Genetic Suppression of Intrinsic +1G Mutations by Compensatory U1 snRNA Changes in Caenorhabditis elegans

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ABSTRACT

Mutations to the canonical +1G of introns, which are commonly found in many human inherited disease alleles, invariably result in aberrant splicing. Here we report genetic findings in C. elegans that aberrant splicing due to +1G mutations can be suppressed by U1 snRNA mutations. An intronic +1G-to-U mutation, e936, in the C. elegans unc-73 gene causes aberrant splicing and loss of gene function. We previously showed that mutation of the sup-39 gene promotes splicing at the mutant splice donor in e936 mutants. We demonstrate here that sup-39 is a U1 snRNA gene; suppressor mutations in sup-39 are compensatory substitutions in the 5′ end, which enhance recognition of the mutant splice donor. sup-6 (st19) is an allele-specific suppressor of unc-73(e309), which contains an intronic +1G-to-A transition. The e309 mutation activates a cryptic splice site, and sup-6(st19) restores splicing to the mutant splice donor. sup-6 also encodes a U1 snRNA and the mutant contains a compensatory substitution at its 5′ end. This is the first demonstration that U1 snRNAs can act to suppress the effects of mutations to the invariant +1G of introns. These findings are suggestive of a potential treatment of certain alleles of inherited human genetic diseases.

The splice donor consensus sequence undergoes base-pairing interactions with the 5′ end of U1 snRNA early in spliceosome assembly (Ares and Weiser 1994). Mutations to the canonical G that begins all intron substrates of the major spliceosome (the +1G) lead to loss of splicing to the wild-type site and often to activation of nearby cryptic splice sites. The cryptic splice sites resemble splice donor consensus sequences but have weaker homology to the consensus than the original splice donor (Roca et al. 2003). Early studies estimated that at least 15% of point mutations responsible for human genetic diseases disrupt signals involved in splicing (Krawczak et al. 1992), and these often disrupt the +1G. This may be an underestimate as extensive analysis of disease alleles of neurofibromatosis type 1 and ataxia telangiectasia indicate that 50% of alleles surveyed affect pre-mRNA splicing (Teraoka et al. 1999; Ares et al. 2000).

Reverse genetic approaches in mammalian cells, Drosophila, and yeast have shown that mutant U1 snRNAs with compensatory substitutions in their 5′ ends can suppress the effects of mutations to splice donor consensus sequences to promote splicing at a mutated donor site (Zhuang and Weiner 1986; Zhuang et al. 1987; Siliciano and Guthrie 1988; Cohen et al. 1993; Lo et al. 1994). U1 snRNA suppressors were developed to suppress mutations that occur at the −1, +3, +5, and +6 positions of the 5′ splice site, positions that vary naturally in metazoans. Studies in yeast and mammalian cells indicated that mutations to the +1G could not be suppressed by compensatory mutations in U1 snRNA (Siliciano and Guthrie 1988; Cohen et al. 1994). It has been proposed that the +1G has additional essential functions in the splicing mechanism through interactions with the terminal nucleotide of the intron and by binding to PRP8 and U6 snRNA (Park and Siliciano 1993; Reyes et al. 1996, 1999). The PRP8 protein of the U5 snRNP, as a component of the U4/U6/U5 tri-snRNP, interacts with this nucleotide early in spliceosome assembly in an ATP-dependent manner, perhaps at the same time as U1 snRNA or as part of the transition from U1 to U6 interactions (Maroney et al. 2000).

In a previous article we analyzed the mechanism of suppression of splicing defects in unc-73(e936) by mutations in sup-39 (Roller et al. 2000). The e936 mutation changes the +1G of intron 15 to U (Steven et al. 1998). sup-39 was identified as an allele-specific suppressor of the uncoordination defect of unc-73(e936) (Run et al. 1996). Therefore we reasoned that an allele-specific suppressor of a splice-site defect might function at the level of splice-site choice. The unc-73(e936) mutation activates three cryptic splice donors, two of which define introns beginning with the canonical GU (Roller et al. 2000). Use of these two cryptic splice sites causes loss of reading.

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frame. The third splice donor, used in 10% of steady-state e936 messages, is the mutated splice donor at the wild-type position. Use of this splice site defines an intron beginning with UU. This was surprising to find because previous work had suggested that mutations to the +1 position of the intron would result, at best, in the first step of splicing but not the second transsterification reaction (Newman et al. 1985; Parker and Guthrie 1985). In the presence of a sup-39 mutation, these same three splice donors are used, but the ratio of messages produced by splicing at these sites changes. The percentage of unc-73(e936) messages containing the wild-type splice junction is increased to 33% with a corresponding increase in the level of UNC-73 protein (summarized in Figure 1A). We did many controls to demonstrate that this effect is due to changes in splicing and not to differential changes in message stability. This sup-39-induced change in cryptic splice-site choice is also observed when the e936 mutant intron region is inserted into a heterologous splicing reporter construct. We propose that mutant sup-39 is a new type of informational suppressor that alters the use of weak splice donors (Roller et al. 2000).

In this article we report the cloning and sequencing of sup-39. sup-39 is a U1 snRNA gene, 1 of 12 in the Caenorhabditis elegans genome. Both known alleles possess the same mutation, which is a compensatory substitution in the 5’ end of this U1 snRNA to match the +1G-to-U mutant splice donor in e936. We have also identified another U1 snRNA gene as a dominant suppressor of a 5’ splice-site mutation. sup-6 is an allele-specific suppressor of a mutation in unc-13(e309), which changes the first nucleotide of an intron from G to A. The sup-6 mutation alters the 5’ end of U1 so that it is complementary to the mutation in the 5’ splice site. These are the first examples in any organism of suppressors of mutations to the +1 position of the intron that activate complete splicing at the proper site. These may have important implications for treatment of specific alleles of human genetic diseases.

MATERIALS AND METHODS

Strains used: All C. elegans strains used in this study, with the exception of SZ4, were obtained from the Caenorhabditis Genetics Center at the University of Minnesota. Mapping of sup-39: sup-39 was reported to map to position +0.22 ± 0.056 cM of LGII (Run et al. 1996). To precisely map the location of sup-39, single nucleotide polymorphisms between CB4856 and the Bristol N2 strain were used to identify regions of chromosomal crossovers. Strain JW104 (Run et al. 1996) has the genotype dpy-10(e128) sup-39(je5) unc-4(e120) II. CB4856 males were crossed to JW104 hermaphrodites. F2 animals that were either Dpy non-Unc or Unc non-Dpy were selected and lines homozygous for the recombinant chromosomes were recovered. A total of 50 Dpy non-Unc and 50 Unc non-Dpy strains were obtained. These were tested for the presence of sup-39(je5) by the ability to suppress unc-73(e936) using crosses described previously (Run et al. 1996). In addition, in selected strains the presence of sup-39(je5) was confirmed through the analysis of splicing of the green fluorescent protein (GFP)-lacZ-unc-73(e936) splicing reporter fusion construct azEx1 (Roller et al. 2000). To construct animals containing the recombinant LGII and azEx1, N2 males were crossed to the animals containing recombinant LGII. F1 males from this cross were then crossed to strain SZ4 containing azEx1. F2 animals from this cross homozygous for the recombinant chromosome were selected and these were screened for the roller phenotype of azEx1 or the GFP phenotype of this array. Animals containing these markers were then grown up on a large plate and RNA was extracted from the animals as previously described (Roller et al. 2000). The splicing of the azEx1 reporter construct was assayed using the 3P reverse transcription-polymerase chain reaction assay as described previously (Roller et al. 2000).

The position of the chromosomal crossover event in each of the recombinant chromosomes was determined through analysis of single nucleotide polymorphisms (SNPs) that lead to restriction fragment length polymorphisms between the CB4856 and N2 strains, as described by Wicks et al. (2001) and Swan et al. (2002). SNPs for mapping sup-39 were chosen from data supplied by Steven Wicks (Hubrecht Laboratory and Center for Biomedical Genetics, Utrecht, The Netherlands), Kat Swan (Exelixis, South San Francisco, CA) and now available on Wormbase. On the basis of this analysis of crossover events, we were able to narrow the position of sup-39 down to a region between position 3335 on cosmid C15F1 and position 7579 on cosmid ZKI127. This region encompasses almost all of cosmids C15F1 and C32E12 and one gene from cosmid ZKI127 (ZKI127.7). Identification of sup-39: We cloned and sequenced a U1 snRNA gene located in the intron of the gene C15F1.3 to determine if this had a lesion in it consistent with it being sup-39. We used the primers 5’-ggaatcttcacacgcgagcactagtc-3’ and 5’-cagaattctaacggtaggtactggag-3’ to amplify the entirety of the 900-base sixth intron of C15F1.3 containing the U1 snRNA coding region. The primers had EcoR1 restriction sites built into them so that the PCR products of the amplification reaction were then cloned into the EcoRI site of pBluescript KS+ (Stratagene, La Jolla, CA). This region was amplified and cloned from N2, sup-39(je5), and sup-39(je6) animals and the inserts were sequenced on both strands using the M13 forward and reverse primers.

RT-PCR assays for unc-13(e309) splicing: To detect changes to splicing induced by the +1G-to-A mutation in e309, RT-PCR reactions were used. Total RNA from was extracted from a large plate of N2, unc-13(e309); dpy-10(e128), and unc-13(e309); dpy-10(e128); sup-6(s119)/dpy-10(e128) sup-6(+)/J animals using methods previously described (Roller et al. 2000). RNA was reverse transcribed with a primer specific for exon 20, 5’-agaatcttcacacgcgagcactagtc-3’, and then PCR reactions were done using the RT primer and a 3P 5’-end-labeled primer that annealed to exon 17 5’-ggaatcttcacacgcgagcactagtc-3’. PCR products were separated on a 6% polyacrylamide urea gel and visualized by autoradiography. To determine the sites of cryptic splice donor usage, RT-PCR reactions were performed as above except that an EcoRI site was added to the 5’ end of the 5’ PCR primer and a BamHI site was added to the 3’ end of the 3’ PCR primer. These products were cloned into pBluescript KS+ (Stratagene). Products from unc-13(e309); dpy-10(e128) and unc-13(e309); dpy-10(e128) sup-6(s119)/dpy-10(e128) sup-6(+)/J animals were sequenced.

Identification of sup-6: The C. elegans gene F58G1.7 contains a U1 snRNA gene located in its fifth intron, and its map position is predicted to be very close to the map position of sup-6. We cloned and sequenced this gene using the primers 5’-acaatccgcatgccgacatgctca-3’ and 5’-aggaatcttcacacgcgagcactagtc-3’ to generate a 280-base-long PCR product containing this U1 snRNA gene. The primers had EcoRI and
BamHI restriction sites built into them and the products were cloned into these sites in pbLeucSript KS+ (Stratagene). This product was amplified from the strain CB2987 in which wild-type moving animals are heterozygous for the \textit{sup-6(st19)} allele. Twelve cloned inserts from this amplification were sequenced. Four had the wild-type gene sequence and 8 of the inserts contained a point mutation near the 5' end of this U1 snRNA gene, consistent with the fact that these clones were derived from heterozygous \textit{sup-6(st19)}/+ animals.

**RESULTS**

\textbf{Identification of \textit{sup-39}:} Using single nucleotide polymorphism mapping (Wicks et al. 2001) we localized \textit{sup-39} to a 79,000-base region of LGII. We found a U1 snRNA gene, 1 of 12 in the \textit{C. elegans} genome, residing in this interval, in the sixth intron of the gene C15F1.5. We cloned and sequenced this U1 snRNA gene from strains containing the two known \textit{sup-39} alleles, \textit{je5} and \textit{je6}. An identical mutation, which changes the eighth nucleotide of this U1 snRNA gene from C to A, was found in the two \textit{sup-39} alleles (Figure 1B). This mutation is a compensatory substitution that allows the 5' end of this U1 snRNA to base pair with a mutant 5' splice site containing a +1G-to-U mutation (Figure 1C). The ability of this U1 snRNA mutant to compensate for the mutation in the 5' splice site in \textit{unc-73(e936)} provides a logical explanation for how \textit{sup-39} mutants act as suppressors. The suppressor U1 snRNA enhances the recognition of the region of the overlapping −1 and wild-type cryptic sites over the +23 site. In both the \textit{unc-73(e936);sup-39(+) and unc-73(e936);sup-39(je5) strains}, the ratio of −1 and wild-type splice-site usage is 1:1. It appears that at a subsequent step of spliceosome assembly, after U1 snRNP recognition, the −1 and wild-type splice sites are chosen with equal efficiency. We made many attempts to suppress \textit{unc-73(e936)} in animals by expression of mutant \textit{sup-39} on a transgene. Even when diluted with carrier DNA, we were unable to generate transgenic lines containing extrachromosomal arrays expressing mutant \textit{sup-39}. However, we could detect co-injected GFP marker genes expressed in dying \textit{C. elegans} embryos (data not shown). This result is consistent with the lethality associated with \textit{sup-39}, as 50% of homozygous \textit{sup-39(je5)} animals die as embryos (Rui et al. 1996).

\textbf{\textit{sup-6} also affects cryptic splice-site choice:} Having identified a U1 snRNA as a dominant suppressor of a +1G mutation, we looked for other suppressors that act in a similar fashion. \textit{sup-6(st19)} is a dominant, allele-specific, extragenic suppressor of \textit{unc-13(e309)}. The e309 allele contains a mutation in the first nucleotide of intron 18, +1G to A (Kohn et al. 2000; Figure 2A). \textit{sup-6 (st19)} was identified in screens for suppressors of \textit{unc-13(e309)} as a suppressor of the uncoordination defect of e309. We have found that homozygous \textit{st19} animals undergo a late-larval developmental arrest. All of the embryos hatch, but larval development is dramatically slowed and very few animals reach adulthood. Those that reach adulthood produce fewer than five viable embryos. \textit{st19} heterozygotes are morphologically and behaviorally indistinguishable from wild-type animals.

To determine whether \textit{sup-6(st19)} acts at the level of the splicing of the e309 message, we isolated RNA from cultures of wild-type, \textit{unc-13(e309)}, and \textit{unc-13(e309);sup-6 (st19)/sup-6(+) animals}. Reverse-transcription-PCR analysis of \textit{unc-13} messages from these strains is shown in Figure 2B. In the presence of the \textit{unc-13(e309)} mutation, all splicing occurs at a cryptic splice site 25 nucleotides 5' of the mutated wild-type site, resulting in an out-of-frame message. In the presence of heterozygous \textit{sup-6 (st19)}, e309 cryptic splicing is altered. In addition to the −25 cryptic splice site, there is significant splicing at the wild-type splice junction even though this splicing defines an intron beginning with an A, instead of the canonical G. This is consistent with a role for \textit{sup-6} in promoting splicing at the mutant splice donor. Two additional minor cryptic sites are also activated by \textit{st19} (Figure 2C).

\textbf{\textit{sup-6} is a U1 snRNA gene:} \textit{sup-6} maps to position 11.96 on LGII. One of the 12 \textit{C. elegans} U1 snRNA genes maps to an interpolated position of 11.61 on LGII. This U1 snRNA gene resides in the fifth intron of the \textit{C. elegans} gene F58G1.7 and has been annotated as F58-G1.10. We found that this U1 snRNA is mutated in \textit{sup-6 (st19)} animals at the eighth position of the snRNA (Figure 3A). This is the same position that is altered in the \textit{sup-39} mutant U1. The mutation in \textit{st19} is complementary to the mutation in e309, allowing for proper base pairing of the mutant U1 snRNA with the mutant 5' splice site (Figure 3B). This compensatory substitution in U1 snRNA promotes recognition of the mutant 5' splice site and activation of both steps of the splicing reaction, suppressing the aberrant splicing due to the e309 mutation.

\textbf{The \textit{C. elegans} U1 snRNA genes:} We have cataloged all of the U1 snRNA genes in \textit{C. elegans}. The sequences of two different \textit{C. elegans} U1 snRNA genes were initially reported by Thomas et al. (1990). Using their initial sequences, we were able to use homology searches of the completed genome to identify 12 different \textit{C. elegans} U1 snRNA genes. The \textit{C. elegans} Wormbase Consortium (Harris et al. 2003) has provided annotations for only 6 of these 12. The alignment of the 12 U1 snRNAs is shown in Figure 4. The 5' ends of all of these U1 snRNAs, the region that base pairs with the 5' splice site, are identical. It may be coincidental, but of these 12 U1 snRNA genes, only \textit{sup-39} and \textit{sup-6} reside in introns of other genes. There is evidence for movement of \textit{C. elegans} U1 snRNA genes within the genome during the period of divergence of \textit{C. elegans} and \textit{C. briggsae}. For example, while \textit{sup-39} resides in the sixth intron of C15F1.5 in \textit{C. elegans}, the \textit{C. briggsae} homolog of C15F1.5 does not contain a U1 snRNA gene in its intron (data not shown). \textit{sup-6} resides in the fifth intron of the \textit{C. elegans} gene F58G1.7. The \textit{C. briggsae} homolog of this gene also contains a U1 snRNA gene at the same position. However, this \textit{C. briggsae} homolog is a U1 snRNA pseudogene because it contains a deletion of nucleo-
Figure 1.—sup-39 is a U1 snRNA with a compensatory substitution for suppression of e936 splicing. (A) Summary of sup-39(je5) effects on unc-73(e936) cryptic splicing. The exon 15 splice donor region that is mutated in e936 is shown. Uppercase letters are exon 15 sequence and lowercase letters are intron sequence. The +1G-to-U mutation of intron 15 is boxed. The different cryptic splice sites activated in both unc-73(e936) and unc-73(e936);sup-39(je5) are indicated along with a table showing their relative usage in different strains as determined by Roller et al. (2000). (B) Predicted secondary structure of the sup-39 U1 snRNA based on the initial secondary structure prediction of C. elegans U1 snRNA genes by Thomas et al. (1990). The site of the mutation in je5 and je6 is indicated. (C) Demonstration of the base pairing of the 5’ end of U1 snRNA (identical in all 12 C. elegans U1 snRNA genes) with the 5’ splice site of unc-73 intron 15. Below that is a demonstration that the mutation in the sup-39(je5) and (je6) alleles acts as a compensatory substitution for the unc-73(e936) mutation, which maintains U1 snRNA base pairing with the splice donor of intron 15. The changed nucleotides in both genes are boxed.

tides 78–110 of the U1 snRNA and thus could not be functional. We could find evidence for 10 full-length C. briggsae U1snRNA genes (data not shown).

DISCUSSION

We have presented evidence that altered U1 snRNAs can suppress the effects of mutations to the +1G of introns. Recovery of such compensatory mutations as suppressors is likely to be very rare for two reasons. First, the mutagenic target in a forward genetic screen is a single base in a U1 snRNA gene. Second, the U1 snRNA mutations themselves have deleterious consequences, likely due to activation of aberrant splicing at additional genes. The sup-39 and sup-6 U1 snRNA mutations cause pleiotropic defects in development: 50% of homozygous sup-39(je5) mutant animals die as embryos (Run et al. 1996) and sup-6(st19) homozygous animals undergo delayed larval development leading to a late-larval arrest. Outside of the dominant suppression of e309, st19/+ animals appear to have no obvious phenotype due to the sup-6 mutation. This difference between the pheno-
types of heterozygous vs. homozygous sup-6 mutants indicates that there is a fine balance between the toxic effects of the mutant U1 snRNA and suppression. That these U1 mutations do not cause more widespread defects may reflect redundancy among the set of 12 U1 snRNA genes in C. elegans; in addition, sup-6 or sup-39 may be expressed at lower levels or only in specific tissues. The extent to which the sup-6 and sup-39 mutations act as general suppressors of similar +1G mutations is unclear; it is possible that only +1G mutations in certain contexts are capable of suppression by this mechanism. Our results suggest the possibility that human diseases due to aberrant splicing at +1G mutations may be treatable with suppressor U1 snRNAs.

Previous studies using reverse genetics have identified U1 snRNA mutations able to activate alternative splice donors in metazoans in vivo (in tissue-culture cells and in flies) (Zhuang and Weiner 1986; Zhuang et al. 1987; Lo et al. 1994). These reverse-genetics studies examined compensatory substitutions in U1 snRNAs only at the −1, +3, +5, and +6 positions of the 5′ splice site, which vary naturally among splice donors in metazoans. Other studies in yeast and mammalian cells that looked specifically at mutations to the +1 position of the intron had suggested that mutations to the first nucleotide of an intron could not be suppressed by compensatory mutations in U1 snRNA (Siliciano and Guthrie 1988; Cohen et al. 1994). In some cases, the first step of splicing could be detected but these were dead-end products, indicating that the G at the first nucleotide of an intron is essential for the second step of splicing (Newman et al. 1985; Parker and Guthrie 1985; Newman and Norman 1991). In another case, suppressor U1 snRNAs caused activation of nearby cryptic splice sites, not splicing at the original splice site (Cohen et al. 1994). From these studies, it appears that the +1G is important for other essential processes in splicing in addition to recognition by U1 snRNA.

The +1 position of the intron is known to interact with the U5 snRNP protein PRP8 and with U6 snRNA at an early step in spliceosome assembly (Reyes et al. 1999; Maroney et al. 2000). In addition, it may have non-Watson-Crick interactions with the terminal nucleotide of the intron (Parker and Siliciano 1993). These studies suggest that an essential role for the +1G in splicing may be that it serves as a point of recognition for essential interactions within the pre-mRNA and with the spliceosome complex. Our studies show that mutations of the canonical G at the first nucleotide of the intron can indeed be suppressed by U1 snRNA mutants and that both of the steps of splicing can be efficiently accomplished for introns not beginning with G. Whether this suppression is limited to the specific mutated splice sites tested remains to be seen. Perhaps the reason that this sort of suppression was unable to be accomplished previously by reverse-genetics approaches may be related to the lethality of these mutant U1 snRNAs that we observed in C. elegans. Transient transfection studies may have led to expression of the U1 snRNA transgenes containing compensatory mutations at too high a level, leading to toxicity.

One of the interesting questions that arises from this work is whether all U1 snRNA genes are expressed equivalently and have equal function. Two independent isolates of extragenic suppressors of unc-73(e936) were both found in the sup-39 gene (Run et al. 1996), and we have found that these two alleles contain identical lesions. If all of the U1 snRNAs are expressed equivalently, then
Figure 3.—Identification and structure of the sup-6(st19) U1 snRNA and demonstration that it contains a compensatory substitution that promotes splicing of the e309 mutation. (A) Predicted secondary structure of the sup-6 U1 snRNA. The site of the mutation in st19 is indicated. (B) Demonstration of the base pairing of the 5' end of U1 snRNA with the 5' splice site of unc-13 intron 18. Below that is a demonstration that the mutation in the sup-6(st19) acts as a compensatory substitution for the unc-13(e309) mutation to maintain U1 snRNA base pairing with the splice donor of intron 18. The changed nucleotides in both genes are boxed.

the odds of finding two rare U1 snRNA suppressors in the same U1 snRNA gene would be 1 in 12. We do know that sup-39 is expressed in multiple tissues because in addition to its ability to alter unc-73(e936) cryptic splicing in neurons, we know from our previous study using splicing reporter transgenes that sup-39(je5) can suppress cryptic splicing in body-wall muscle cells (Roller et al. 2000). Perhaps sup-39 is expressed at much lower levels than the other U1 snRNA genes, and therefore mutations in this gene can be tolerated because the toxicity will not be as high. There is evidence for developmental stage-specific expression of U1 snRNA genes in Drosophila and in Xenopus (Forbes et al. 1984; Lo and Mount 1990). It will be interesting in the future to determine whether developmental stage or tissue specificity is involved in the expression of the 12 C. elegans U1 snRNA genes.

It is estimated that splicing consensus sequences are disrupted in 15% of inherited genetic human diseases caused by point mutation (Stenson et al. 2003). Of the disease-causing point mutations that disrupt splicing consensus sequences, the most common disruptions alter the canonical G at the +1 position of the intron, consistent with the essential nature of this G for proper splicing (Krawczak et al. 1992). Our discovery of U1 snRNA mutants in C. elegans that suppress +1G mutations by causing splicing at the wild-type site is suggestive of a method to repair this type of frequently occurring disruption through the use of suppressor U1 snRNAs. However, no previous attempts to suppress +1G mutations with suppressor U1 snRNAs in yeast, Drosophila, and mammalian tissue-culture cells have succeeded. Our demonstration that this suppression can indeed work in C. elegans, along with our demonstration of the toxicity of these specific U1 snRNA suppressors, suggests a new hope that this type of suppression may work in
human cells. Perhaps suppression of +1G mutations can occur if experiments are done to modulate the U1 snRNA level so that the proper balance between toxicity and suppression can be achieved. An alternative hypothesis is that there exists a fundamental difference in the requirement for a G at the +1 position of the intron between worms and other species that would account for the ability of sup-6 and sup-39 to function in suppression. Because wild-type *C. elegans* introns begin with G, any potential differences in the requirement of an intronic +1G between mammals and worms would be evident only during cryptic splice-site activation caused by splice donor mutation.

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**LITERATURE CITED**


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