, shifted to nonpermissive temperature by the addition of prewarmed or prechilled medium, and incubated for the indicated times before RNA extraction or structure probing.

RNA extraction and analysis

Total RNA was extracted essentially as described previously (Ares and Igel 1990). Cell pellets were resuspended in 0.5 ml of AK buffer (AK buffer = 1 gram of triisopropylnaphthalene sulfonic acid, 6 grams of sodium *p*-aminosalicylate, 1.17 gram of sodium chloride, and 6 ml of phenol per 100 ml). Hot (65°C) phenol (0.5 ml) was added, and the mixture was vortexed for 30 sec, incubated at 65°C for 30 min, and vortexed twice for 30 sec each. The mixture was spun in a microcentrifuge for 5 min, and the aqueous phase was removed and extracted once with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and once with chloroform. Sodium acetate (pH 5.2) was added to a final concentration of 0.3 M, and the RNA was precipitated by the addition of 3 volumes of 95% ethanol. The pellet was resuspended in water to a nucleic acid concentration of 1.0 mg/ml (A_{260} = 25).

Primer extensions were as follows for a 20-μl reaction: 5–12 μg of total RNA and 0.2 ng of end-labeled oligonucleotide primer in 13 μl of water plus 1 μl of 10× RT buffer [1.25 M Tris-Cl, 175 mM KCl (pH 8.3)] was incubated at 65°C for 5 min and then at the appropriate annealing temperature (determined empirically) for an additional 30 min. Six microliters of extension cocktail (per reaction: 1 μl of 10× RT buffer, 1 μl of 0.1 M dithiothreitol, 2 μl of 0.1 M $MgCl_2$, 1 μl of either 2.5 mM all dNTPs or, in the case of dideoxynucleotide replacement, a mix containing 100 μM of the appropriate ddNTP in place of the corresponding dNTP, 0.5 μl of 1.0 mg/ml actinomycin D, and 0.25 μl of AMV reverse transcriptase (Life Sciences, 17–22 U/μl) was added, and the mixture was incubated at 42°C for 30 min. Five microliters of RNase solution [10 mg/ml of RNase A, 30 mM EDTA, and 0.6 M sodium acetate (pH 5.2)] was added. After 5–10 min incubation at 42°C, 15 μl of a proteinase K solution (10 mg/ml of proteinase K, 0.2% SDS, and 0.6 M sodium acetate) was added, and samples were incubated for 10 min at 65°C. The samples were extracted once with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and precipitated with 95% ethanol. Samples were resuspended in 98% formamide, 0.1× TBE, and dyes and electrophoresed on 7.5 M urea 6% or 8% polyacrylamide gels. Reference U2 sequence ladders were generated by doping each of four primer extension reactions containing all dNTPs with 1 μl of a different ddNTP stock as follows: ddATP and ddGTP, 0.75 mM; ddCTP, 1 mM; ddTTP, 1.5 mM.

Oligonucleotide primers were labeled at their 5′ ends with [^{32}P]phosphate using [γ-^{32}P]rATP and polynucleotide kinase. The sequences and the annealing temperatures used are as follows:

23T: GTCTCTTCCCGTCCATTTTATTA (37°C)
MATa*1*: GTTGCTCTACTTTAGTCAAATTACTTTCC (57°C)
U3 21-mer: CCAAGTTGGATTCAGTGGCTC (58°C)
YSTR: ATTATTTTGGGTGCCAA (37°C)
YU1: TTAACGTCCTTCTACTATTGGAAGCGC (58°C)

Splicing extract and substrate preparation

Yeast whole-cell extracts were prepared as described by Lin et al. (1985) from strain BJ81 derivatives grown in YEPD at 30°C to an A_{600} = 3.0–5.0. Actin pre-mRNA substrate was synthesized in vitro using T7 RNA polymerase as described by Milligan and Uhlenbeck (1989). The template was constructed as follows. A derivative of pUC12 carrying a 24-bp insertion of a T7 promoter in the *Eco*RI site that maintains the *lacZ'* reading frame for blue/white selection and recreates the *Eco*RI site in the polylinker was a gift of Dr. Jim Stefano (National Jewish Hospital, Denver, CO). A fragment of the yeast actin gene (Gallwitz and Sures 1980; Ng and Abelson 1980; gift of P. Novick, Yale) was isolated, which extends from the *Bst*YI site at position −81 from the AUG in exon 1 through the 309-nucleotide intron to the *Hpa*II site at position +366 in exon 2, and cloned between the *Bam*HI and *Acc*I sites of the pUC12T7 plasmid. The plasmid DNA was isolated, cleaved with *Hin*dIII, and used as a template. The 488-nucleotide transcript was gel purified and used at a sp. act. of 500–2000 cpm/fmole.

Splicing reactions, complex gels, and detection of splicing intermediates

Splicing reactions were carried out essentially as described by Lin et al. (1985): A 10-μl reaction contained ~10–100 fmoles of substrate, 40% in splicing extract, 3% polyethylene glycol, 60 mM potassium phosphate (pH 7.0), 2.5 mM magnesium chloride, 2 mM spermidine, and 2 mM ATP. GTP was also included at 2 mM. For analysis of splicing substrate reactions, 5-μl samples were incubated in 100 μl of stop buffer (0.3 M sodium acetate, 5 mM EDTA, 0.2% SDS, and 100 mg/ml of proteinase K) at 65°C for 15 min, followed by extraction with an equal volume of acid phenol and precipitation with ethanol. Samples were separated on 6% polyacrylamide–7.5 M urea gels, dried, and autoradiographed.

For analysis of splicing complexes, procedures described by Seraphin and Rosbash (1989, 1991) were used. Splicing reactions (5 μl) were quenched by the addition of 10 μl of cold R buffer [2 mM magnesium acetate and 50 mM HEPES–Na^+ (pH 7.5)] and 5 μl of total yeast RNA at 1 mg/ml. After 10–15 min on ice, 5 μl of loading buffer (50% glycerol, 5 mM EDTA, 0.01% xylene cyanol) was added, and the samples were immediately loaded and electrophoresed at 100 V for 15 hr in the cold (4°C–6°C) on composite acrylamide–agarose gels (3% 60 : 1 acrylamide/bisacrylamide, 0.5% agarose, 5% glycerol in 0.5× TBE; Seraphin and Rosbash 1989). During the time course experiments, the quench time is maintained at 10–15 min and the total electrophoresis time is therefore less for the later time points. The complex gels were dried and autoradiographed. Analysis of substrate reactions within the different complexes was done using electroelution to NA45 DEAE membrane according to Pikielny and Rosbash (1986).

U2 RNA representation in the splicing extracts was monitored by extracting RNA from 100-μl aliquots of wild-type and mutant extracts with 400 ml of AK buffer as described above for whole cells. Primer extension was performed as described above, except that two end-labeled oligonucleotide primers (23T for U2 and YU1 for the U1 internal control) were added to the reaction.

In vivo structure probing

In vivo structure probing was performed essentially as described (Ares and Igel 1990). Strains were grown at permissive temperature and then shifted to restrictive temperatures for 4 hr. Cultures were maintained at A_{600} between 0.5 and 1.0 during this time. Ten-milliliter aliquots of culture were incubated for 2 min at the appropriate temperature with 200 μl of a 1 : 1 dilution of DMS in 95% ethanol. Ten-milliliter ice-cold 2 M 2-mercaptoethanol and 5 ml of water-saturated isoamyl alcohol were added to quench the reactions. Cells were pelleted and washed once with 10 ml of ice-cold 1 M 2-mercaptoethanol, and total RNA was isolated by using AK buffer as above. To demonstrate that little or no modification occurred during RNA extraction, stop controls were done. The cells were grown at 30°C and treated identically, except that the DMS was added after the addition of the isoamyl alcohol and 2-mercaptoethanol.

The sites of modification were mapped by primer extension (Inoue and Cech 1985; Moazed et al. 1986) with the YSTR or 23T oligonucleotides as described above.

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