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Limited functional equivalence of phylogenetic variation in small nuclear RNA: Yeast U2 RNA with altered branchpoint complementarity inhibits splicing and produces a dominant lethal phenotype

(spliceosome assembly/small nuclear ribonucleoproteins/RNA structure)

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ABSTRACT **U2 is a highly conserved small nuclear RNA essential for pre-mRNA splicing in mammals and yeast and for trans-splicing in trypanosomes. To test the function of variant U2 RNA structures from different organisms, we conducted phylogenetic exchanges of U2 domains. Replacing nucleotides 1–120 of yeast U2 with the corresponding region of human U2 generates a U2 RNA that is correctly folded and functions in yeast. In contrast, replacement of the branchpoint interaction region of yeast U2 with the corresponding region from trypanosome is dominant lethal. Using a *GAL*–U2 promoter fusion, we show that the dominant phenotype can be made conditional and that the accumulation of mutant U2 is followed rapidly by inhibition of nuclear pre-mRNA splicing. The results suggest that U2 small nuclear ribonucleoprotein particles normally participate in stable complexes with a limiting splicing factor prior to formation of U2–intron branchpoint base pairs.**

U2 small nuclear RNA (snRNA) is an essential component of the nuclear pre-mRNA splicing machinery (for reviews, see refs. 1 and 2) and is conserved in a wide range of organisms (3, 4). In spite of this, U2 function seems to differ in different species. For example, although an identical sequence near the 5′ end of U2 pairs with the intron (5–7) during splicing in yeast and humans, U2–pre-mRNA binding requirements differ. In mammals, the polypyrimidine tract associated with the branchpoint region and ATP (1), as well as factor U2AF (8), are required. Other factors also influence polypyrimidine tract-dependent binding (8–10). In yeast, binding of U2 small nuclear ribonucleoprotein particles (snRNPs) to pre-mRNA is also ATP-dependent (11, 12); however, yeast introns do not always contain an obvious polypyrimidine tract (13), placing the need for factors analogous to mammalian U2AF in question. Instead, factors requiring the conserved yeast branchpoint sequence UACUAAC prior to U2 binding have been detected (11, 14). Although mammalian U2 snRNPs have a branchpoint sequence-specific binding activity (15), a yeast intron is spliced in a human extract using sequences resembling a mammalian branchpoint, rather than at the more U2-complementary UACUAAC sequence (16).

Dependence on assembly factors notwithstanding, the contributions of U2 structural variation and U2–intron pairing to U2 function are unclear. Mammalian splicing efficiency is influenced by but is not dependent on U2–branchpoint complementarity (6, 7, 17–19) whereas in yeast this requirement seems more rigid (2). Precise selection of the branched nucleotide may also differ because in yeast, only the sixth position of the branchpoint sequence can form a branch (2), while in mammals multiple residues can be used (e.g., see ref. 20).

Trypanosome mRNAs are matured by a trans-splicing process (21) that is U2-dependent (22). *Trypanosoma brucei* U2 is not conserved in the region of yeast and metazoan branchpoint interaction (3, 4). This may be peculiar to trypanosomes rather than to trans-splicing, because nematodes perform a similar trans-splicing reaction (23), but have the metazoan and yeast branchpoint-interaction region (24). The few trans-splicing branchpoints mapped show no consistent complementarity to the cognate U2 (25, 26).

To test species-specific requirements for U2 function, we constructed phylogenetic exchange mutants of U2 and introduced them into yeast. Yeast carrying human U2 sequences are unimpaired for splicing and growth, indicating that little of the U2 sequence variation between these organisms is essential for function in yeast (see also ref. 27). In contrast, substitution of the branchpoint-interaction region with a trypanosome sequence results in a dominant lethal phenotype and inhibition of splicing. Based on the dominant phenotype, we suggest that the U2 snRNP is assembled into a complex with a limiting splicing factor prior to the requirement for U2–intron branchpoint pairing.

MATERIALS AND METHODS

Construction of U2 Mutants. Cloning followed standard procedures (28). For the human exchange, the human U2 coding region (29) was subjected to oligonucleotide-directed mutagenesis (30) using oligodeoxynucleotide HK (5′-CTCCCTGGTACCAAAAATCC-3′) to insert a *Kpn* I site 3′ of the Sm site in human U2. The yeast U2 gene (31) was similarly treated with YK (5′-ACATTTTTTGGTAC-CCAAAA-3′). The *Kpn* I–*Hin*dIII fragment from the 3′ half of the yeast gene was inserted 3′ of the human sequences, and the yeast U2 promoter was inserted upstream using polylinker sites. Polylinker sequences near the start site of transcription and the internal *Kpn* I site were removed using oligonucleotide YPF (5′-AGGCCGAGAAGCGATGGG-GATTTAAAAAAAAAAACAAGTAG-3′) to repair the promoter and YSTR (5′-ATTATTTTGGGTGCCAA-3′) to remove the *Kpn* I site, resulting in precise replacement of yeast nucleotides 1–120 with human nucleotides 1–106. The fusion was inserted into YCpU2-3′ to regenerate a complete U2

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Abbreviations: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein particle; UAS, upstream activating sequences.

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gene on a *LEU2* centromere plasmid (32, 33). The trypanosome exchange was constructed using oligonucleotide TRYP (5′-AAGACAGTTTAATAACTTGATCT-3′). For the GAL-regulated promoter, the U2 gene was cloned into pEMBL yex4 (34). Sequences from the *Dra* III site just upstream of the presumed TATAA sequence were fused to the *GAL* upstream activating sequence (UAS) by using the *Xho* I site of pEMBLyex4 (34). From this, an *Nco* I–*Hin*dIII fragment containing the 3′ half of the *URA3* gene in pEMBLyex4 through the *GAL* UAS and the U2 promoter into the U2 coding region was ligated to a fragment derived from the 5′ half of a U2::*URA3* disruption allele (31). A U2 gene resulted that had the selectable *URA3* gene and a *GAL* UAS inserted at −107. To construct the *GAL*-controlled trypanosome-exchange U2 gene, the *Cla* I fragment between −67 and 432 was replaced with that from the trypanosome-exchange mutant. Additional mutants were constructed using oligonucleotides GΔ (5′-CAGATACTAACTTGAT-3′), A36U and G37U [5′-AACAGATA(C or A)(T or A)ACACTTGAT-3′], and U35C and G37A [5′-TGAAAAGAACAGATA(C or T)T(A or G)CACTTGATCTAAGCC-3′].

Yeast Strains. Yeast strains were constructed and cultured by standard techniques (28). Plasmid shuffling of mutant U2 genes was as described (33). Strains used for the experiments in Table 1 are based on MA80 (*MAT***a**, *ura3-52, leu2-3,112, his4-619, lys2*$^-$), which carries a single full-length wild-type U2 gene. MA90 is the same except that it carries a single chromosomal mini U2 gene derived by deletion of sequences between 123 and 1081 (32). A strain with two copies of wild-type U2 was obtained by transforming MA80 with the *LYS2*, U2 plasmid (33). A strain with 30–50 copies of U2 was obtained by transforming MA80 with the complete U2 gene in pEMBLyex4 (34).

A strain expressing only the human–yeast fusion RNA was constructed by transformation of IH1097 (*MAT***a**, *GAL2, ura3-52, leu2, trp1, pep4-3, prb1, prc1*; obtained from Beth Shuster, University of California, San Francisco) with the linear *Hin*dIII fragment carrying the U2–*URA3 GAL*–U2 segment and selection on galactose medium lacking uracil for one-step gene replacement (28). After transformants were screened for stable sensitivity to 5-fluoroorotic acid (28) and for galactose dependence, DNA was isolated and analyzed by blot hybridization, and a strain (BJ81) in which the *URA3 GAL*–U2 promoter has replaced the wild-type U2 promoter was identified and transformed with the human–yeast fusion plasmid. When this strain is grown on glucose, the synthesis of wild-type U2 is repressed, and only the fusion U2 RNA is expressed.

Galactose induction experiments were performed (35) using MA90 carrying a galactose-inducible full-length U2 gene (wild-type or trypanosome exchange).

Analysis of RNA. RNA extraction, *in vivo* RNA structure probing, and primer extension reactions were performed as described (33).

RESULTS

Function of U2 Phylogenetic Exchange Mutants. We tested two mutations replacing conserved yeast U2 sequences with corresponding sequences from human or trypanosome (Fig. 1). The first substituted nucleotides 1–106 of human U2 for nucleotides 1–120 from yeast U2. This region is the only absolutely indispensable portion of yeast U2 *in vivo* (33, 37) or *in vitro* (38). We found that the human–yeast U2 fusion could replace wild-type U2 in yeast without affecting growth rate or splicing (data not shown, see below). This indicates that all essential yeast U2 functions, including spliceosome assembly, interactions with protein factors or other snRNPs, and the splicing reactions themselves, can be supplied by this part of human U2 (see also ref. 27).

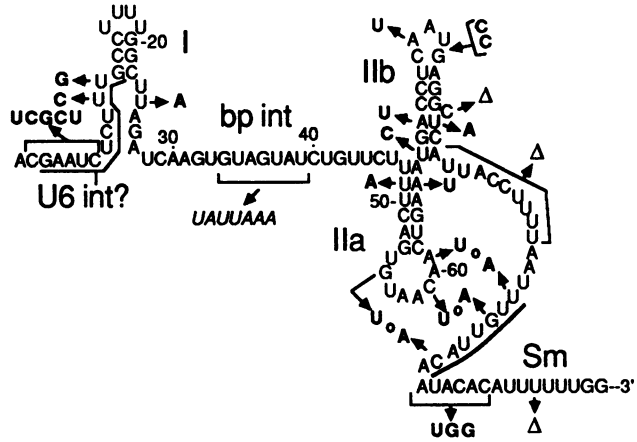

FIG. 1. Phylogenetic exchange derivatives of the 5′ end of yeast U2. The sequence of yeast U2 (31) is shown. Indicated are the branchpoint-interaction region (bp int); stem–loops I, IIa, and IIb; the Sm site; and a proposed interaction with U6 snRNA (U6 int?) (36). The heavy line indicates a phylogenetically conserved complementarity to the loop of IIa. Changes between the human and yeast U2 are in bold letters. Circles indicate compensating base changes between loop IIa and the dispensable complementarity (33). The trypanosome-exchange mutation is indicated in italics. Nucleotides between the Sm site and the 3′ end are not shown.

A second phylogenetic exchange replaced the yeast branchpoint-interaction sequence GUAGUAU with the UAUUAAA sequence found in *T. brucei* (Fig. 1). Introduction of this U2 gene on a centromere plasmid into a strain containing a full-length wild-type U2 gene caused a slow growth phenotype. Occasional spontaneous revertant colonies were observed and fell into two classes distinguishable after wild-type U2 plasmid shuffling (33). One contains recessive lethal U2 alleles that produce no detectable trypanosome-exchange U2 (data not shown), suggesting that the reversion events inactivate expression of the dominant allele. The other class contains functional U2 alleles that support growth after loss of the wild-type U2, *LYS2* plasmid. Direct sequencing of U2 RNA from 24 such revertants showed that all had replaced the trypanosome sequence with GUAGUAU, probably by gene conversion.

An Inducible Dominant Lethal U2 Allele Inhibits Splicing. To prove that expression of the mutant U2 causes the dominant

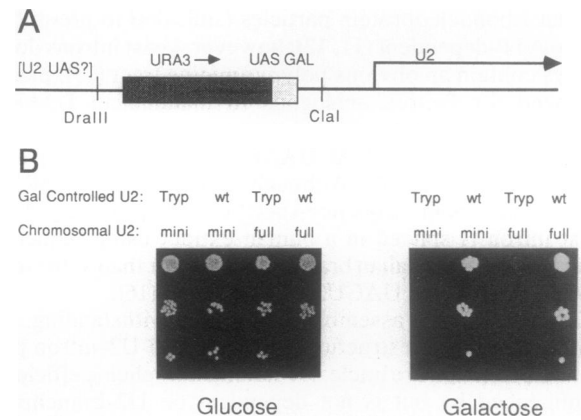

FIG. 2. Galactose-dependent dominant lethality. (*A*) Structure of the galactose-inducible U2 promoter fusion. (*B*) Phenotype of cells carrying the galactose-inducible dominant U2 mutation. Yeast carrying either a full-length or a mini U2 gene in the chromosome and either the wild-type (wt) or the trypanosome-exchange (Tryp) U2 gene under *GAL* control on a centromere plasmid were spotted on plates containing glucose or galactose as a carbon source and were incubated at 30°C for 3 days.

lethality, we constructed a repressible U2 promoter by fusing the *GAL* UAS immediately upstream of the U2 TATAA sequence (ref. 14, Fig. 2*A*). When the trypanosome-exchange U2 gene was brought under *GAL* control, the dominant growth defect became dependent on galactose (Fig. 2*B*). This experiment eliminated uncertainties in interpreting the negative results of direct transformation experiments with dominant lethal alleles and suggested that transcription of the trypanosome-exchange U2 RNA is required for dominance.

We measured the induction of U2 transcription from the *GAL*-regulated wild-type and trypanosome-exchange U2 plasmids in a strain carrying a U2 gene lacking sequences between 123 and 1081 of U2 (32). Cells were shifted to galactose-containing medium and RNA was isolated at various times and used in a primer-extension assay specific for *GAL*-controlled U2 (Fig. 3*A*). Consistent with *GAL* regulation, induction of synthesis of wild-type or trypanosome-exchange U2 was dependent on galactose. We saw reproducibly lower accumulation of trypanosome-exchange U2 as compared with wild-type U2, suggesting that the trypanosome-exchange transcript was less stable.

We monitored accumulation of intron-containing pre-U3 RNA by primer extension (Fig. 3*B*) and observed no effect on the splicing of U3 after expression of the wild-type U2. In contrast, induction of the trypanosome-exchange U2 resulted in the rapid accumulation of pre-U3. The trypanosome-exchange transcript was detectable within 20 min of galactose addition (Fig. 3*A*), and accumulation of unspliced U3 was apparent by 40 min after induction, before peak levels of the trypanosome-exchange transcript were reached (Fig. 3*A*). The rapid kinetics of splicing inhibition following expression of the trypanosome exchange U2 RNA argues that the effect is direct and due to deleterious function of the trypanosome exchange U2 RNA. We conclude that the dominant lethal phenotype is caused by the splicing-inhibitory effects of the mutant U2 RNA.

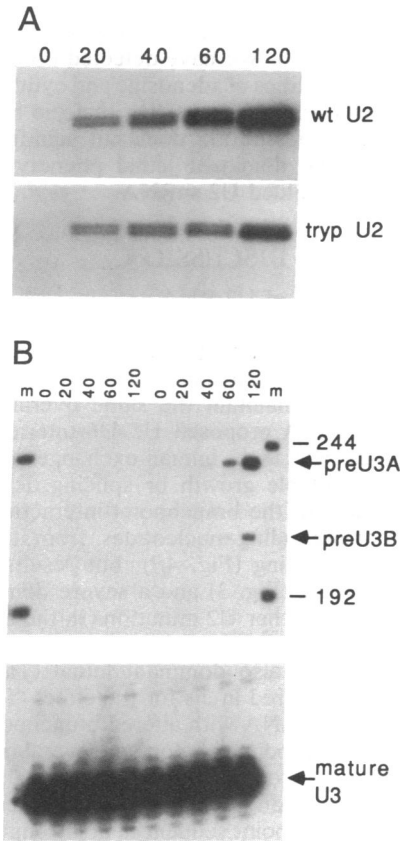

FIG. 3. Galactose induction of U2 accumulation and splicing inhibition. Wild-type (wt) or trypanosome-exchange (tryp) U2 expression was induced by addition of galactose. RNA was isolated at indicated times (minutes) after galactose addition. (*A*) Time course of induction of U2 expression. Reverse transcription with a primer at positions 130–152 of U2 RNA was used. Since the chromosomal mini U2 gene lacks sequences between 123 and 1081, the primer is specific for the *GAL*-controlled U2. (*B*) Inhibition of pre-U3 splicing in cells expressing trypanosome exchange U2. A primer complementary to the second exon of U3 was extended using RNA from cells expressing wild-type (left lanes) or trypanosome-exchange (right lanes) U2. Lanes m, size markers (244 and 192 nucleotides long).

Point Mutations with Dominant Lethal Phenotypes of Varying Severity. The dominance of the trypanosome-exchange U2 is reminiscent of an effect reported with the U2 suppressor mutation A36U (5). We tested A36U as well as other mutations for dominance in strains carrying different U2 genes (Table 1). Although A36U is not dominant in our wild-type strain background, isogenic derivatives carrying the mini U2 gene cannot be transformed with a plasmid carrying a full-length A36U U2 gene (Table 1). G37U, which is not dominant to a full-length U2 allele (ref. 5 and Table 1), is dominant to the mini U2 allele. Two additional alleles show similar effects. Interestingly, the two different alterations of G37 have dominant phenotypes of different severity. All mutations we have made in the branchpoint interaction region are lethal as the only copy of U2 in the cell (data not shown; see also ref. 5).

We tested a single base deletion predicted to increase base pairing while removing the ability of the branched adenosine residue to be bulged, according to a commonly discussed model for U2–intron base pairing (see Table 1). This mutation, G34Δ, is dominant to the full-length wild-type gene (Table 1). While G34Δ could act through a different mechanism, perhaps by interfering with recognition of the adenosine to be branched, the results show that mutations predicted to increase as well as decrease branchpoint complementarity in yeast are dominant.

Suppression of the Dominant Lethal Phenotype. U2 stem–loop IIa is an essential structure (33), but its function is unknown. Mutations in this stem–loop are recessive, render cells temperature-sensitive for growth, and do not affect the stability of U2 RNA (33). By testing the phenotype of a dominant branchpoint-interaction-region mutation in combination with a recessive stem–loop IIa mutation, we could

Table 1. Mutations in the branchpoint-interaction region

Name	Sequence (nucleotides 32–40)	Growth			
		Mini	1	2	30–50
	A CAUCAU				
Wild type	GUGUAGUAU	+	+	+	+
tryp YT3	GU<u>UAUUAAA</u>	–	SG	SG	+
tryp YT4	GUG<u>AUUAAA</u>	–	SG	SG	+
G34Δ	GU_UAGUAU	–	SG	SG	+
U35C	GUG<u>C</u>AGUAU	SG	+		
G37U	GUGUA<u>U</u>UAU	SG	+		
G37A	GUGUA<u>A</u>UAU	–	SG		
A36U	GUGU<u>U</u>GUAU	–	+		
G53C	GUGUAGUAU	+			
A36U, G53C	GUGU<u>U</u>GUAU	+			

Sequence changes in the mutants are underlined. Growth of cells carrying a mutant U2 gene and the wild-type mini U2 gene or 1, 2, or 30–50 copies of the wild-type full-length gene was scored after 3 days at 30°C: +, colonies > 1.5 mm in diameter; SG (slow growth), colonies < 1.5 mm in diameter; –, only revertant colonies (see text). The branchpoint consensus is shown above the wild-type U2 sequence in the pairing configuration that bulges the branched adenosine residue (5).

order the requirements for the two elements during splicing. A normally dominant A36U branchpoint mutant carrying an additional mutation (G53C) in stem–loop IIa becomes recessive (Table 1). The G53C mutation acts as a second-site intragenic suppressor of the dominance, suggesting that the function of stem–loop IIa is required in cis to produce the dominant effect.

We also tested the ability of extra copies of the wild-type U2 gene to suppress the dominant lethal phenotype (Table 1). A centromere plasmid carrying the full-length wild-type U2 gene fails to suppress the most severe dominant alleles, while the U2 gene on a 2-μm plasmid completely suppresses (Table 1). U2 RNA accumulates to only about 3- to 5-fold over single-gene levels in these strains (data not shown), suggesting that fewer transcripts per gene enter the U2 snRNP pool. Although other mechanisms are not strictly excluded, multicopy suppression by wild-type U2 can be explained by changes in the representation of the mutant RNA in U2 snRNPs.

The Phylogenetic Exchange U2 RNAs Are Correctly Folded. An axiom of the phylogenetic comparison method of RNA structure model building is that analogous RNAs from different organisms will fold similarly despite primary sequence differences. To test this directly, we examined the secondary structures of the phylogenetic exchange mutants in yeast by *in vivo* structure probing (ref. 33 and Fig. 4). Single-stranded adenosine and cytidine residues react with dimethyl sulfate and give rise to strong stops upon subsequent reverse transcription, allowing unpaired nucleotides to be identified (39, 40). The secondary structure for yeast U2 shown in Fig. 1 is consistent with phylogenetic (3, 4), genetic (33), and chemical probing (33) data. Because the model is phylogenetically consistent, human U2 sequences have the potential to fold in a similar fashion (Fig. 1). If this folding is important for correct function, we expect the bulk of the human–yeast fusion RNA to adopt a similar secondary structure in yeast cells. Nucleotides in the human homologs of stem–loops IIa and IIb and in the phylogenetically conserved but dispensable region of complementarity to the IIa loop (ref. 33; see Fig. 1) have reactivities consistent with their position in the structure. These results indicate that yeast has all the necessary factors required to fold this region of human U2, and provide direct evidence for the phylogenetic generality of U2 structure.

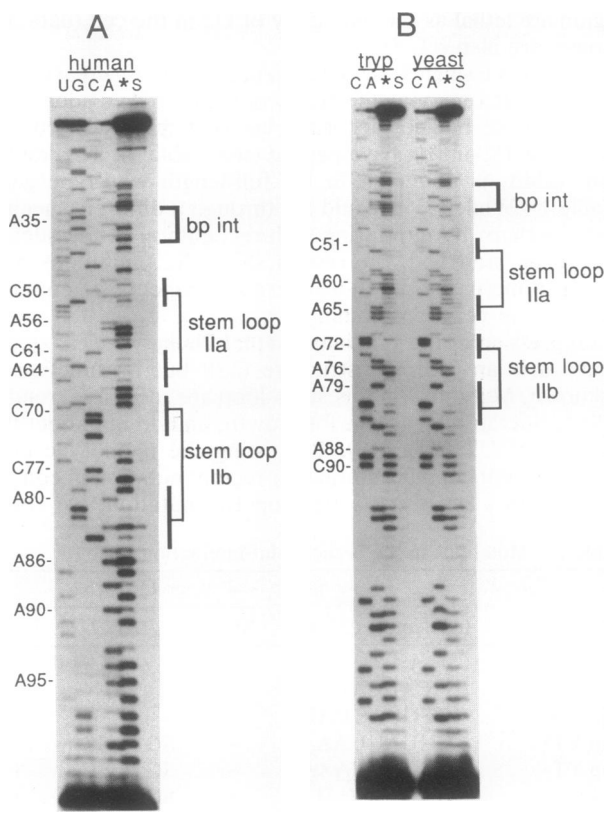

FIG. 4. *In vivo* structure probing of phylogenetic exchange RNAs. (*A*) Human U2 sequences in yeast. Yeast expressing only the human–yeast U2 fusion gene were incubated with dimethyl sulfate as described (33). A primer (oligonucleotide YSTR) was extended on either unmodified RNA in the presence of ddATP (lane U), ddCTP (lane G), ddGTP (lane C), or ddTTP (lane A), modified RNA (lane ★), or a "stop control" RNA (lane S) isolated from cells treated with dimethyl sulfate after the addition of the quench solutions. (*B*) Structure probing of galactose-induced wild-type (yeast) or trypanosome-exchange (tryp) RNA. Cells were induced with galactose as described for Fig. 3, incubated for 120 min, and treated as above. Note that modification stops appear 1 nucleotide shorter than corresponding dideoxynucleotide stop (39, 40). bp int, Branchpoint interaction region.

Interpretations of dominant lethal phenotypes often rely on the assumption that the mutant gene product is at least partially functional (41). To eliminate the possibility that the altered nucleotide sequence of the trypanosome branchpoint-interaction region in the context of yeast U2 sequences causes an alternative RNA folding inconsistent with function, we examined the structure of the trypanosome exchange RNA after *GAL* induction, using a probe specific for *GAL*-controlled U2 (Fig. 4*B*). We have detected no major differences in the accessibilities of adenosine and cytidine residues outside the altered region, suggesting that the branchpoint-interaction region substitution does not significantly alter folding and that the dominant lethal phenotype is not a consequence of misfolded U2 snRNA.

DISCUSSION

Phylogenetic variants of U2 RNA are not necessarily functionally equivalent in yeast. Sequences from human and yeast U2 vary by several deletion/insertions, transitions, and transversions but are identical in the branchpoint-interaction region (Fig. 1) and maintain the same overall secondary structure (Fig. 4*A*). A proposed U2–U6 interaction (36) is reduced to 4 base pairs in the human-exchange strain, yet this produces no detectable growth or splicing deficiencies *in vivo*. Replacement of the branchpoint-interaction region of yeast with corresponding nucleotides from trypanosome does not disturb folding (Fig. 4*B*), but results in a rapid inhibition of splicing (Fig. 3) and a severe dominant lethal phenotype (Fig. 2). Other U2 mutations in the branchpoint-interaction region predicted to improve or reduce intron complementarity are also dominant lethal (Table 1), and stem–loop IIa is required in cis for the effect (Table 1).

Dominance of U2 RNA with altered branchpoint complementarity could be produced by one of several mechanisms. Missplicing due to incorrect branchpoint recognition seems unlikely given the strong dependence on the conserved 5′ splice site and branchpoint sequences in introns for splicing-complex assembly in the absence of U2 *in vitro* (11, 14). Competition for a limiting intrinsic U2 snRNP protein seems unlikely as well, since numerous recessive lethal mutant U2 RNAs accumulate efficiently enough to represent more than half of the U2 snRNP pool, without causing dominant effects (33), and a mini U2 RNA that never achieves wild-type RNA levels is sufficient for growth (32).

Most explanations of how a mutation can create a new gene product that actively interferes with wild-type activity are based on the multimeric nature of biological catalysts and structures (41). If the mutant gene product makes a stable, nonproductive interaction with a limiting substrate or subunit, the wild-type gene product is prevented from function-

ing. In this case, U2 snRNA with altered branchpoint complementarity could participate in a stable complex that sequesters something essential from productive interaction with wild-type U2 snRNP. Since pre-mRNA accumulates rapidly after the dominant mutation is expressed (Fig. 3), we propose that the limiting essential factor is a component of the splicing apparatus rather than an essential pre-mRNA.

A model for yeast U2 action follows from the dominant lethal phenotype of mutations in the branchpoint-interaction region. In this model, U2 snRNP entry into splicing complexes is independent of branchpoint complementarity but requires U2 stem–loop IIa (Table 1). Once assembled, the complex containing U2 cannot disassemble and includes at least one limiting splicing component (not U2 snRNP; see above). When U2 is required to pair with the branchpoint, the complex either proceeds and the limiting component is recycled (in the case of wild-type U2) or fails to proceed and is degraded or only slowly disassembled, leading to loss of limiting component activity and inhibition of splicing. The varying phenotypes of the different dominant U2 alleles could be explained by partial function; mutations in the branchpoint interaction region allow splicing of mutant introns (5–7) and could conceivably operate on wild-type branchpoints with varying efficiency.

A model in which stable U2 binding precedes base pairing may reconcile apparent differences between yeast and metazoan branchpoint selection. UACUAAC functions most efficiently as a branchpoint in yeast introns but is not absolutely required (2). Similarly the efficiency of mammalian splicing is influenced by U2 snRNA–branchpoint region complementarity (6, 7, 16–18), but very poor complementarity is tolerated (19). Mammalian U2 snRNP binding to pre-mRNA could involve an initial step dependent on polypyrimidine followed by selection of the branchpoint based on complementarity (16–18, 20). In both yeast and metazoan extracts, factor dependence of U2 assembly with pre-mRNA has been demonstrated (1, 8–11, 14, 16), and base pairing need not be proposed to explain specific U2 binding. The model is consistent with data suggesting that conservation of the yeast intron branchpoint sequence has a role in pre-mRNA recognition by splicing factors (2, 11, 14), and it is not inconsistent with the base-pairing requirement for efficient splicing *in vivo* (5). The intragenic suppressor of dominance in stem–loop IIa of U2 suggests that this structure mediates U2 participation in stable splicing complexes, possibly by interacting with factors that bind U2 snRNP to discrete sites on pre-mRNA (33). A late requirement for U2–intron pairing in splicing may suggest a catalytic or proofreading role for this interaction.

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