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Studies of Point Mutants Define Three Essential Paired Nucleotides in the Domain 5 Substructure of a Group II Intron

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Domain 5 (D5) is a highly conserved, largely helical substructure of group II introns that is essential for self-splicing. Only three of the 14 base pairs present in most D5 structures (A2 U33, G3 U32, and C4 G31) are nearly invariant. We have studied effects of point mutations of those six nucleotides on self-splicing and in vivo splicing of aI5γ, an intron of the *COXI* gene of *Saccharomyces cerevisiae* mitochondria. Though none of the point mutations blocked self-splicing under one commonly used in vitro reaction condition, the most debilitating mutations were at G3 and C4. Following mitochondrial Biolistic transformation, it was found that mutations at A2, G3, and C4 blocked respiratory growth and splicing while mutations at the other sites had little effect on either phenotype. Intra-D5 second-site suppressors showed that pairing between nucleotides at positions 2 and 33 and 4 and 31 is especially important for D5 function. At the G3 U32 wobble pair, the mutant A U pair blocks splicing, but a revertant of that mutant that can form an A^+ C base pair regains some splicing. A dominant nuclear suppressor restores some splicing to the G3A mutant but not the G3U mutant, suggesting that a purine is required at position 3. These findings are discussed in terms of the hypothesis of Madhani and Guthrie (H. D. Madhani and C. Guthrie, Cell 71:803–817, 1992) that helix 1 formed between yeast U2 and U6 small nuclear RNAs may be the spliceosomal cognate of D5.

Self-splicing group II introns have conserved secondary-tertiary structures that participate in the splicing reactions (21). We have concentrated on studies of the domain 5 (D5) substructure, using chiefly the self-splicing group II intron aI5γ of *Saccharomyces cerevisiae* mitochondrial DNA (mtDNA). By using a *trans* assay for D5 function, it has been found that D5 is essential for self-splicing and binds to the intron via as-yet-unspecified tertiary interactions (14). It is now clear that D5 is essential for both steps of self-splicing (7). Studies of forms of aI5γ deleted for multiple domains showed that the D5 binding site is in D1 (16). More recently, additional *trans*-splicing experiments that support the earlier inference (14) that D5 interacts with D6, the adjacent substructure that contains the branch site used in the first step of splicing, and plays a role in 3′-splice site selection have been reported (10). It has also been shown that a minimal D5 of 36 nucleotides (nt) is sufficient for *trans* complementation and that this small D5 RNA promotes the release of the first exon with multiple turnover (12, 26).

As illustrated in Fig. 1, D5 of aI5γ is 34 nt long and is capable of forming a secondary structure that is a 14-bp helix interrupted by a 2-base bulge and capped by a 4-base loop. Analysis of the sequences of D5 elements in other introns reveals considerable sequence variation, especially at most paired residues (21). There are examples of D5 sequences that differ at over 50% of the positions (in comparison with D5 of aI5γ), and no single base or base pair is invariant. Chimeric versions of aI5γ in which its natural D5 has been replaced by the D5 from other group II introns have been studied separately (4), and the results indicate that the nucleotide bases of those variable positions play no essential role in the function of D5. Heterologous *trans* assays of D5 function, however, showed clearly that each D5 functions best in its natural intron context, suggesting that some determinants of D5 function are intron specific.

Three base pairs in D5 structures, A2 U33, G3 U32, and C4 G31 (Fig. 1), are very highly conserved, and this study tests the effects of point mutations of those nucleotides on splicing of aI5γ. All of the mutant introns had at least some self-splicing activity under one standard self-splicing condition, but mutations at G3 and C4 had the most severe in vitro defects. Representative mutations at each site were studied in vivo following mitochondrial transformation, with the finding that mutations of A2, G3, and C4 but not G31, U32, or U33 strongly inhibited splicing in vivo. Spontaneous revertants of the splicing-defective mutants included second-site suppressors within D5 that provided further information about these three paired positions.

MATERIALS AND METHODS

Plasmids. Plasmid pJD20 is the template for the wild-type precursor RNA containing aI5γ (14). pJD20 was the template for site-directed mutagenesis (17) of positions A2, G3, C4, U9, C29, G31, U32, and U33 of D5 of aI5γ. Each construction was confirmed by DNA sequencing, and each resulting plasmid was used as a template for precursor RNAs with those mutated D5s. These plasmids are referred to as pJD20-D5G3A and pJD20-D5G3U, etc.

In vitro transcription and RNA splicing methods. In vitro precursor RNAs were transcribed with T7 RNA polymerase by using *Hin*dIII-cleaved plasmid DNAs. Transcription was carried out at 30°C to eliminate detectable self-splicing during transcription. All reaction buffers used in this study for self-splicing reactions have been described previously (15, 16, 23). In vitro splicing reactions were carried out at 45°C for the lengths of time indicated below. Electrophoresis and gel processing conditions were described previously (16).

Relative rates of splicing were estimated by performing at least duplicate incubations for an appropriate time with each preparation of mutant and wild-type precursor RNA (13, 22). The level of products formed was quantitated after electrophoresis and PhosphorImager (Molecular Dynamics) scanning. Because each RNA was assayed under reaction conditions in which the first step of

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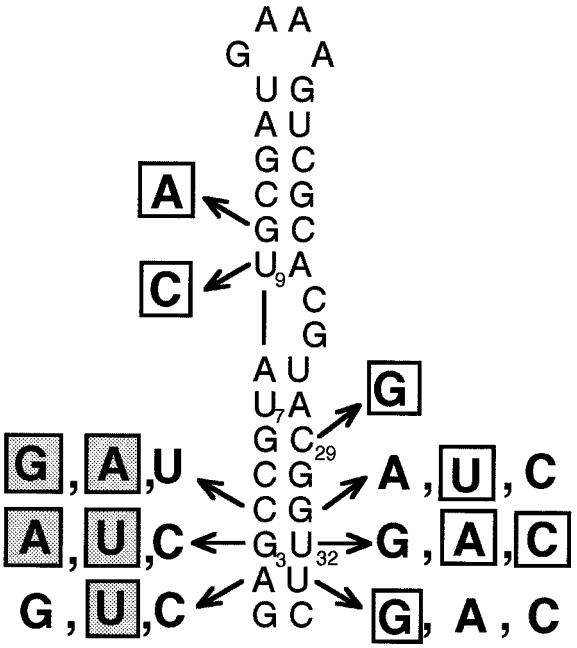

FIG. 1. Diagram of point mutations of D5 of aI5γ. The sequence and proposed secondary structure of D5 of aI5γ are shown. Specific point mutations of nucleotides A2, G3, C4, U9, G10, C29, G31, U32, and U33 that were made and analyzed in this study are indicated (see Table 1 and Fig. 2 and 3). The 12 mutations that were also analyzed in vivo, following mitochondrial transformation, are indicated (shaded boxes, mutations that block respiratory growth in vivo; unshaded boxes, mutations that permit growth on glycerol-containing medium).

splicing occurs almost exclusively via branch formation, and because none of the mutations changed that, the level of the intron lariat product was measured. Each intron allele was analyzed with at least two different preparations of precursor RNA, and all measurements were normalized for loading and compared with wild-type transcripts of the same specific radioactivity and analyzed in the same experiment.

The time of incubation chosen for the control RNA was within what is known to be an apparently linear part of the progress curve for this reaction (<20% conversion to product) (e.g., see reference 23). Mutant RNAs that spliced relatively well in preliminary experiments were reacted for the same time of incubation as the control RNA (5 or 8 min for high-salt reactions and 30 or 45 min for low-salt reactions). Mutant RNAs that spliced very slowly were incubated for 45 min under high-salt reaction conditions and for 45 or 90 min under low-salt conditions. In low-salt reactions in which no reaction products were detected, even in dark exposures of the gels, the detection limit was estimated to be about 4% of the control under the same conditions. The lowest extent of splicing observed in high-salt conditions was about 1% of the control level of intron lariat RNA and, in that case, intron lariat and spliced-exon products were plainly evident.

Mitochondrial genetics and transformation. Mitochondrial transformation was carried out essentially as described previously, using plasmid pPA100 as the basic vector (1, 2, 22). The fragment of mtDNA containing aI5γ and flanking exon sequences from pJD20 was transferred to pPA100 as an *Eco*RI fragment to make plasmid pSMB20. The same transfer to pPA100 of mtDNA sequence from the derivatives of plasmid pJD20 containing the point mutations which are boxed in Fig. 1 was carried out to make pSMB20-D5A2U, etc. Plasmid DNAs prepared from cultures of *Escherichia coli* DH5α were precipitated onto gold particles, and aliquots containing 2 to 4 μg of DNA were then shot at lawns of approximately 2×10^8 cells of [*rho*^{0}] strain DBY947 (*MAT*α *ade2-101 ura3-52*) by using a PD1000-He Biolistic particle delivery system. Media and the screening procedure used to identify and purify [*rho*$^-$] mitochondrial transformant strains were as described previously. Cells shot with pSMB20-D5A2U, pSMB20-D5G3A, pSMB20-D5G3U, pSMB20-D5C4A, and pSMB20-D5C4G were screened until a stable [*rho*$^-$] transformant of each was obtained. Those mutations were then transplaced into an intact mtDNA by mating to strain MY375 (*MAT***a** *kar1 ura3-52 his4*Δ*34*) containing the GII-0 [*rho*$^+$] mtDNA (29) in which the *COXI* gene contains only introns 3α and 4α or a related mitochondrial genome, GII-5γ, in which wild-type aI5γ is also present. Haploid respiration-deficient (recombinant) cytoductants were identified as tan colonies on yeast extract-peptone-dextrose (YEPD) medium, isolated, subcloned, and tested for nuclear markers. As a final confirmation of each transplacement, PCR was used to amplify aI5γ from cellular DNA with primers complementary to sites flanking D5, and the main product was purified and sequenced as described in reference 22. The A2U, G3A, G3U, C4A, and C4G mutations were studied here in a *COXI* gene containing three introns (3α, 4α, and 5γ).

A simpler version of this protocol was used to transfer the mutations U9C, C29G, G31U, U32C, U32A, and U33G to an intact mtDNA. Ura$^+$ areas of petri dishes shot with pSMB20-D5U9C or pSMB20-D5C29G, etc., that were found to be *COXII*$^+$ in the cross with a *COXII*-deficient tester strain (JC3/TF145) were inoculated in liquid YEPD medium, grown overnight, and mated to strain ID41-6/161 (*MAT***a** *ade1 lys1*) containing the C1067 mutation of aI5γ (20), a nonreverting mutant which has two point mutations in D5 and two more in D6. Diploid recombinants capable of growing on glycerol medium were isolated, and the presence of the mutated position of D5 in each construct was confirmed as described above. Those (functional) mutations were thus obtained in a *COXI* gene with seven introns. A Gly$^+$ strain containing only the G10A mutation in D5 of aI5γ in the seven-intron form of *COXI* was obtained as a rare diploid recombinant of a cross between mutant C1067 (which contains the G10A mutation in addition to three other changes nearby) and a pMIT strain containing a mutation of the 2-nt bulge of D5 (27).

Most revertants of respiration-deficient mutants were obtained as papillae overgrowing lawns or colonies of mutant strains on petri dishes containing YEP medium with 2% glucose; a few revertants were obtained by plating cells from a glucose culture on dishes containing YEP medium containing 2% glycerol. Revertants were subcloned at least once on YEPD medium, and then several subclones were suspended in wells of microtiter dishes and tested for their nuclear markers and growth phenotypes on glycerol medium at 23, 30, and 36°C. Standard genetic procedures were used to characterize selected revertants, including cytoduction and meiotic analysis to distinguish nuclear suppressors from mitochondrial back mutations and second-site suppressors (summarized in reference 25). A *ura3* mutant derived from strain ID41-6/161, called 161-U7, was used in some experiments; the other strains used are described in the legend to Fig. 4.

Other biochemical methods. Wild-type or mutant yeast cultures were grown in YEP medium with 2% raffinose, and total cell RNA was extracted from spheroplasts by the method described in reference 8. The RNA was fractionated by electrophoresis on agarose gels containing 4% formaldehyde, and the gels were blotted onto Nytran membranes and hybridized with various probes. RNA samples were balanced according to their content of the mRNA of the *OLI2* gene (based on scanning of gels with a PhosphorImager system). Oligonucleotide probes complementary to short segments of these mitochondrial RNAs were synthesized and 5′ end labeled with [γ-^{32}P]ATP (Amersham) (see reference 22 for details). Restriction enzymes were obtained from Promega. Plasmid DNAs were sequenced by using Sequenase version 2.0 (United States Biochemical Corp.).

PCR was used to generate DNA templates for sequencing experiments that defined the D5 mutation in each mutant or revertant reported here. Upstream primers used for PCR contained nt 8537 to 8553 (in the upstream exon) or 8622 to 8639 (at the beginning of the intron), and the downstream primer was complementary to nt 9561 to 9579 in the last exon (refer to reference 3 for the primary sequence). Upstream primers contained a biotinylated nucleotide to permit the purification of one strand of the amplified material with Dynabeads (Dynal, Inc.) prior to sequencing.

RESULTS

Effects of mutations of highly conserved nucleotides of D5 on self-splicing. All possible point mutations of the six most highly conserved paired nucleotides of D5 of aI5γ were constructed. Plasmids carrying mutant alleles were transcribed, and the self-splicing activity of each RNA sample was measured under several conditions (Table 1). Some mutants altered at several positions (A2, G31, and U32) splice under the low-salt condition (100 mM Mg^{2+}), while the other mutants are inhibited at least 25-fold under that condition. All of the mutant RNAs that have some activity in the low-salt buffer were found to be very reactive in the high-salt buffer [100 mM Mg^{2+} plus 500 mM $(NH_4)_2SO_4$]. Several of the mutants that yielded no visible products in low-salt reactions (A2U, U33N, U32G, and G31A mutants) are quite reactive in high-salt reactions. It is striking that the mutations at G3 and C4 are the least reactive in the high-salt condition. Several other high-salt reaction conditions have been employed in previous studies (for examples, see references 10, 15, 16, and 22); since the relative activities of these mutant alleles are essentially the same under the other conditions [e.g., 500 mM NH_4Cl or KCl

TABLE 1. Relative rates of splicing by precursor RNAs with point mutations in D5 of aI5γ[a]

Intron form	Rate of splicing (mean ± SE) under the following condition(s):	
	100 mM $MgSO_4$	100 mM $MgSO_4$ and 0.5 M $(NH_4)_2SO_4$
Wild type	1.0	1.0
A2C	0.31 ± 0.04	0.74 ± 0.08
A2G	0.1 ± 0.03	0.71 ± 0.07
A2U	<0.04	0.67 ± 0.1
U33A	<0.04	0.69 ± 0.04
U33C	<0.04	0.52 ± 0.12
U33G	<0.04	0.32 ± 0.12
G3A	<0.04	0.12 ± 0.03
G3C	<0.04	0.08 ± 0.02
G3U	<0.04	0.21 ± 0.01
U32A	0.85 ± 0.08	0.83 ± 0.12
U32C	0.38 ± 0.06	1.03 ± 0.04
U32G	<0.04	0.85 ± 0.14
C4A	<0.04	0.08 ± 0.02
C4G	<0.04	0.02 ± 0.01
C4U	<0.04	0.33 ± 0.06
G31A	<0.04	0.81 ± 0.13
G31C	<0.04	0.38 ± 0.01
G31U	0.53 ± 0.08	0.79 ± 0.11
U9C	<0.04	0.74 ± 0.08
C29G	<0.04	0.09 ± 0.02

[a] See Materials and Methods.

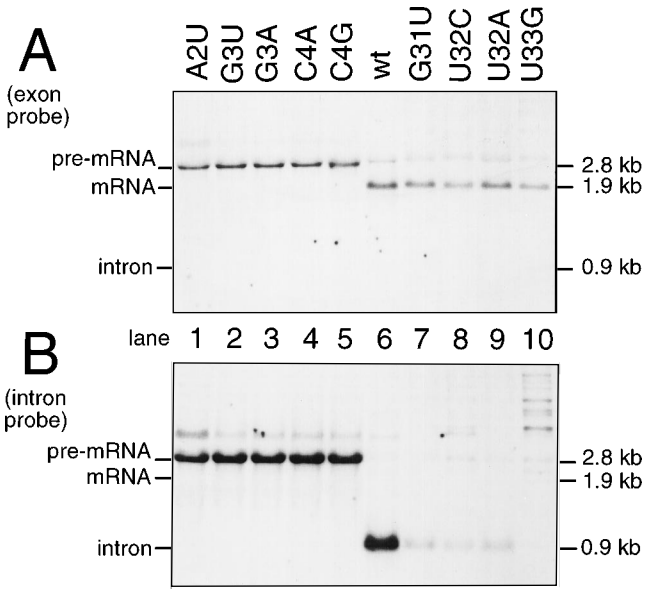

FIG. 2. In vivo splicing phenotypes of point mutations of the three nearly invariant base pairs of D5 of aI5γ. Cellular RNA was purified from raffinose-grown cells of strains carrying the mutations of positions 2, 3, 4, 31, 32, and 33 of D5 shown in Fig. 1. The amount of mitochondrial RNA in each sample was balanced by determining the relative amounts of a mitochondrial mRNA that is not affected by these mutations (the *OLI2* mRNA). A probe specific for exon 4 of the *COXI* gene (A) and a probe specific for aI5γ (B) were used for hybridization. The source of RNA used is shown above each lane in panel A. The positions of the 2.8-kb pre-mRNA not yet spliced for aI5γ, the 1.9-kb mRNA, and the 0.9-kb excised intron lariat RNA are shown in each panel. The minor bands migrating more slowly than the 2.8-kb pre-mRNA in panel B are partially spliced precursors that contain aI5γ plus one or more other introns. The sizes of the minor bands vary with the number of introns present in the *COXI* gene of the strain; A2U, G3U, G3A, C4A, and C4G are in the three-intron configuration of the *COXI* gene, while the other mutations are in the seven-intron form (see Materials and Methods). Note that the precursor RNA seen in the wild-type (wt) sample (panel A, lane 6) does not contain unspliced aI5γ, since a strong signal is not present in panel B, in which an aI5γ-specific probe was used. Intron 4α splices somewhat less efficiently than aI5γ in that wild-type strain, and that accounts for the presence of a precursor RNA larger than 2.8 kb in lanes 1 to 5 of both panels (especially panel B) and in Fig. 3, 5, and 6.

in place of $(NH_4)_2SO_4$] (9, 26a), we report here data from only one of those conditions.

Although some other mutations of aI5γ lead to self-splicing with reduced or no branching in the first reaction step (5, 16, 22), none of the D5 mutations described above has that effect. Also, none of these mutants has a detectable defect in the second reaction step, since none accumulates detectable intron–3′-exon reaction intermediate. These in vitro data indicate that the G3 U32 and C4 G31 base pairs are quite important for self-splicing while the A2 U33 pair appears to be relatively unimportant. Under the high-salt reaction condition, asymmetry is apparent in the G3 U32 and C4 G31 pairs, in which mutations of G3 and C4 are more inhibitory to splicing than are mutations of their pairing partners, U32 and G31, respectively.

In vivo phenotypes of D5 point mutants define nucleotides essential for splicing. Interpreting in vitro experiments of this sort is complicated by the use of several reaction conditions, all of which are nonphysiological. Regardless of how clear-cut a given in vitro result might be, some reservations about the biological significance of each finding persist. So far, site-directed mutations of group II introns have been analyzed in vivo in only one study (22). In vitro and in vivo phenotypes of a mutant intron may depend on different factors, in which case a given mutation may have one effect on splicing in vitro but a different effect in vivo. However, when mutations affect portions of the RNA that may be fundamental to the RNA-catalyzed reactions (as appears to be the case here [7, 24, 26]), it is possible or even likely that there will be a general concordance. Therefore, one goal of the following experiments was to learn whether these D5 mutations have similar effects on splicing in vitro and in vivo. It should not be overlooked that the two phenotypes were similar in a previous in vivo study of mutations of the first intron nucleotide (22).

We used Biolistic transformation to place representative D5 point mutations in yeast mitochondria. Recipient [*rho*^{0}] strains lacking all mtDNA sequences were transformed to synthetic petite mutants (pMIT strains) (1, 11) with mtDNA containing the A2U, G3A, G3U, C4G, C4A, G31U, U32C, U32A, and U33G mutations of D5 of aI5γ used above. These mutant introns were then transferred into otherwise intact mitochondrial genomes by recombination, and the resulting strains were analyzed phenotypically (see Materials and Methods).

The strains carrying the mutations A2U, G3A, G3U, C4A, and C4G are respiration deficient and unable to grow on glycerol-containing medium, while the strains with the G31U, U32C, U32A, and U33G alleles respire and grow well on glycerol medium (Fig. 1). Cellular RNA was purified from raffinose-grown cultures of the wild-type control and each mutant strain for RNA hybridization analysis (Fig. 2). RNA samples were balanced on the basis of the amount of the mitochondrially encoded *OLI2* gene mRNA (not shown, but see reference 22) and hybridized with a probe specific for *COXI* exon 4 (Fig. 2A). The data show that all of the respiration-deficient strains (Fig. 2A, lanes 1 to 5) lack detectable spliced mRNA and so are substantially defective for aI5γ splicing; on the basis of mRNA levels, the respiration-competent mutant strains all splice the intron efficiently (Fig. 2A, lanes 7 to 10). The phenotypes of these strains were also analyzed by using an intron-specific probe (Fig. 2B); in that experiment, mutants

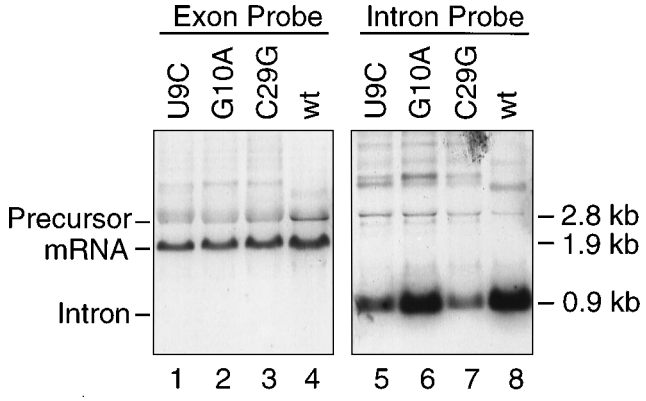

FIG. 3. In vivo splicing phenotypes of point mutants with changes at three highly variable base pairs of D5 (U9C, G10A, and C29G) analyzed as in Fig. 2. A probe specific for exon 4 (lanes 1 to 4) and a probe specific for aI5γ (lanes 5 to 8) were used for hybridization. wt, wild type.

that lack mRNA in Fig. 2A lack excised intron RNA with the second probe and accumulate mostly the 2.8-kb precursor RNA containing the exons and aI5γ. Interestingly, the four mutants that splice as determined from Fig. 2A (lanes 7 to 10) have only a low level of excised intron RNA in Fig. 2B. This is a first indication that the postsplicing metabolism of this intron RNA is influenced by the sequence or structure of D5. Data in Fig. 3, 5, and 6 show that this defect in accumulating excised intron RNA is present in nearly all of the tested mutant strains that can still splice the intron.

These in vivo phenotypes indicate that the G3 and C4 positions of D5 are essential for aI5γ splicing in vivo, while mutations of U32 and G31 are tolerated. While the in vitro data indicated that neither A2 nor U33 is critical for self-splicing under the high-salt reaction condition, the in vivo data show clearly that A2 is much more important for splicing than is U33. Overall, we conclude that three of the six most highly conserved paired nucleotides of D5 are crucial for in vivo splicing of this intron, while the other three equally well-conserved nucleotides can be altered with retention of substantial splicing. In vivo, each of these base pairs exhibits an asymmetry in which the 5′ nucleotide appears to be more important for D5 function than the 3′ one.

Splicing phenotypes of three mutations at highly variable sites in D5. We have also constructed three yeast strains carrying mutations of nonconserved paired positions in D5 (U9C, G10A, and C29G; Fig. 1). Each of these mutants grows well on glycerol medium, and RNA hybridization experiments (Fig. 3) show that they splice efficiently. While all three mutants splice about equally well, it is evident (Fig. 3, lanes 5 to 7) that the level of excised intron RNA varies widely among them: mutant G10A has about as much of the 0.9-kb species as does the wild-type control (relative to the amount of mRNA), mutant U9C has somewhat less, and mutant C29G has still less.

Self-splicing data for U9C and C29G are provided in Table 1. Since the C29G mutant self-splices poorly but splices well in vivo, this is another case in which the in vivo and in vitro data do not agree. The G10A mutant was analyzed qualitatively in vitro with the finding that it splices in low-salt buffers and is indistinguishable from the wild-type control in the high-salt buffer used here (see reference 9). These findings indicate that the two base pairs just above the 2-nucleotide bulge are probably not critical; we will show elsewhere that the bulge is a key determinant of D5 function both in vivo and in vitro (27).

TABLE 2. Summary of revertants analyzed[a]

Mutant	No. of revertants analyzed	No. of back-revertants (phenotype)	No. of nuclear suppressors (phenotype)	No. of mitochondrial suppressors (phenotype)	No. of nuclear and mitochondrial suppressors (phenotype)
A2U	26	0	22 (cs, hs)	0	4 (tr)
G3U	38	38 (tr)	0	0	0
G3A	38	20 (tr)	12 (ts)	6 (tr)	0
C4A	3	1 (tr)	2 (ts)	0	0
C4G	10	5 (tr)	0	5 (tr)	0

[a] cs, cold sensitive; hs, heat sensitive; tr, temperature resistant; ts, temperature sensitive.

Spontaneous revertants of splicing-defective mutants. Spontaneous revertants of the five splicing-defective point mutants of D5 described above were isolated and characterized in detail. Initial screening by direct sequencing of the 3′ end of the intron from each revertant quickly identified backrevertants and second-site suppressor mutations located in D5. In some cases, revertants that retained the original mutation and had no additional mutation in the region sequenced were found. Representatives of this type of revertant were characterized in detail, and each was found to contain a dominant nuclear suppressor. In the following paragraphs, we summarize our studies of these five sets of revertants (Table 2).

(i) Revertants of the A2U mutant. Initial revertants of the A2U mutant all grew slowly on glycerol medium at 30°C and were temperature sensitive, being unable to grow at 23 or 36°C. Twenty-two revertants of this type were found by DNA sequencing to have no change in D5. Four other revertants that were first analyzed only some months after the initial screening were found to grow more vigorously on glycerol medium at 30°C than the original revertants and to be able to grow at 23 and 36°C. The genetic and phenotypic analyses of strain A2U-R10 illustrate the properties of such revertants (Fig. 4 and 5). A2U-R10 was found to contain two suppressor mutations, one nuclear and dominant (su^+, where su^- denotes the wild-type allele) and the other at a second site in D5 of aI5γ. In addition to the original A2U mutation, strain A2U-R10 contains a second mutation in D5 that changes the base pair from U2 U33 to U2 A33.

As outlined in Fig. 4, we used cytoduction methods to separate the nuclear and mitochondrial suppressor mutations of strain A2U-R10 (strain 2). Strain 1 is the A2U mutant, and it has the su^- nuclear background of the untransformed parent of all of the synthetic petite mutants made in this study (strain DBY947, shown as strain 0 in Fig. 4). Strain 3 has the mtDNA of the original A2U mutant (via strain II) and the nuclear background of strain 2 (su^+, via strain 2′ in Fig. 4). Strain 4 has mtDNA from strain 2 (A2U-R10) and the nuclear genome of strain 0 (su^-). Strain 3 is both heat and cold sensitive for glycerol growth, while strain 4 grows on glycerol medium at 23, 30, and 36°C. Several control strains were included in this experiment: strain 5 has the su^- nuclear genome of strain 0 and the wild-type aI5γ (A2 U33), and strain 6 has wild-type mtDNA in the su^+ nuclear background. Since strain 6 is temperature resistant for glycerol growth, we conclude that the temperature-sensitive nature of strain 3 results from an interaction between the mutant intron and the nuclear suppressor.

Northern blots were used to analyze the extent of aI5γ splicing in these strains (Fig. 5A [exon probe] and B [intron probe]). The nuclear suppressor restores a relatively low level of splicing by itself (compare lanes 1, 3, and 4 in Fig. 5A). The

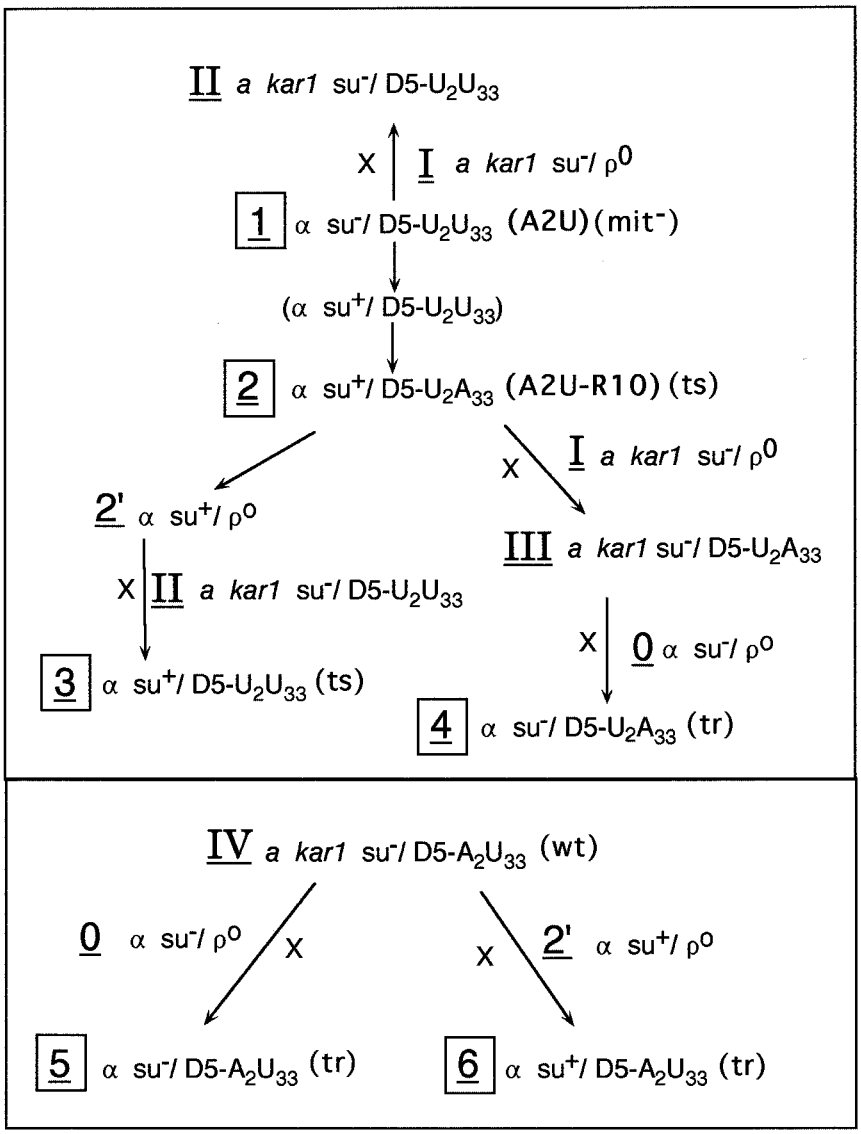

FIG. 4. Genetic analysis of a revertant of the A2U mutant. Strain constructions carried out to define the two mutations present in revertant A2U-R10 are summarized. The resulting strains are characterized phenotypically in Fig. 5. Strain 1 is the A2U mutant strain constructed as described in Materials and Methods. That mutant mtDNA is present in the nuclear background of strain DBY947 (*MAT*α *ade2-101 ura3-52*); it lacks any suppressor of D5 defects and so is shown as su$^-$. Strain 0 is the [*rho*^{0}] derivative of DBY947 [*rho*$^+$]. Strain 2 is revertant A2U-R10, and strain 2′ is the [*rho*^{0}] derivative of strain 2. On the basis of DNA sequencing data reported in the text and the results of genetic experiments, strain 2 was deduced to contain two suppressor mutations, one in nuclear DNA (su$^+$) and another in mtDNA (U33A). In this revertant lineage, we did not obtain the strain containing only the su$^+$ nuclear mutation (shown in parentheses between strains 1 and 2). The construction from strain 2 leading to strain 3 provided a strain with the same nuclear genome as strain 2 but the mtDNA of strain 1. The construction from strain 2 leading to strain 4 provided a strain with the nuclear DNA of strain 1 but the mtDNA of strain 2. The final constructions provide strains with a wild-type D5 having the nuclear genome of strain 1 (strain 5) or of strain 2 (strain 6). Strains I to IV all have the nuclear genome of strain MY375 (*MAT***a** *kar1-1 ura3-52 his4*). Strain I is the [*rho*^{0}] version of MY375, and strains II to IV have mtDNA with the A2U mutation, the A2U and U33A mutations, and a wild-type D5, respectively. ts, temperature sensitive; tr, temperature resistant; wt, wild type.

faint band in Fig. 5B, lane 4 (arrow), is the splicing intermediate containing the intron and the 3′ exon, indicating that this combination of nuclear and mitochondrial mutations causes a detectable defect in the second step of splicing. The mitochondrial second-site suppressor mutation restores quite efficient splicing (compare lanes 1 and 5 in Fig. 5A), indicating that restoring a Watson-Crick base pair at that site in D5 is sufficient for splicing. When lanes 1 and 5 in Fig. 5B are compared, it is clear that the excised intron RNA is greatly destabilized in the double-mutant strain. The nuclear suppressor has little or no effect on splicing of the wild-type or the double-mutant intron (compare lanes 1 and 2 and 5 and 6 in Fig. 5A), although it somewhat improves the accumulation of excised intron RNA in the double mutant (compare lanes 5 and 6 in Fig. 5B). We also tested strain 2 (su$^+$ A2U) for its splicing phenotype when cells are grown at 23 and 36°C (not shown). While there was no detectable splicing in the cells grown at 23°C, consistent with the growth phenotype, there was at least as much mRNA in the cells grown at 36°C as in the culture grown at 30°C (Fig. 5A, lane 4). This indicates that the suppressor is cold sensitive for splicing but owes its heat-sensitive growth defect to some other problem.

Each haploid strain used above was mated to strain 161-U7 (su$^-$ [*rho*^{0}]) to form diploid strains that were then tested for

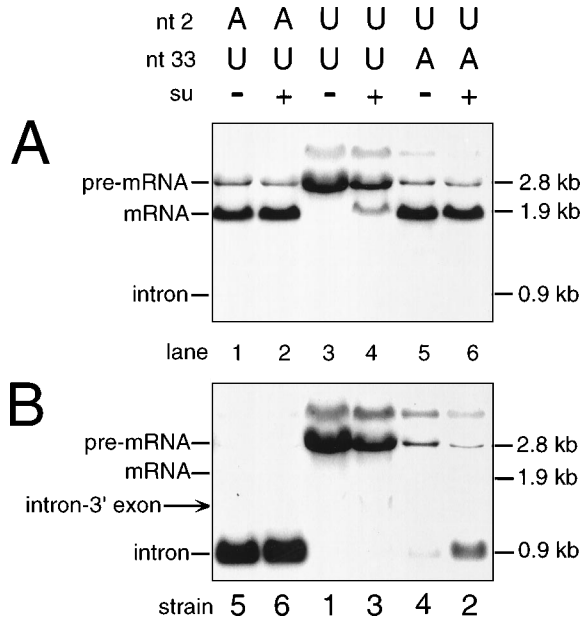

FIG. 5. Splicing phenotypes of strains related to A2U-R10. RNA samples were prepared from strains 1 to 6 of Fig. 4 and were analyzed as for Fig. 2, with a probe specific to *COXI* exon 4 (A) and a probe specific for aI5γ (B). Major transcripts are identified as in Fig. 2. The suppressor allele and the sequence of the second base pair of D5 (A2 U33 in the wild type) of each strain are shown above the lanes. Strain 3 exhibits a partial step 2 splicing defect, since it accumulates some of the 1.5-kb splicing intermediate containing the intron plus 3′ exon (arrow).

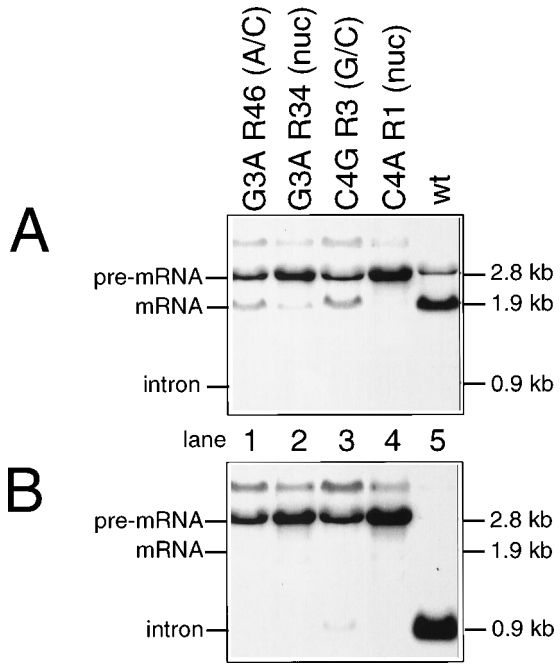

FIG. 6. Splicing phenotypes of revertants of G3A, C4G, and C4A. RNA samples were prepared from the strains listed above the lanes and were analyzed as for Fig. 2. Major transcripts are identified as in Fig. 2. The mitochondrial or nuclear (nuc) mutations present in each strain are given in parentheses. wt, wild type.

their growth phenotypes. All of the haploid strains that were temperature resistant for glycerol growth yielded temperature-resistant diploids. The diploid which contained the A2U mutation and was heterozygous for the suppressor mutation grew on glycerol medium at 36°C but was still cold sensitive, not growing at 23°C. We conclude that the suppressor mutation is dominant and that the reason for its inability to support glycerol growth at 36°C in a haploid strain is probably unrelated to the splicing of aI5γ. Some nuclear genes that influence splicing of other mitochondrial introns are known to have other functions unrelated to splicing (18).

Overall, these genetic manipulations define a dominant nuclear suppressor of the A2U mutation of D5 of aI5γ and demonstrate that efficient splicing can be restored to the A2U mutant by a second mutation in D5 that changes the U2 U33 pair to U2 A33. While the studies of a point mutant at each of these two positions indicated that A2 is more important than U33, this second-site suppressor shows that pairing at that site in D5 is more important than the identity of the nucleotide bases. Curiously, none of 26 revertants of A2U that we sequenced was a backrevertant, and all of the strains carrying the mitochondrial second-site suppressor mutation also have a nuclear suppressor. The primary temperature-sensitive revertants of A2U were found to have just a nuclear suppressor mutation; they retain the U2 U33 allele of D5, and in all other respects are indistinguishable from strain 3 of Fig. 4. It appears that the nuclear suppressor somehow facilitates the recovery of the mitochondrial second-site suppressor.

(ii) Revertants of G3 mutants. All revertants of the G3U mutant grew well on glycerol medium at 30 and 36°C. Six revertants of G3U were sequenced, and each was found to be a backrevertant. Thirty-two additional revertants of G3U were also screened for loss of an *Mbo*I site created by the mutation, and none that still had the site (and therefore the G3U mutation) was found (summarized in Table 2; see reference 2 for details). Because we found nuclear or mitochondrial second-site revertants for all of the other mutants analyzed, in some cases from screening just a few isolates, it appears that a U at the third nucleotide of D5 of this intron is incompatible with splicing in vivo.

Revertants of G3A were more diverse. Of 34 that were sequenced, 16 were backrevertants and 12 still had the G3A mutation. One of these, G3A-R34, was studied in detail. It was found to have no other mutation in the intron and to owe its glycerol growth and splicing of aI5γ to a dominant nuclear suppressor mutation. We also carried out meiotic analysis of a diploid formed by mating G3A-R34 to the [*rho*^{0}] of strain 161-U7 and observed 2:2 segregation of the glycerol growth phenotype (not shown) (2).

Interestingly, six revertants (including G3A-R46, reported here) that retain the G3A mutation and contain a second-site suppressor mutation at U32, converting the mutant A2 U32 pair to the non-Watson-Crick base pair A3 C32, were found. Diploid derivatives of G3A-R46 remain Gly$^+$ and, upon sporulation and tetrad dissection, yield exclusively 4 Gly$^+$:0 Gly$^-$ complete asci (not shown) (2). This shows that the regained ability to grow on glycerol medium results from the new mutation in mtDNA. As shown in Fig. 6A, lane 1, the mitochondrial double mutant (G3A-R46) splices about 20% of its *COXI* transcripts, compared with at least 95% for the wild-type control (lane 5). The strain with the nuclear suppressor (G3A-R34) (Fig. 6A, lane 2) splices aI5γ from about 10% of the *COXI* transcripts present in the strain. As was noted for a number of other mutants and revertants reported here, the level of excised intron RNA in these revertants of G3A is quite low relative to the amount of mRNA (Fig. 6B), indicating that

the mutant intron RNA is less stable than the wild-type intron lariat.

(iii) Revertants of C4 mutants. Revertants of both mutants altered at C4 yielded several classes of revertants, including some backrevertants (Table 2). C4A yielded one nuclear suppressor (C4A-R1) that grows on glycerol medium but splices very poorly (Fig. 6A, lane 4). No nuclear suppressor of C4G was obtained among 10 revertants, but five mitochondrial second-site revertants in which the C4G mutation remains in D5 but is compensated by a mutation of G31 to yield a C4G G31C allele of D5 were found. As shown in Fig. 6A, lane 3, a G4 C31 double mutant (C4G-R3) splices about 25% of *COXI* transcripts.

DISCUSSION

These experiments demonstrate that two of the three most highly conserved base pairs of D5 of a group IIB intron are important for self-splicing and that all three are essential for splicing in vivo. In vitro, only a minority of the mutants were active under the low-salt condition of self-splicing. However, with a commonly used high-salt reaction condition, one that yields 10- to 20-fold-higher rates of self-splicing of the wild-type pre-mRNA than are obtained under low-salt conditions (15), all of the mutant introns self-spliced to some extent. In the high-salt buffer, the effects of mutations at the third and fourth base pairs of D5 (G3 U32 and C4 G31) appeared to be asymmetric: mutations of one nucleotide of each pair had a greater effect on self-splicing than did mutations of the other. Under high-salt conditions, the mutations of the A2 U33 base pair had modest effects on self-splicing, with no apparent asymmetry of effects; including the low-salt condition results, it appears that mutations of A2 are somewhat less debilitating than mutations of U33. Since all six nucleotides analyzed are highly and nearly equally well conserved, we were somewhat surprised to find that only two of the six nucleotides had relatively large effects on self-splicing under both reaction conditions reported here.

These same mutant alleles were analyzed separately by using an in vitro *trans* assay for D5-dependent release of the first exon by hydrolysis (24), with a similar outcome. In that study, all three G3 mutants and both C4R mutants were inactive within the limits of that assay, while all of the other mutant alleles supported splicing, with allele-specific effects on K_m and k_{cat}. An important finding of that study was that D5 RNAs with the G3 and C4R mutations are competitive inhibitors of the *trans* assay when both wild-type and mutant D5 RNAs are present: this indicates that those mutant D5s still bind to the intron but are strongly debilitated in their ability to promote the splicing reaction. Further, from an entirely different set of in vitro experiments, Chanfreau and Jacquier (7) have reached essentially the same conclusions regarding these six nucleotides of D5, including asymmetric effects of alterations at the three base pairs.

Mitochondrial transformation was used to place representative mutations of each of those six positions in mtDNA, and the resulting glycerol growth and aI5γ splicing phenotypes were determined. The in vivo phenotypes of the tested mutants with alterations at the G3 U32 and C4 G31 base pairs resemble the high-salt in vitro results, including a very clear indication of asymmetry of phenotypes. In vivo, mutations of G3 and C4 blocked both glycerol growth and the accumulation of detectable mRNA, while mutations of U32 and G31 permitted respiratory growth and splicing. Both in vitro assays of the effects of mutations at the A2 U33 base pair indicated that neither nucleotide is crucial for self-splicing; however, the in vivo analysis of representative mutations indicated that the pair is essential and revealed the same type of asymmetry as was seen in vivo for the other two pairs. Clearly, we have identified three adjacent nucleotides of D5 of aI5γ (A2, G3, and C4), each of which is essential for splicing in vivo. Point mutations at three other paired positions of D5 (U9, G10, and C29) had, at most, slight effects on splicing in vivo, indicating that those positions are not critical for D5 function.

While there are some differences, these data indicate that the low-salt in vitro phenotypes are generally good predictors of the in vivo phenotypes (compare the data of Table 1 with those of Fig. 2 and 3). The four mutants tested that retained some splicing activity under the low-salt condition (G10A, G31U, U32A, and U32C) also spliced in vivo. Eight of the mutations studied in vivo inhibit self-splicing at least 25-fold under the low-salt reaction condition. Five of those mutations (A2U, G3A, G3U, C4A, and C4G) also inhibit splicing substantially in vivo; however, three mutants (U9C, C29G, and U33G) yield no detectable products in low-salt reactions but splice well in vivo.

The in vitro phenotypes in 0.5 M $(NH_4)_2SO_4$ also correlate generally well with the in vivo data. Four of five mutants that splice at <22% of the control level (G3A, G3U, C4A, and C4G) block respiratory growth and the accumulation of detectable mRNA in vivo, while five of six mutants that splice at or above 67% of the control rate (U32C, U32A, G31U, U9C, and G10A) splice well in vivo. One mutant, U33G, self-splices at about 32% of the control level and was found to splice enough in vivo to grow well. Two exceptions to this correlation were encountered among the 12 mutants analyzed here: the A2U mutant self-splices very well (67%) under high-salt conditions but does not splice detectably in vivo, and the C29G mutant is inhibited about 10-fold in the high-salt buffer but splices well in vivo. There is a generally good agreement between the in vitro and in vivo phenotypes reported here (9 of 12 for low-salt conditions or 10 of 12 for high-salt conditions). The exceptions show that the sequence (and presumably the structure) of D5 is not the only factor influencing the splicing of some of the mutant introns.

Our analysis of revertants of A2U, G3A, and C4G provided further information about the roles of these three base pairs in splicing. Among spontaneous revertants of A2U and C4G were second-site suppressors within D5 that restore Watson-Crick pairing at those positions by creating base pairs, U2 A33 and G4 C31, not yet observed in natural D5 structures. We conclude that base pairing is more important than the identities of the nucleotide bases at those positions. It should be noted that the G4 C31 double mutant does not splice as well as the U2 A33 double mutant; this may mean that there is some preference for a pyrimidine at position 4. However, pairing does not fully explain the roles of those nucleotides, because we have found nuclear suppressor mutations that restore splicing to the A2U and C4A mutants.

A different situation was obtained at the G3 U32 wobble pair. There, one Watson-Crick pair (A3 U32) blocks splicing while another (G3 C32) supports efficient splicing. Interestingly, a non-Watson-Crick pair (A3 C32) partially substitutes for the G U wobble pair. It has been suggested previously that the A^+ C pair resembles a G U wobble pair (6); thus, these data may mean that the local distortion of the helix that is a feature of a wobble pair may be functionally important. As noted above, we found that the A2U mutant (causing a mutant U U pair) is suppressed by a nuclear gene mutation, while none of the 38 revertants of the G3U mutant (also causing a U U pair) contained a nuclear suppressor. We also found that the nuclear suppressor of G3A reported here does not sup-

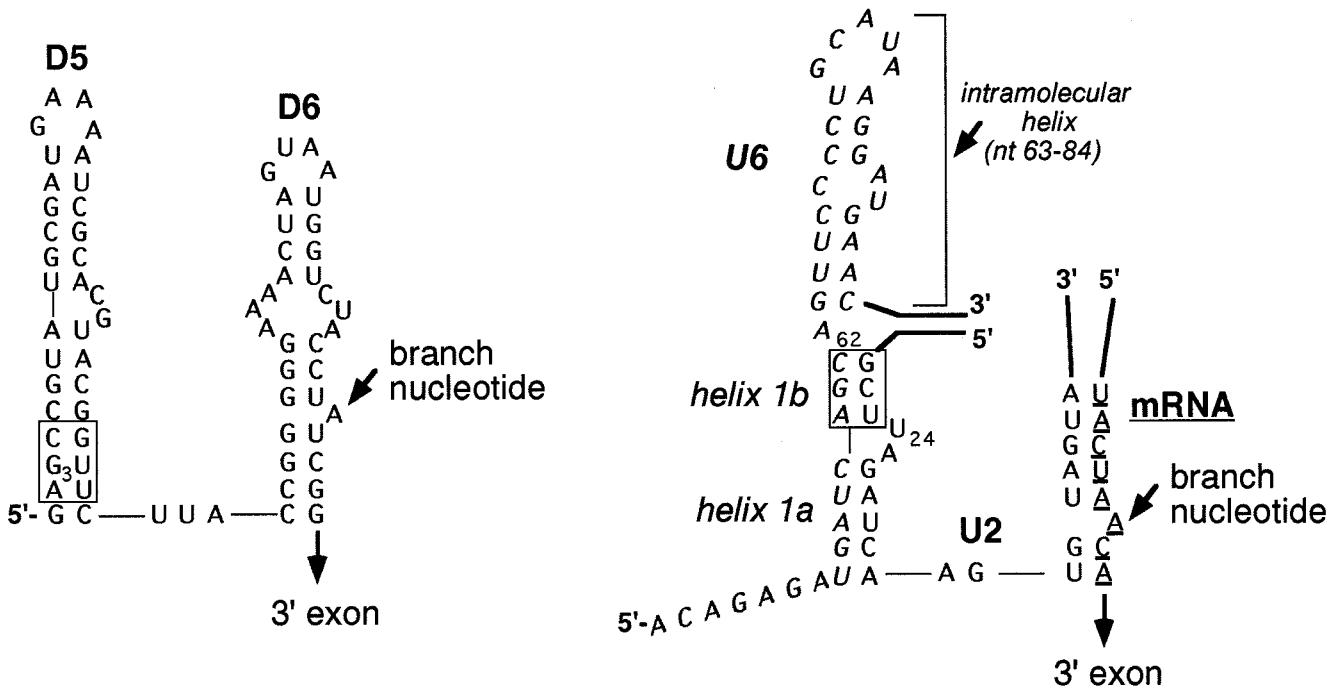

FIG. 7. Comparison of D5 and D6 of a group II intron and the U6-U2 mRNA branch site complex of the nuclear spliceosome. The sequence and proposed secondary structure of the 3′ end of aI5γ containing D5 and D6 are shown on the left. The three nearly invariant base pairs that are shown in this paper to be crucial for D5 function in vivo are boxed. The third nucleotide of D5 is numbered (G_3) to aid comparison with Fig. 1. The sequences of portions of U6 snRNA (italics), U2 snRNA (boldface), and a typical mRNA (underlined) from *S. cerevisiae* and the interactions proposed to occur among those three sequences (19) are shown on the right. Helix 1 and its components helix 1a and 1b are indicated; helix 1b is boxed to emphasize its similarity to the three nearly invariant base pairs of D5 of group II introns. Several nucleotides are numbered to define their location in each yeast snRNA. The intramolecular helix of U6 snRNA, with a diagram illustrating a further base-pairing interaction between other parts of U6 and U2 snRNAs, and the branch nucleotides in D6 of group II introns and in the helix formed by pairing between sequences of U2 snRNA and mRNA are shown.

press the G3U mutation. The absence of mitochondrial second-site suppressors of G3U, such as U3 G32 and U3 A32, indicates that a purine may be required at position 3. Since a G U pair is wild type at that position of D5, and since mutations to G A or G C splice very efficiently in vivo, while the double mutant, A C, and the strain carrying the nuclear suppressor of A U splice less efficiently, we conclude that G is preferred at that site. These data may mean that the C-2 amino group of the purine ring of guanine influences the function of D5; it will be interesting to test whether an inosine at position 3 can support self-splicing. Despite equally strong conservation of all three base pairs, the other two lack such a strong requirement for purine or pyrimidine at either site.

It has been known for some time that D5 is essential for group II intron self-splicing (14), and the present study shows clearly that D5 is essential for splicing in vivo. Only recently have data indicating that D5 has a catalytic function in splicing that can be distinguished from its required binding to D1 been reported (7, 24, 26). Since D6 contains the nucleophile that attacks the 5′ splice junction in vivo, a direct catalytic role for D5 would appear to involve the activation of D6 or the splice junction. A recent model (28) proposes that D5 may function by positioning one or more magnesium ions that catalyze splicing. We have proposed elsewhere that D5 interacts with D6 in a functionally important way (10). However, since D5 activates 5′-exon release in complementation experiments when there is no D6 present (14), it follows that D5 does not act solely through its contact with D6. Two mutations that change the number of nucleotides between D5 and D6 of aI5γ permit self-splicing under the high-salt condition used here but without branching in the first reaction step (5); we interpret that phenotype as a result of a change in the relative orientations of D5 and D6. Because the D5 mutations studied here reduce the in vitro rate of the first splicing reaction but none of them leads to splicing with less branching, we conclude that none of them chiefly interferes with the D5-D6 interaction.

None of these D5 mutations led to a detectable defect in the second step of self-splicing. That is not surprising, because the second splicing reaction is reported to be much faster than the first reaction in the *cis* splicing reactions analyzed here (13). Chanfreau and Jacquier have recently studied effects of base modifications in D5 on the second splicing reaction and concluded that nucleotides G1, A2, and G3 are important for that reaction (7). We have previously reported that mutations of the first nucleotide of aI5γ affect both splicing reactions and lead to the accumulation of the intron–3′-exon reaction intermediate in vivo. Among the 15 strains that splice aI5γ to some extent in vivo (including point mutants and revertants) analyzed in this study, only 3 strains appear to have a defect in the second splicing reaction. The reaction intermediate is noted in Fig. 5B (lane 4) for the A2U mutant strain that has the nuclear suppressor mutation; some of that species is seen in two other strains in darker exposures of Fig. 6 (lanes 2 and 4). All three of these strains splice partially because of a nuclear suppressor mutation, so the origin of the second-step defect may not be a direct result of the D5 mutation.

Chanfreau and Jacquier have recently reported initial studies of mutations of the phosphodiester backbone of D5 (7). They found that Rp phosphorothioates substituted at the phosphate 5′ from A2 and G3 strongly inhibit both steps of splicing; they noted that addition of Mn^{2+} to the splicing reaction mixtures can rescue part of the splicing defect of several of

those atomic mutations. Those data may mean that the phosphodiester backbone of the D5 helix is involved in Mg^{2+} ion binding. While nothing in the current database excludes the possibility that some feature of the nucleotide bases A2, G3, and C4 plays a direct role in D5 function, it is likely that those nucleotides influence aspects of D5 structure needed for its function. This might involve the manner in which one or more Mg^{2+} ions is bound to D5; perhaps the precise location of the metal ion(s) relative to the other elements present at the reaction center of this catalytic RNA is strongly influenced by the G3 U32 wobble pair and, possibly, by stacking interactions influenced by the required purine at position 3.

Madhani and Guthrie (19) showed that a specific base-pairing interaction, called helix 1 (subdivided into two substructures, helix 1a and 1b), between short segments of U2 and U6 small nuclear RNAs (snRNAs) of the yeast nucleus (Fig. 7) is essential for splicing of nuclear pre-mRNA introns. They proposed that helix 1 resembles D5 of group II introns and suggested that the two structures may have similar functions in splicing. It had previously been suggested that the pairing between part of U2 snRNA and the branch sites of nuclear introns resembles D6 of group II introns (30); the portions of U2 snRNA involved in helix 1 and in the D6-like pairing are separated by just 2 nucleotides.

If that analogy to D5 is valid, then it follows that similar mutations at functionally analogous sites in the two structures should have similar consequences. Madhani and Guthrie reported that three base pairs of helix 1a are critical for splicing and that point mutations of either nucleotide (one from U6 and the other from U2) have similar inhibitory effects. Since compensating mutations forming a different base pair at those sites restored splicing, they concluded that pairing, but not the identity of those nucleotide bases, was important. A different situation was found for helix 1b, in which the nucleotides contributed by U6 snRNA (A59, G60, and C61) are required to various degrees for yeast viability, while their presumptive pairing partners in U2 snRNA (G21, C22, and U23) are more tolerant to mutation (Fig. 7). While several mutations of U23 of U2 snRNA restore splicing by compensating for a pairing defect caused by mutations of A59 of U6 snRNA, the other two base pairs appeared to be asymmetric in that compensating mutations in the U2 sequence did not suppress mutant alleles of U6.

Because helix 1 has eight base pairs while D5 has 14, it is not obvious how to compare the two structures. When the two helices are compared relative to their 2-base bulges, it is clear that our study deals primarily with the portion of D5 that is positioned like helix 1a of the U6 U2 structure. However, the effects of mutations of the second through fourth base pairs of D5 are quite different from the effects of similar single and double mutations in helix 1a. It also appears that the nucleotides just above the 2-nt bulge of D5 (U9 and G10) are not important for splicing of this group II intron, while the similarly situated nucleotides of U6 in helix 1b (A59 and G60) are essential. It appears unlikely that U6-U2 helix 1a is functionally equivalent to the base of the D5 helix.

We noticed that the crucial portion of D5 defined by this study appears very similar to the three base pairs of helix 1b. The A2 U33 pair of D5 can be substituted by a different base pair (e.g., U2 A33), and likewise, the A59 U23 pair of helix 1b can be substituted by a different pair. Similarly, mutation of A2 of D5 or A59 of U6 blocks splicing while mutation of U33 of D5 or U23 of U2 does not block splicing. Like the G3 U32 pair of D5, the G60 C22 pair of helix 1b depends on the G residue, and mutations of G3 are not suppressed by formation of alternative Watson-Crick base pairs. Finally, mutation of the C61 G21 pair of helix 1b shows the same sort of asymmetry we found for the C4 G31 pair of D5. Changing the C G pair to G C does not support spliceosomal splicing, but G C functions partially in the group II intron.

We find it remarkable that U6-U2 helix 1b contains three adjacent, critical nucleotides (A59, G60, and C61) that are the same as the three nucleotides of D5 identified in this study as essential for splicing in vivo (A2, G3, and C4). These two strings of nucleotides appear to have similar functions in splicing but occupy different locations in the helical structure, both relative to the 2-nt bulge and relative to the proposed D6 cognates. Since helix 1b may be functionally analogous to the bottom part of D5, perhaps the intramolecular helix of U6 snRNA (nt 63 to 94; Fig. 7) may be analogous to the top part of D5.

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