ing to Newport and Spann (*6*) by the use of extraction buffer [EB; 80 mM β glycerophosphate, 20 mM EGTA, 5 mM $MgCl_2$, 20 mM potassium Hepes (pH 7.5)] and supplemented with magnesium adenosine triphosphate (ATP) and creatine phosphate before use [A. W. Murray and M. W. Kirschner, *Nature* **339**, 275 (1989)]. An antiserum for histone H1X was raised in a rabbit against H1X protein that had been purified from egg extracts as described (*10*). Antibody-loaded protein A–Sepharose beads were prepared according to Hirano and Mitchison (*9*). For immunodepletion of H1X, an egg extract was incubated with an equal volume of packed beads at 4°C for 30 min with rotation. The beads were pelleted (1500*g*, 5 min) and the extract was reincubated with the equal volume of fresh beads for an additional 30 min. Demembranated sperm were prepared and incubated with egg extracts as described (*10*). To monitor the state of sperm nuclei, a 2-μl sample was removed onto a glass slide and added with 3 μl of fixative [10% formalin, 50% glycerol in EB containing 4,6-diamino-2-phenylindole (DAPI) (10 μg/ml)] and then squashed with a coverslip for observation under an epifluorescence microscope. For immunostaining, a 20-μl sample was fixed with 200 μl of 5% formalin in EB. Metaphase chromosomes were fixed on a polylysine–coated glass slide as described (*9*). After blocking with 20% fetal calf serum, preparations were incubated with the 1:500 diluted anti-H1X serum, followed by incubation with fluoroscein isothiocyanate–conjugated goat anti-rabbit immunoglobulin G, and counterstained with DAPI. For immunoblotting, samples were diluted 10-fold into an SDS-PAGE sample buffer, run on 15% polyacrylamide gels [U. K. Laemmli, *Nature* **227**, 680 (1970)], and then transferred onto an Immobilone membrane (Millipore). H1X was probed with the 1:1000 diluted anti-H1X serum and visualized with a 5-bromo-4-chloro-3-indolyl-phosphate–nitro blue tetrazolium phosphatase substrate system.

18. Demembranated sperm (5×10^5) were incubated with 100 μl of egg extracts for 90 min. The mixture was diluted 10-fold with EB, and metaphase chromosomes were sedimented (10,000 *g*, 10 min) onto 2.0 M sucrose in EB through 100 μl of 1.2 M sucrose in EB. Metaphase chromosomes at the 1.2 to 2.0 M interphase were suspended in EB and then pelleted (5000*g*, 10 min). Chromatin proteins were extracted from metaphase chromosomes and erythrocyte nuclei with 0.4 N H_2SO_4 as described (*10*) or in SDS-PAGE sample buffer and analyzed on 15% polyacrylamide gels.
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Role of U6 snRNA in 5′ Splice Site Selection

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Two models describing the interaction between U6 small nuclear RNA (snRNA) and the 5′ splice site of introns have been proposed on the basis of cross-linking experiments. Here it is shown that a conserved sequence present in U6 snRNA forms base pairs with conserved nucleotides at the 5′ splice junction and that this interaction is involved in 5′ splice site choice. These results demonstrate a specific function for U6 snRNA in splicing and suggest that U6 snRNA has a proofreading role during splice site selection. A model is presented in which this new interaction, in concert with previously described interactions between U6 snRNA, U2 snRNA, and the pre–messenger RNA, would position the branch point near the 5′ splice site for the catalysis of the first splicing step.

Splicing of nuclear introns occurs by a two step pathway (*1*). In the first step, the phosphodiester bond at the 5′ splice site is attacked by the 2′-OH of an adenosine residue in the intron, the branch point. This reaction produces a free upstream exon and a lariat intermediate molecule containing both the downstream exon and the intron with its 5′ end covalently linked to the branch nucleotide. The selection of the 5′ splice site phosphodiester bond to be cleaved by this reaction must be highly accurate. Indeed, most errors would introduce frame shifts into the coding part of mRNAs thereby impairing protein production. Accurate splice site choice probably results from the numerous interactions established between the pre-mRNA and splicing factors during spliceosome assembly (*1*, *2*).

The U1, U2, U4, U5, and U6 small nuclear RiboNucleoProteins (snRNPs) are splicing factors that function in spliceosome assembly and splicing catalysis. U1 snRNA forms base pairs with 5′ splice sites, and in some species, with 3′ splice sites (*3*, *4*, *5*), whereas U2 snRNP interacts with the branch point (*6*). U1 snRNA recognizes pre-mRNAs containing introns and commits them to the splicing pathway (*3*). For alternatively spliced pre-mRNA, U1 snRNP will thus select the splice site to be used from the potential 5′ splice sites. However, U1 snRNA is not involved in the selection of the precise 5′ splice site bond to be cleaved (*7*, *8*). The U5 snRNA interacts with exon sequences in the pre-mRNAs, intermediates, and products of the splicing reaction (*9*, *10*) and is one of the factors implicated in the choice of the 5′ phosphodiester bond to be cleaved. However, additional unidentified factors are also postulated to play a role in 5′ cleavage site choice (*3*, *7*, *8*).

Because U6 is the most conserved of the snRNAs involved in splicing, it is assumed that it has an important, but unidentified, function (or functions) in the splicing reaction (*2*). Cross-linking experiments in mammalian and yeast systems indicate that U6 snRNA and the pre-mRNA are in close proximity (*11*, *12*, *13*). Because of limitations of the cross-linking technique, it has been unclear whether U6 snRNA forms base pairs with the 5′ splice site, or if the cross-linking results from the proximity of the two RNAs in the spliceosome (*12*, *13*). After these studies, two mutually exclusive models proposed that base pairing occurs between U6 snRNA and the 5′ splice site (*12*, *13*). In the first model (*12*), a region of yeast U6 flanking C48 would form base pairs with intron sequences flanking G5 (Fig. 1, A and B). This model suggests an interaction between conserved sequences in U6 and at the 5′ end of introns. Furthermore, several U6 nucleotides near C48 are critical for U6 snRNP function (*14*, *15*). In a second proposal (*13*), U6 snRNA C43 and flanking nucleotides would form base pairs around intron position 5 (Fig. 1C). Nucleotide C43 and some flanking nucleotides are neither evolutionarily conserved nor essential for U6 function (*14*, *15*). Interactions predicted by both models would extend only over a few nucleotides.

Yeast introns mutated at position 5 display two phenotypes (*16*). (i) They have a reduced splicing efficiency, which is reflected by an increase in pre-mRNA levels in

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vivo, and a low ability to assemble spliceosomes in vitro. In the presence of complementary U1 mutants, this defect is partly corrected (*7, 8*). (ii) Mutations at position 5 of introns induce aberrant cleavage of the pre-mRNA either upstream or downstream of the normal 5′ splice site. The products of these aberrant cleavage events accumulate as lariat intermediates (*16*). We have shown, however, that if a G follows the cleaved phosphodiester bond, the second splicing step will occur to produce an aberrantly spliced message (*8*). Thus, intron mutants at position 5 induce inaccuracy in the selection of the 5′ splice site. In the presence of complementary U1 mutants, splicing at both the normal and aberrant sites increases in a parallel manner indicating that U1 snRNA base pairs only with the normal site (*8*). Splicing inaccuracy can be attributed to the inability of a factor (other than U1) to recognize the mutant position 5 at a later step of spliceosome assembly but the nature of this factor (or factors) is unknown. In light of the cross-linking results (*11, 12, 13*), we decided to test if U6 mutants at position 43 or 48 would affect the splicing of introns with position 5 mutations.

Several mutations were made in the U6 gene (*17*): position 43 was changed from C to T to give U6-43U, position 48 was changed to T or A to create U6-48U and U6-48A, respectively, and as a control, positions 69/70 were changed to GA. Mutants at position 43 and 69/70 are able to support growth in yeast (*18*). As predicted (*15*), strains homozygous for any of the mutations at position 48 were not viable under the conditions analyzed (*18*). Furthermore, when expressed from a centromeric plasmid, these U6 mutants accumulated to less than one-tenth of the level of the wild-type chromosomal U6 (*19*). In contrast, primer extension analyses indicated that the same mutants inserted into multicopy vector accumulated to an amount similar to the endogenous wild-type U6 (*19*). However, it is not clear what fraction of the mutant U6 snRNA is functional.

Reporter genes with RP51A intron derivatives inserted into the lacZ gene were introduced into these strains (*20*). We used reporters containing G nucleotides at positions −3 and −4 in the upstream exon and different substitutions at position 5 of the intron (*8, 21*). In the presence of the two exonic G residues, intron position 5 mutants show some residual splicing at the normal site, but significant amounts of aberrantly spliced RNA are also produced. Most of the aberrant splicing events use the site at position −4 although small amounts of mRNA spliced at position −3 can also be detected. Because spliced mRNA resulting from splicing at the normal 5′ splice site (or for a small proportion at position −3) are in a different reading frame compared to mRNA spliced at position −4, use of these sites can be independently monitored by β-galactosidase assays with constructs in which lacZ is in one or the other reading frame (*8*).

With the β-galactosidase assay (*22*), we analyzed the effect of wild-type U6 (WT-U6), U6-48U, U6-43U, and U6-69G/70A, on the splicing of introns with either a G or an A at position 5. The only significant effect observed was that U6-48U increased the usage of the normal 5′ splice site of the mutant reporter that produced 2.4 ± 0.2– (average ± standard error, five experiments) fold more β-galactosidase activity (*18*) (Fig. 2A). This effect did not result from a greater splicing efficiency of this pre-mRNA in the presence of U6-48U because splicing at the aberrant site was not affected. This interaction between U6 position 48 and intron position 5 is specific because the splicing of the mutant intron was not affected by U6 mutant at other positions (*18*). To eliminate the possibility that U6-43 interacts with the 5′ splice site but is recessive, we analyzed splicing of the same set of reporters in a strain expressing only U6-43U. In that case, the splicing of all constructs, including those with an A at intron position 5, was slightly reduced (*18*). This indicates that U6 position 43 does not interact specifically with the 5′ splice site and that U6-43U is slightly defective for splicing even though it does not impair growth. Our data are consistent with the model proposing that U6 position 48 interacts with the 5′ splice site (*12*) (Fig. 1B).

To determine the nature of the interaction between U6 and the 5′ splice site, we analyzed the splicing of introns with G (wild type), A, or U at position 5 in the presence of WT-U6, U6-48U, or U6-48A. The wild-type reporter gene was efficiently spliced at the normal site, but only a minute amount of splicing could be detected at the aberrant site. Splicing of the wild type reporter was not affected by the U6 mutants because each strain also contains a WT-U6 gene (Fig. 2A and B, WT columns). The construct with an A at position 5 spliced poorly and used the normal and the aberrant site similarly (*8*). The U6-48U mutant significantly increased splicing at the normal site for the construct with an A at intron position 5 (Fig. 2A) but did not affect (or reduced slightly) splicing at the aberrant site (Fig. 2B). Splicing of the same construct was not affected by the presence of the U6-48A mutant. For the intron mutant with a U at position 5, splicing at

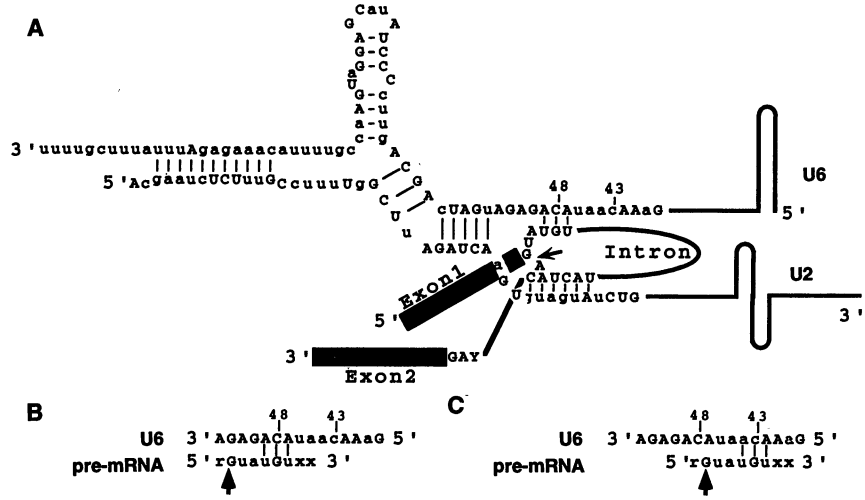

Fig. 1. Models of interaction between U6 snRNA, U2 snRNA, and the pre-mRNA before the first splicing step. (**A**) Model depicting the location of U6 position 43 and 48 with respect to U6-U2 interaction regions and conserved sequence elements. The sequences depicted are part of yeast U2 and U6 snRNAs. snRNA nucleotides that are conserved between various species are indicated by uppercase letters. This part of the model has been adapted from Madhani and Guthrie (*28*). In the pre-mRNA, exons are indicated by boxes while the intron is drawn as a line. The consensus sequences at the branch point and the 5′ and 3′ splice sites are indicated. The U6 snRNA-5′ splice site interaction described in the text is depicted. An arrow points to the location of the first nucleotide of the intron and the adenosine at the branch point. The proposed U2 snRNA–U6 snRNA–pre-mRNA interactions would bring the two nucleotides that will react during the first splicing step into proximity. (**B**) The model of interaction between U6 and the pre-mRNA proposed by Wassarman and Steitz (*12*). The upper line represents the sequence of the yeast U6 and the bottom line the consensus yeast 5′ splice site with conserved nucleotides in upper case. An arrow indicates the location of the 5′ splice site. (**C**) The model of interaction between U6 and the pre-mRNA proposed by Sawa and Abelson (*13*). The same symbols as in part (B) are used. C43 is not phylogenetically conserved.

the normal site was strongly enhanced and splicing at the aberrant site was unaffected by the complementary U6-48A (Fig. 2A and B). A weaker effect was observed in the presence of the noncomplementary U6-48U. We extracted RNAs from the set of strains described above and mapped 5′ cleavage sites by primer extension with an intron primer (*23*). The resulting gel is shown (Fig. 2C). This analysis confirms the results of the enzymatic assay; U6-48U increases the usage of the normal splice site when intron position 5 has been changed to an A (Fig. 2C, compare lane 8 with lanes 7 and 9) whereas the construct with a U at position 5 is more affected in a strain expressing U6-48A (Fig. 2C, compare lane 12 with lanes 10 and 11).

Because U6-48U affected splicing of constructs with an A or a U at intron position 5, this could imply that the U6-pre-mRNA contact is not a regular Watson-Crick interaction. However, because the U6-48U effect on constructs with a U at intron position 5 is weak, it is also possible that it results from a U-U base pairing interaction. Indeed, U-U base pairing is quite stable and well tolerated in RNA helices (*24*). To solve this ambiguity, we constructed double mutants in U6 (U6-47G/48A and U6-48U/49U) and measured their effect on the efficiency of splicing at the normal site for reporters harboring complementary mutations in the intron (5U/6C and 4A/5A). For these reporters, splicing at the normal site was only increased in the presence of the complementary U6 snRNA (Table 1). This confirms that the U6–pre-mRNA interaction occurs by base pairing and suggests that it involves U6 positions 47 to 49. On the basis of the limited extent of sequence conservation at 5′ splice site, this pairing is unlikely to extend further into the intron than position 6 (*25*). To affect 5′ splice site choice, this pairing has to be established during spliceosome assembly before, or concomitant with, the first splicing step.

The suppression of intron position 5 mutants by complementary U6 mutants was always observed to be weak. This is probably because mutants in an essential region of U6 are poorly incorporated into functional snRNPs (*15*). Thus, the fraction of mutant snRNP is probably low to begin with and might be further reduced by inefficient competition with the wild-type snRNP at other stages during splicing. This possibility is supported by two experiments. (i) When U6-48U RNA levels were titrated in vivo, a concomitant change in the usage of the normal 5′ splice site of a position 5A mutant was observed (*18*). (ii) We constructed a U6 mutant at positions 44 to 46 (U6-44/46) that would extend the U6 base pairing with our reporter gene to include intron positions 7 to 9 (Fig. 3A). We expected that this mutant would anchor the U6 snRNA in the appropriate position even in the presence of a mutation at intron position 5 (*26*). This mutant U6 is viable (*18*) and experiments were conducted without competition by the WT-U6. We analyzed the splicing of reporter genes with either a G or an A at intron position 5 in the U6-44/46 strain. Results of the β-galactosidase assay and RNA analysis are shown (Fig. 3, B and C). The mutant U6-44/46 did not affect the splicing of our wild-type reporter gene (Fig. 3, A and B). However, it increased the usage of the normal site by 10-fold and decreased usage of the aberrant site by 100-fold as measured by the β-galactosidase assay (Fig. 3B, logarithmic scale). This is confirmed by primer extension analysis of the corresponding RNAs (Fig. 3C, compare lanes 5 and 6). This demonstrates in a definitive manner that U6 is involved in 5′ splice site selection and strengthens the idea that the partial restoration of usage of the normal 5′ splice site observed with the other U6 mutants is the result of their low activity.

Our results demonstrate that U6-position 48 can specifically interact with position 5 of the intron. We also demonstrate that U6 snRNA is involved in 5′ cleavage site selection. Indeed, restoring or increas-

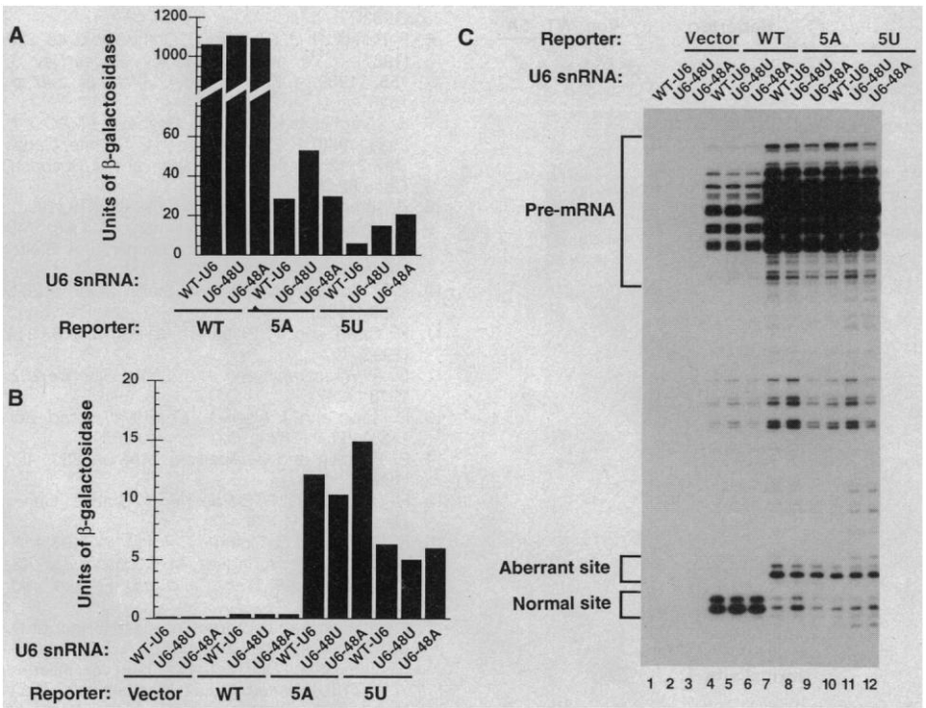

Fig. 2. U6 position 48 forms base pairs with intron position 5. (**A**) Usage of the normal 5′ splice site for reporter genes with, at intron position 5, either a G (WT), an A (5A), or a U (5U) was determined with the use of the β-galactosidase assay in the presence of either WT-U6, U6-48U, or U6-48A. (**B**) An analysis identical to the one described in part (A) was done for the aberrant splice site. The background levels of β-galactosidase were determined in strains carrying a vector without lacZ (Vector). (**C**) Primer extension analysis of the 5′ splice site used. RNAs from strains described above were extracted and analyzed by primer extension with the use of an intron primer to map the 5′ splice site used (*23*). Bands corresponding to extension of molecules cleaved at the normal or aberrant 5′ splice site as well as pre-mRNA species are indicated. The pre-mRNA heterogeneity results from the use of numerous transcription start sites (*19*). RNA extracted from cells carrying the vector alone (lanes 1 to 3) are used as a control for extension of endogenous RP51A messages. The reporters used carry either the wild-type (WT, lane 4 to 6), position 5A (5A, lanes 7 to 9) or position 5U (5U, lanes 10 to 12) intron. The plasmid-encoded U6 genes were WT-U6 (lanes 1, 4, 7, and 10), U6-48U (lanes 2, 5, 8, and 11), or U6-48A (lanes 3, 6, 9, and 12). The additional faint stops present in lanes 11 and 12 were not reproducibly detected.

Table 1. Analysis of double mutants. Double mutants were constructed in U6 (U6-47G/48A, U6-48U/49U) and complementary mutations in the reporter gene (5U/6C, 4A/5A). The wild-type reporter (WT) was used as a control. For each construct, the increase in the usage of the normal site by U6 mutant relative to the wild-type U6 is tabulated. Values are average ± error of two experiments.

Reporter	U6-47G/48A	U6-48U/49U
WT	0.94 ± 0.08	1.01 ± 0.07
5U/6C	1.69 ± 0.02	1.13 ± 0.02
4A/5A	1.04 ± 0.02	1.95 ± 0.02

ing base pairing between U6 and the 5′ splice site leads to an increased splicing at the normal site with no effect (or a decreased splicing) at the aberrant site. None of the U6 mutants reduced the high quantities of pre-mRNA seen with the intron position 5 mutants (for example, Fig. 3C). Thus, U6 snRNA has significant effects on splicing accuracy but little effect on the splicing efficiency. This is opposite to what is observed for U1 snRNA (*3*, *7*, *8*). The importance of the U6–pre-mRNA base pairing interaction helps to explain the strong conservation of G at the position 5 of introns (*25*) and why U6-C48 is also absolutely conserved and essential for U6 function (*14*, *15*, *18*).

In summary, we would like to propose the structure depicted in Fig. 1A as a model of the active spliceosome. In this model, known U6 snRNA–U2 snRNA (*27*, *28*) and U2 snRNA–pre-mRNA (6) interactions as well as the U6 snRNA–pre-mRNA base pairing described here, would bring the branch nucleotide and the 5′ splice site into close contact. The presence of U6 snRNA close to the catalytic site of the spliceosome is supported by recent cross-linking experiments (*10*). In the absence of the U6 snRNA–pre-mRNA interaction (for example, intron mutant 5A), 5′ splice site choice would rely on other interactions between the pre-mRNA and splicing factors, leading to the increased selection of aberrant sites. Base pairing between U5 snRNA and exon sequences is a likely candidate for such an interaction (*9*, *29*). Currently it is not known how the U5 snRNP interacts with the other snRNPs or protein splicing factors, therefore U5 snRNA is not depicted in our model. U1 snRNA is probably no longer base-paired with the 5′ splice site at this stage, because previous studies showed (i) that U1 snRNA does not select the 5′ splice site (*3*, *8*), (ii) that U1 snRNA cross-linking with the 5′ splice site diminishes during the formation of the mature spliceosome (*12*), and (iii) that U5 and U6 snRNAs base pair with some of the nucleotides that have been shown to base pair with U1 snRNA at an early stage of spliceosome assembly (*3*, *5*, *9*). U6 snRNA could thus be involved in a proofreading step at the 5′ splice site. The early U1 snRNP interaction with the 5′ splice site is important to recruit RNA sequences into commitment complexes and pre-spliceosomes (*3*). However, this initial interaction could be checked by pairing of U6 snRNA with the 5′ splice site. In the presence of a faithful 5′ splice site, U6 snRNA could properly position the 5′ splice junction in the catalytic center thereby increasing the splicing rate relative to the rate of spliceosome disassembly or degradation.

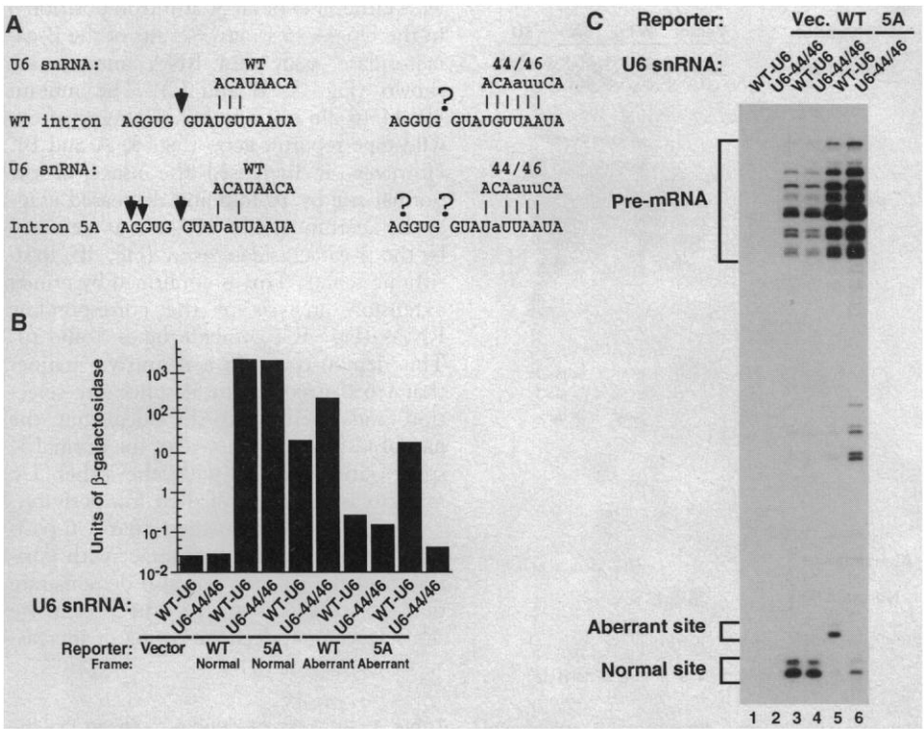

Fig. 3. Anchoring of U6 snRNA through extended base pairing affects splice site selection. (**A**) U6-44/46 has extended complementarity to the RP51A intron. The predicted base pairing interaction between WT-U6 or U6-44/46 and reporter genes with either a G or an A at position 5 of the intron are depicted. Note that mutations at position 5 of the intron would probably disrupt the WT-U6–pre-mRNA interaction whereas U6-44/46 might remain associated with the intron through base pairing with nonconserved intron sequences. (**B**) Usage of the normal and aberrant site in strains harboring either the WT-U6 or U6-44/46. The background levels of β-galactosidase were determined in strains carrying a vector without lacZ (Vector). As a result of the wide range of β-galactosidase levels, the results are plotted on a logarithmic scale. (**C**) Primer extension analysis of the 5′ splice site used. Bands corresponding to extension of molecules cleaved at the normal or aberrant 5′ splice site as well as pre-mRNA species are indicated. RNAs were extracted from cells carrying the vector alone (Vec., lanes 1 and 2) or reporters with either wild-type (WT, lanes 3 and 4) or position 5A intron (5A, lanes 5 and 6). A single U6 gene was present in these strains: WT-U6 (lanes 1, 3, and 6) or U6-44/46 (lanes 2, 4, and 6).

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19. Expression of position 48 mutants was analyzed by primer extension (*5*) with oligonucleotide EM75 (5′ CTTATGCAGGGGAACTGCTG 3′) in the presence of dideoxyG.
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construct (*20*). Mutagenesis, sequencing, and recloning was as described (*5*). Reporters were introduced into *Saccharomyces cerevisiae* strains BSY17 (*7*), BSY398 [*MATα, CAN1, his4, leu2, trp1, ura3, snr6::LEU2*, pBS521 (2 μm, WT-U6, TRP1)], or BSY399 [*MATα, CAN1, his4, leu2, trp1, ura3, snr6::LEU2*, pBS605 (2 μm, U6-44/46, TRP1)].

22. β-galactosidase assays were as described [L. Guarente and M. Ptashne, *ibid.* **78**, 2199 (1981)] or conducted with a modification of the original protocol in which the chloroform-SDS mixture was replaced by 200 μl of ether. In the latter case, ether was evaporated before the assay. Assays were done in duplicate and data represent average values. The variation between duplicate samples was at most 25%.
23. RNA extraction and primer extension were as described (*5*). The RB27 primer complementary to intron sequences downstream of the 5′ splice was used to map the cleavage site on lariat molecules (*8*). Because the efficiency of the second splicing step is different for RNAs cleaved at the normal or aberrant splice site, it is not possible to compare quantitatively the analyses by primer extension and enzymatic assay.
24. J. J. SantaLucia, R. Kierzek, D. H. Turner, *Biochemistry* **30**, 8242 (1991).
25. Analysis of 74 different yeast introns indicate that the yeast consensus sequence at the 5′ splice site is rr/GuauGuwx, in which uppercase letters represent completely conserved nucleotides, r: purine, w: A or U, x: any nucleotide and /: the 5′ splice junction. Thus, there seems to be no selection after position 6 in the intron for pairing with nucleotides upstream of position 47 in U6 snRNA. However, a U that can not base pair with U1 snRNA but can with U6 snRNA is conserved at position 4. In human, the 5′ splice site consensus is kag/Gtragtr (symbols as above with k being A or C) [R. M. Stephens and T. D. Schneider, *J. Mol. Biol.* **228**, 1124 (1992)]. G at position 5 of the intron is the third most highly conserved nucleotide in that consensus. There could be pairing between U6 position 46 to 48 and nucleotides 5 to 7 of 5′ splice sites.
26. A similar strategy with a different reporter gene and similar results are reported by C. Lesser and C. Guthrie [*Science* **262**, 1982 (1993)].
27. B. Datta and A. M. Weiner, *Nature* **352**, 821 (1991); J. Wu and J. L. Manley, *ibid.*, p. 818.
28. H. D. Madhani and C. Guthrie, *Cell* **71**, 803 (1992).
29. Analysis of constructs with an A at intron position 5 and various substitutions in the last two bases of the upstream exon indicate that the level and location of the aberrant cleavages' events are consistent with base pairing of U5 snRNA with nucleotides preceding the cleaved phosphodiester bond (*18*).
30. We thank D. Brow for plasmids and yeast strains, P. Legrain for suggestion on the β-galactosidase assay, C. Lesser, C. Guthrie, E. Sontheimer, and J. Steitz for exchange of information before publication, and the EMBL services for their help. We are grateful to K. Bohmann, C. Dingwall, E. Izaurralde, S. Gunderson, J. Lewis, Z. Lygerou, I. Mattaj, F. Mauxion, and K. Weis for their thoughtful discussions and comments on the manuscript. We thank I. Mattaj and EMBL for support.

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Connexin Mutations in X-Linked Charcot-Marie-Tooth Disease

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X-linked Charcot-Marie-Tooth disease (CMTX) is a form of hereditary neuropathy with demyelination. Recently, this disorder was mapped to chromosome Xq13.1. The gene for the gap junction protein connexin32 is located in the same chromosomal segment, which led to its consideration as a candidate gene for CMTX. With the use of Northern (RNA) blot and immunohistochemistry technique, it was found that connexin32 is normally expressed in myelinated peripheral nerve. Direct sequencing of the connexin32 gene showed seven different mutations in affected persons from eight CMTX families. These findings, a demonstration of inherited defects in a gap junction protein, suggest that connexin32 plays an important role in peripheral nerve.

Charcot-Marie-Tooth disease (CMT) is a pathologically and genetically heterogeneous group of disorders that cause progressive degeneration of peripheral nerves. Affected patients have distal weakness, atrophy, sensory loss, and decreased tendon reflexes. CMT has traditionally been classified by whether the primary pathological defect is degeneration of the myelin (CMT1) or of the axons (CMT2) in the peripheral nerves. Within the demyelinating type of CMT, there is genetic heterogeneity, with similar dominantly inherited disease manifestations produced by genetic defects on chromosomes 17 (CMT1A), 1 (CMT1B), and X (CMTX). Recently, mutations in the chromosome 17– and chromosome 1–linked forms of CMT have been found in the genes for peripheral myelin protein 22 (PMP22) and myelin protein zero (P_0), respectively (*1*, *2*). Here, we report that patients with X-linked CMT have mutations in the gene for the gap junction protein, connexin32 (Cx32, *GJB1*).

Linkage studies and analysis of recombinants were initially used to map CMTX to the proximal long arm of the X chromosome (*3*, *4*) and subsequently to refine the localization to band Xq13 (*5*). Analysis of additional recombinations in CMTX families placed CMTX in a small interval between the markers DXS106 and DXS559 in Xq13.1 (*6*). The gene for Cx32 is known to map to this interval (*7*), and we therefore evaluated it as a candidate gene for CMTX. We performed Southern (DNA) blots of patient DNA to look for rearrangement of the Cx32 gene in CMTX and Northern blots to determine the extent of expression in peripheral nerve RNA. The Southern blots showed no abnormality, but Northern analysis showed expression of the Cx32 gene in peripheral nerve at a level comparable to that in liver and greater than that present in most other tissues (Fig. 1).

Because we found expression of Cx32 in peripheral nerves, we directly sequenced the translated portion of the gene in samples from CMTX patients. We discovered seven variations from the control sequence in eight CMTX families (Figs. 2 and 3): six single-base changes that predict nonconservative amino acid substitutions, and one single-base insertion that shifts the translational reading frame at position 175 and predicts a premature stop signal at position 241. Two families (221 and K1905) from the midwestern United States shared the same mutation at position 139. One other CMTX family (family 63) (*5*) had no detectable mutation in the translated portion of the Cx32 gene.

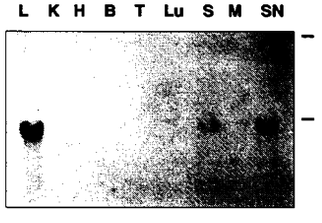

Fig. 1. Northern blot showing expression of Cx32 in peripheral nerve (*19*). The lanes contain 10 μg of total RNA from the following rat tissues; L, liver; K, kidney; H, heart; B, brain; T, thymus; Lu, lung; S, spleen; M, muscle; and SN, sciatic nerve. The hatch marks at right indicate the positions of 28*S* and 18*S* ribosomal RNA, respectively.

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