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Nearby Stop Codons in Exons of the Neurofibromatosis Type 1 Gene Are Disparate Splice Effectors

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Summary

Stop mutations are known to disrupt gene function in different ways. They both give rise to truncated polypeptides because of the premature-termination codons (PTCs) and frequently affect the metabolism of the corresponding mRNAs. The analysis of neurofibromin transcripts from different neurofibromatosis type 1 (NF1) patients revealed the skipping of exons containing PTCs. The phenomenon of exon skipping induced by nonsense mutations has been described for other disease genes, including the CFTR (cystic fibrosis transmembrance conductance regulator) gene and the fibrillin gene. We characterized several stop mutations localized within a few base pairs in exons 7 and 37 and noticed complete skipping of either exon in some cases. Because skipping of exon 7 and of exon 37 does not lead to a frameshift, PTCs are avoided in that way. Nuclear-scanning mechanisms for PTCs have been postulated to trigger the removal of the affected exons from the transcript. However, other stop mutations that we found in either NF1 exon did not lead to a skip, although they were localized within the same region. Calculations of minimum-free-energy structures of the respective regions suggest that both changes in the secondary structure of the mRNA and creation or disruption of exonic sequences relevant for the splicing process might in fact cause these different splice phenomena observed in the NF1 gene.

Introduction

The effects of stop mutations have been studied in different genes, and three possible outcomes have been demonstrated. In genes such as the β-globin gene, the triosephosphate-isomerase gene, the methylmalonyl-CoA mutase gene, and the T-cell receptor-β gene, it has been shown that stop mutations are able to affect mRNA metabolism and to reduce the amount of detectable mRNA (Daar and Maquat 1988; Baserga and Benz 1992; Ogasawara et al. 1994; Maquat 1995; Carter et al. 1996). Also, in the neurofibromatosis type 1 (NF1) gene, a correlation between a high proportion of stop mutations and unequal expression of the two alleles could be found (Hoffmeyer et al. 1994, 1995). Less commonly, mRNA containing a nonsense mutation is translated and results in a truncated protein (Lehrman et al. 1987; Fojo et al. 1989). The third possible outcome is an abnormally spliced mRNA induced by a premature-termination codon (PTC) in the skipped exon. This has been demonstrated previously in several human genetic diseases, such as Fanconi anemia, cystic fibrosis, Marfan syndrome, gyrate atrophy, and Hurler syndrome (Bach et al. 1993; Dietz et al. 1993; Gibson et al. 1993; Hull et al. 1994). Very recently, PTC-induced skipping of exon 37 of the NF1 gene has been reported by Messiaen et al. (1997).

NF1, or von Recklinghausen neurofibromatosis (MIM 162200 [http://www3.ncbi.nlm.nih.gov:80/htbin-post/Omim/dispmim?162200), is one of the most common autosomal dominant disorders, affecting ~1/3,000 individuals. The disease is characterized by café-au-lait spots, multiple neurofibromas and Lisch nodules of the iris, and an increased predisposition to certain malignancies (Huson and Hughes 1994). The NF1 gene (GDB 120231 [http://www.gdb.org]) was identified on human chromosome 17 by positional cloning in 1990 (Cawthon et al. 1990; Viskochil et al. 1990; Wallace et al. 1990). It spans ~350 kb of genomic DNA and contains 60 exons (Danglot et al. 1995; Li et al. 1995). Numerous screening methods have found different types of mutations in the NF1 gene. The great majority (60%–70%) of the NF1 gene mutations reported to the NNFF International NF1 Genetic Analysis Consortium (Korf 1997) and to the Human Gene Mutation Database (Cardiff) (1997) are truncating mutations. The consequence

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of all these nonsense mutations should be a shortened neurofibromin, which, however, has not been detected by western blotting thus far.

The large number of stop mutations found in the NF1 gene prompted us to look specifically for this type of mutation, by applying the protein-truncation test (PTT) (Roest et al. 1993). We detected nearby stop mutations in exon 7 of the NF1 gene, one of which leads to the skip of the exon in which the mutation is located. Likewise, reanalysis of different stop mutations in exon 37 of the NF1 gene previously found by temperature gradient–gel electrophoresis (TGGE) revealed PTC-induced exon skipping in some cases. In all cases the known splice-relevant genomic DNA sequences flanking the exons were unaltered. In addition, the PTCs leading to exon skipping did not seem to be located in sequence motifs that are known to function as splicing enhancers.

In the present article, examples of exon skipping as a consequence of stop mutations in the respective exons of the NF1 gene are reported. Special emphasis is laid on the differing effects that various PTCs that are short distance from each other exert on the splicing of the affected exon. Our data suggest that both changes in the secondary structure of the mRNA and creation or disruption of exonic sequences relevant for the splicing process might cause these different splice phenomena.

Material and Methods

RNA Preparations and Reverse Transcription (RT)

Material.—Fresh blood or cell cultures (fibroblast-like cells from neurofibromas or melanocytes from normally pigmented skin and from café-au-lait macules) from NF1 patients were used.

RNA isolation.—RNA isolation was performed with the RNeasy total RNA purification kit (Qiagen). A solution of 600 μl RLT-lysis buffer and 1.5 ml fresh blood (~10^7 cells) was microspun to produce 1.5–7.5 μg total RNA.

cDNA synthesis.—cDNA synthesis was performed by the SuperScript preamplification system (BRL Gibco). The cDNA reaction was performed in a 20-μl reaction volume containing 2–3 μg total RNA, 1.5 μg random hexamers (BRL Gibco), 0.5 μg single-stranded binding protein (Promega), 20 units RNAsin (Promega), 4 mM each dNTP, and 300 units Superscript II RT (BRL Gibco). cDNA was stored at −20°C.

PTT

RT-PCR.—Hot-start PCR reactions to amplify each of the five overlapping segments required that 2.5–3 μl cDNA be added to a lower-phase PCR mixture containing 1 × PCR buffer (50 mM KCl, 10 mM Tris ph 8.3, and 2 mM $MgCl_2$), 0.8 mM each dNTP, and 10 pmol each primer (primer sequences were as described elsewhere [Heim et al. 1995]), in a total volume of 40 μl. A wax gem (Perkin-Elmer) was added to each tube. Reaction mixtures were heated to 80°C for 5 min, followed by cooling to room temperature. A 10-μl upper-phase mixture containing 1 × PCR buffer, 0.25 μg single-stranded binding protein, and 2.5 units *Taq* polymerase (Pharmacia) was layered onto the solidified wax. Reactions were performed in a Perkin Elmer Cetus thermal cycler (model 9600), under the following conditions: 95°C for 1 min; 40 cycles of 95°C for 30 s, 61.5°C for 30 s, and 72°C for 90 s; and 10 min at 72°C. The PCR products were analyzed by agarose gel electrophoresis.

In vitro transcription/translation and analysis of peptides.—For each in vitro transcription/translation reaction, 4–9.5 μl PCR product (volume was dependent on the quality of the PCR product) and 0.5 μl ^{35}S-methionine (specific activity >1,000 Ci/mmol; Amersham) were added to the commercially available TNT™ Coupled Reticulocyte Lysate System (Promega). Reactions were performed under conditions recommended by the manufacturer, with one exception: for all components, only a one-half volume was added to the PTT reaction (e.g., 12.5 μl TNT Rabbit Reticulocyte Lysate). After incubation of the complete reaction for 1.5 h at 30°C, 5 μl of the reaction was added to 5 μl loading buffer (10% glycerol, 5% 2-mercaptoethanol, 2% SDS, and 0.1% bromphenol blue), was heated for 5 min, and then was placed on ice. Samples were subjected to electrophoresis in a 14% SDS-polyacrylamide gel for 1.5–2 h at 40 mA (Rainbow ^{14}C methylated protein [Amersham] was used as a protein-weight marker). The gel was fixed in a solution of 40% methanol and 10% acetic acid and dried for 1.5 h at 80°C in a gel dryer. The synthesized polypeptides were visualized by means of a PhosphoImager (Molecular Dynamics) after the gel had been exposed overnight to a special film slide (Molecular Dynamics).

Sequence Analysis

Single-stranded DNA was obtained by alkali denaturation of biotinylated PCR products and separation with streptavidin-coated magnetic beads (Dynal). Solid-phase sequencing was performed with a Sequenase V. 2.0 kit from United States Biochemicals.

RFLP Analysis and AccI Restriction

RT-PCR fragments were produced by means of primer sequences that have been described elsewhere (Hoffmeyer et al. 1995). The PCR products were digested with *Acc*I in a reaction volume of 15 μl containing 10 U restriction enzyme and 1 × restriction buffer (50 mM

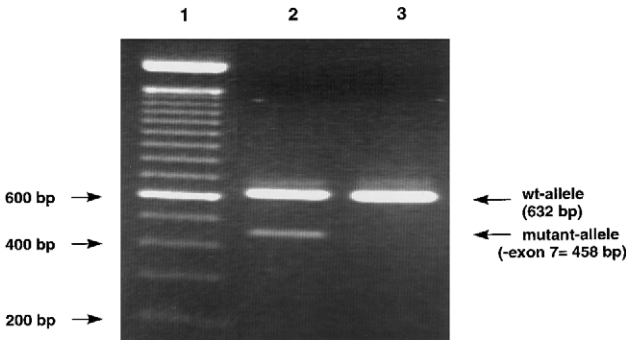

Figure 1 Exon 7 skipping: RT-PCR analysis. The agarose gel was stained by ethidium bromide. PCR products were derived from cDNA of NF1 patients U-NF259 and U-NF262 (lanes 2 and 3). Lane 1, 100-bp ladder.

Tris-HCl pH 8.0 and 10 mM $MgCl_2$) for 1 h at 37°C. The restriction fragments were analyzed by means of agarose gel electrophoresis.

Minimum-Free-Energy Structures

The calculation of minimum-free-energy structures is based on a dynamic programming algorithm originally developed by Zucker and Stiegler (1981). The partition-function algorithm is based on the work of McCaskill (1990). The energy parameters are taken from Turner et al. (1988). The version of the program used was RNAfold 1.00.42

Results

PTT Analysis

In the PTT, which is based on mRNA analysis, not only nonsense mutations but also aberrant splice products are detected as additional RT-PCR fragments prior to the actual check of the reading frame. After RT-PCR, which is the first step of the PTT analysis, NF1 patient U-NF259 showed a shortened PCR fragment in addition to the expected one (fig. 1). Further analyses of the respective cDNA region revealed that the size differences of the PCR fragments were based on deletion of exon 7. The common causes of splicing defects are mutations either (*a*) in the consensus sequences of the donor or acceptor splice sites or (*b*) around the branch point. In the case of this NF1 patient, however, a stop mutation was identified in exon 7 when the genomic DNA was sequenced. This finding prompted us to reanalyze, at the mRNA level, NF1 patients in whom TGGE had identified stop mutations in exon 37 (Robinson et al. 1995; Böddrich et al. 1997). Among the nine patients from five families, all patients with the Y2264X mutation showed skipping of exon 37, whereas the others did not (see table 1 and fig. 2). Likewise, PTT identified, in exon 7 in patient U-NF262, a further stop mutation that did not cause exon skipping (table 1 and fig. 1). Both the removal of exon 7 and the removal of exon 37 result in an in-frame coupling of the flanking exons, as has been described for other genes (Dietz et al. 1993; Gibson et al. 1993).

Analysis of Splicing-Relevant Sequence Motifs

Although all of the nonsense mutations found in exon 37 are located within only a few base pairs (fig. 3), they exert quite different effects on splicing. In the case of the direct stop mutation (C6792A) in exon 37, as well as in the case of the small deletion (6789delTTAC) and the small insertion (6790insTT), sequence analyses at the DNA level showed that all sequence motifs (e.g., splice sites or branch points), which are normally involved in the splicing process, are unaltered (data not shown). This applies also to the mutations found in exon 7. In addition, we also quantified the splice-site scores for exon 7, as well as those for exon 37, using a scoring system described by Shapiro and Senapathy (1987); but all scores are within a normal range (exon 7 had a 3′ splice-site score of 79.5 and a 5′ splice-site score of 79.9; and exon 37 had a 3′ splice-site score of 87.7 and a 5′ splice-site score of 86.6).

These results indicated that exon skipping apparently has to be attributed to the mutations themselves. As shown in figure 3, the mutations 6790insTT and 6789delTTAC cause a frameshift that, in both cases, results in a stop codon at position 6806–6808 (UGA).

Table 1

Stop Mutations and Their Effect on RNA Splicing

Patient	Exon	Mutation	mRNA Level	Expression of Two NF1 Alleles
U-NF259	7	C910T (R304X)	Exon 7 skipping	Unequal expression
U-NF262	7	918delT; stop codon 947–949	Normal splicing	Not determined
B-NF2/3	37	C6792A (Y2264X)	Exon 37 skipping	Equal expression
B-NF4/5	37	C6792A (Y2264X)	Exon 37 skipping	Equal expression
B-NF6/7/8	37	6790insTT; stop codon 6806–6808	Normal splicing	Equal expression
B-NF9	37	6789delTTAC; stop codon 6806–6808	Normal splicing	Equal expression
U-NF33	37	6789delTTAC; stop codon 6806–6808	Normal splicing	Not determined

RT-PCR of the affected area revealed, exclusively, transcripts of normal size (except for the few inserted/deleted base pairs; fig. 2), indicating that the stop codon at this position does not interfere with the subsequent splicing process. In contrast, the recurrently found direct stop mutation C6792A (UAA) in exon 37 leads to a skipping of this exon.

Analysis of Expression of the Two NF1 Alleles

As in our previous studies (Hoffmeyer et al. 1994, 1995), we looked also for the expression of the two NF1 alleles in these cases. The expression studies for the mutations in exon 37, which did not cause exon skipping (table 1), were performed by means of an expressed polymorphic *Rsa*I restriction site in exon 5 of the NF1 gene (Hoffmeyer and Assum 1994). In the case of exon skipping, we measured the band intensities of the two types of transcripts after RT-PCR (wild-type and mutant alleles). Interestingly, in the case of the C6792A mutation in exon 37, the level of the shortened transcript was not reduced (fig. 2).

In contrast, in the case of the stop mutation (C910T) detected in exon 7, a diminished level of the shortened transcript was found (fig. 1). However, the longer of the two RT-PCR products could be a homogeneous wild-type product, or, alternatively, it could contain unknown portions of an amplification product from the cDNA of the mutant message that was not abnormally spliced. To clarify this possibility, we utilized, in exon 7, an *Acc*I restriction site that was affected by the stop mutation (C910T; see table 1). This enabled us to distinguish the two types of amplification products of equal size. After RT-PCR and a subsequent *Acc*I restriction, the larger PCR product was totally digested, indicating that it represents only the wild-type allele of this NF1 patient.

Calculations of Minimum-Free-Energy Structures

Calculations of minimum-free-energy structures, by application of the computer program RNAfold (Zucker and Stiegler 1981; Turner et al. 1988; McCaskill 1990) have revealed the following remarkable results with regard to exons 7 and 37. In the case of exon 7 both the wild-type sequence and the sequence including the mutation that did not lead to exon skipping (i.e., 918delT) showed comparable secondary structures (fig. 4*a* and *b*). In contrast, the direct stop mutation (C910T) causes a secondary structure that is completely different from the other two structures calculated for exon 7 (fig. 4*c*). Hence, a dramatic change of the secondary structure might in fact cause the skipping of exon 7. The calculations for all three mutations in exon 37, including the wild-type sequence, showed no differences between the secondary structures, apparently because the bases involved form an impaired loop (data not shown). However, as shown in figure 5, because of the C→A substitution in exon 37 (i.e., C6792A), a new branch-point sequence apparently emerges.

Discussion

There have been a considerable number of reports of PTC-induced exon skipping (e.g., see Bach et al. 1993; Dietz et al. 1993; Gibson et al. 1993; Hull et al. 1994). Different hypotheses about the mechanism of this phenomenon have been proposed (Dietz and Kendzior 1994; Carter et al. 1996; Maquat 1996), but at present a unifying model that reconciles all observations does not exist.

In the case of the C6792A mutation in exon 37 of the NF1 gene, expression studies revealed equal expression of the two alleles. This finding also indicates that the prevention of normal splicing by this mutation is complete—that is, the mutation C6792A causes a nonleaky splicing anomaly. The uniformity of the wild-type copy was also ascertained by sequence analysis (data not shown). If, as well, the corresponding shortened neurofibromin and the normal neurofibromin are being synthesized in equal amounts, a dominant negative mechanism may cause the disease in these particular cases. The in-frame splicing of exon 37 renders this a real possibility. Similarly, Dietz et al. (1993) have reported a relatively high amount of an abnormally spliced transcript of the fibrillin gene (FBN1), caused by a stop mutation in exon 51. This gene encodes a structural protein, the expression of which, in both allelic forms, is in accordance with the dominant negative mechanism proposed for Marfan syndrome (Dietz et al. 1992). To our knowledge, there exist no data suggesting that polypeptides translated from messages lose individual exons by abnormal skipping.

These results are in contrast to observations made in other genes, such as the ornithine aminotransferase gene, where the amount of cDNA amplified from the abnormally spliced transcript has been seen to be significantly lower than that amplified from the normal-size transcript (Dietz et al. 1993). Likewise, in the case of the stop mutation (C910T) in exon 7 of the NF1 gene, a diminished level of the abnormally spliced transcript was found. This finding does not, however, allow any conclusion with respect to the intranuclear events. The diminished amount of the shortened mutant transcript, which lacks exon 7, along with its stop codon, can still be explained in at least two ways. The alternative, abnormal splicing that leads to the skipping of exon 7 could be incomplete, and the mutant variety of RNA molecules still containing exon 7 could become degraded. Alternatively, abnormal splicing of the mutant RNA precursor could be complete, but part of the ab-

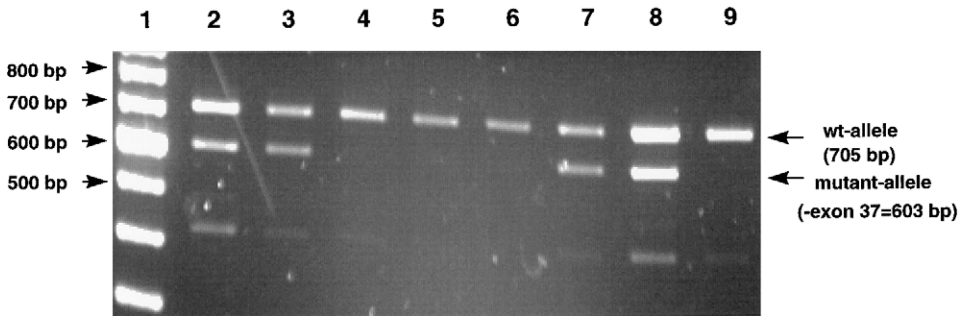

Figure 2 Exon 37 skipping: RT-PCR analysis. The agarose gel was stained by ethidium bromide. PCR products are derived from cDNA of NF1 patients B-NF2, B- NF3, B-NF6, B-NF7, B-NF8,B-NF4, B-NF5, and B-NF9 (lanes 2– 9, respectively). Lane 1, Size marker.

normally shortened message could be subjected to intranuclear or cytoplasmatic degradation.

The skipping of constitutive exons has, however, been demonstrated not only for stop mutations but also for an intraexonic deletion and a missense mutation that activates a cryptic splice site (Matsuo et al. 1991; Wakamatsu et al. 1992). In the case of the 52-bp intraexonic deletion found in exon 19 of the dystrophin gene, near the 5′ splice junction of intron 19, the recognition of this site could be hampered by the small size of the truncated exon (Matsuo et al. 1991). The deletion (6789delTTAC) and the insertion (6790insTT) found in exon 37 of the NF1 gene do not have an effect on splicing (whereas the intraexonic deletion in the dystrophin gene does have this effect), and these mutations are located distant from each of the splice sites and do not significantly change the size of the exon. In addition, an intraexonic silent mutation has recently been shown to cause skipping of the comparable exon in FBN1 (Liu et al. 1997). That a PTC can also inhibit the normal splicing of the affected exon has been demonstrated by Milstein and colleagues, who analyzed the splicing phenotypes of minigene constructs from a κ light-chain gene, using nuclear extracts in a cell-free system (Aoufouchi et al. 1996).

The phenomenon of PTC-induced exon skipping has given rise to a debate about the underlying mechanism (Dietz and Kendzior 1994; Carter et al. 1996; Maquat 1996). The discussion is primarily concerned with the problem of the intranuclear recognition of the PTCs, which followed by their partial or complete removal via illegitimate skipping of the exon containing the affected codon. Urlaub et al. (1989) have suggested such a nuclear-scanning model, in which specific intranuclear factors are able to scan the primary transcript for PTCs. A

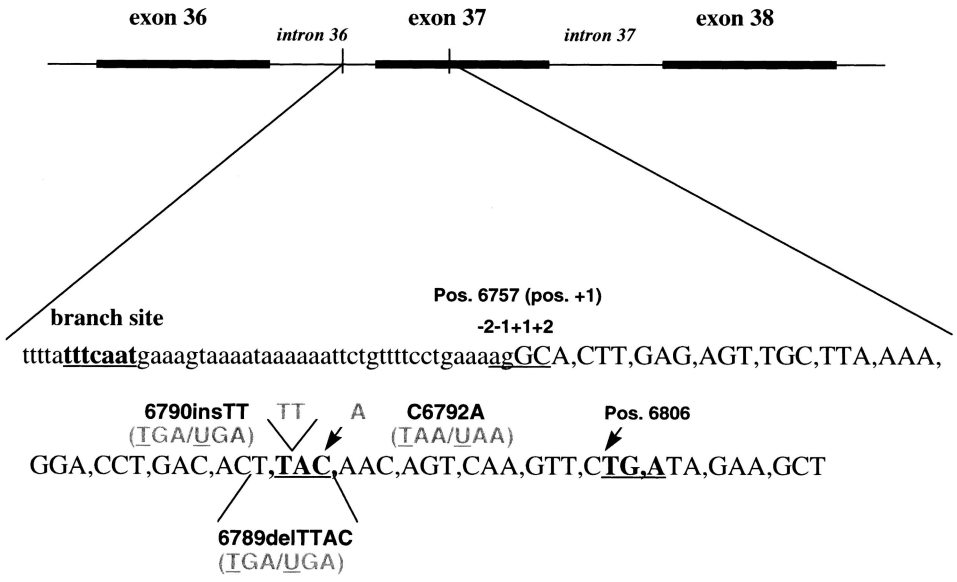

Figure 3 Localization of different stop mutations within exon 37 of the NF1 gene.

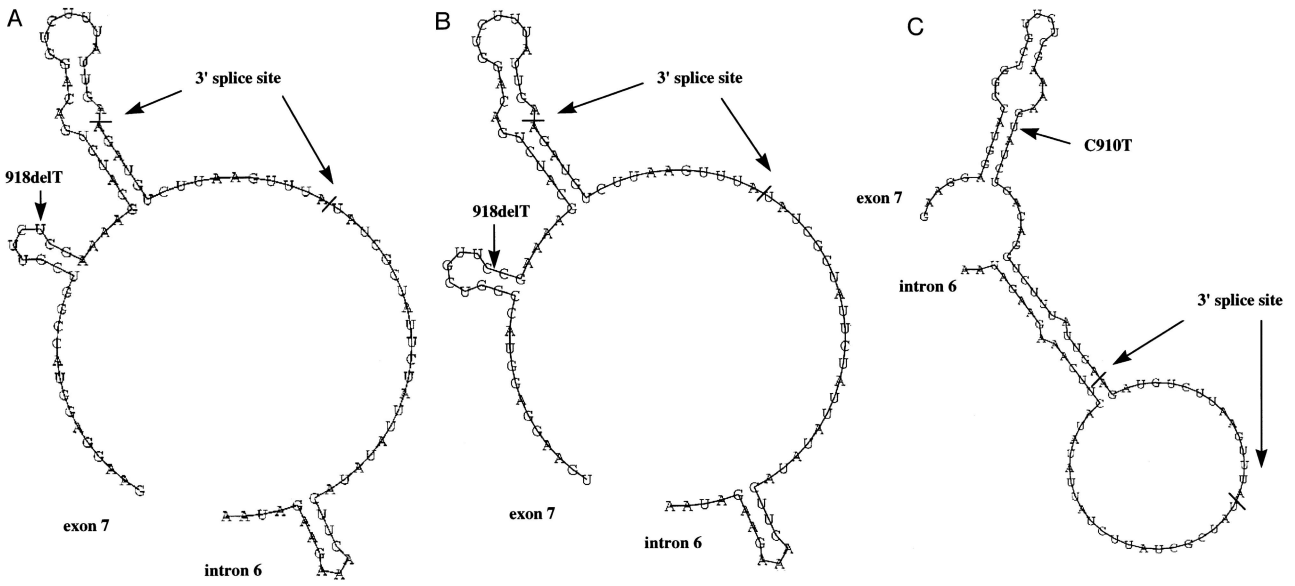

Figure 4 Minimum-free-energy structures for exon 7. The sequences contain +/− 50 bases downstream and upstream, respectively, of the 3′ splice site. *a,* Wild-type sequence of intron 6 and exon 7. *b,* Sequence of intron 6 and exon 7, including mutation 918delT. *c,* Sequence of intron 6 and exon 7, including mutation C910T.

similar but more far-reaching concept has been developed by Dietz and Kendzior (1994), on the basis of their studies utilizing minigene constructs from exons 50–52 of FBN1, with experimental variation of a naturally occurring nonsense codon. The fact that, with one exception, normal splicing was completely restored when the reading frame was shifted, so that the three bases of the former PTCs came to belong to consecutive sense codons, was considered to be strong evidence in favor of an intranuclear-scanning mechanism based on the recognition of the reading frame. Dietz and Kendzior (1994) assigned to this mechanism the important function of the maintenance of an open reading frame. A similar proposal has been made by Carter et al. (1996). The common denominator of these ideas is the existence of a scanning mechanism that recognizes PTCs and triggers exon skipping, but a role for alterations of the secondary structure of the transcript, which result from the base exchanges, is also being taken into consideration, either as a contributory factor (Dietz and Kendzior 1994) or as an alternative explanation (Maquat 1996).

If primarily concerned with the maintenance of an open reading frame, such a scanning mechanism should be capable of recognizing the PTCs that have arisen downstream of the three frameshift mutations here described (table 1), if not these frameshifts themselves. Our findings, together with the aforementioned exceptions, suggest that (*a*) the scanning mechanism either is leaky or has a context specificity with yet unknown preferences or (*b*) does not exist at all.

Purin-rich sequences that serve as splicing enhancers by interacting with splicing factors and with *cis*-acting intronic elements have been described for exons of several genes, such as the adenosine deaminase (ADA) gene and the fibronectin gene (Lavigueur et al. 1993; Santis-

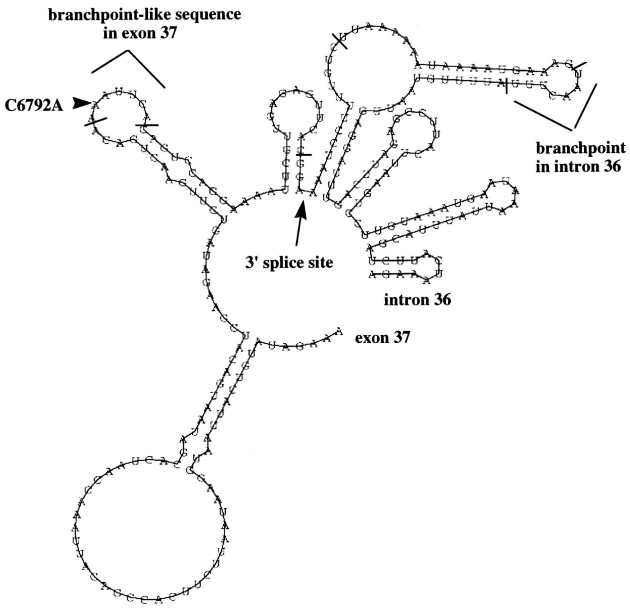

Figure 5 Minimum-free-energy structure for exon 37, including mutation C6792A. The sequence contains +/− 120 bases downstream and upstream, respectively, of the 3′ splice site.

teban et al. 1995). Some types of nonsense mutations localized in such enhancer sequences are able to cause the skipping of the respective exon, because of a disruption of the interaction between an exonic splicing enhancer and serine arginine proteins (Watakabe et al. 1993; Santisteban et al. 1995; Chiara et al. 1996). For example, a nonsense mutation (R142X; CGA→TGA) in a patient with severe combined immunodeficiency disease has been found to cause skipping of exon 5 of the ADA gene. This mutation is located within a purine-rich segment of exon 5 in the ADA gene, distant from both splice junctions. There is, however, in the putative enhancer sequence, no change of the number of purines that is due to the mutation in exon 5 (Santisteban et al. 1995). In this regard, a reduction of the splicing-enhancer effect could be observed in vitro by introducing a thymidine (T) into purine-rich sequences (Tanaka et al. 1994). Furthermore, in the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene, five of six exonic point mutations that caused exon skipping were substitutions by T, two of which also created nonsense codons (Steingrimsdottir et al. 1992).

In addition to purine-rich enhancers, non–purine-rich sequences can also function as splicing enhancers (Staknis and Reed 1994; Lynch and Maniatis 1995). Thus, although all the mutations found in exons 7 and 37 of the NF1 gene are localized in a heterogeneous sequence of bases, the possibility that such splicing enhancer sequences exist in these regions cannot be excluded.

Besides the known splicing-relevant sequences, such as splice sites and branch-point motifs, additional intronic sequences have been described as playing a role in the splicing process. Sirand-Pugnet et al. (1995) reported, in the chicken β-tropomyosin gene, an (A/U)GGG repeat with a potential role as an enhancer of the splicing reaction at the level of spliceosome assembly. Recently, Cogan et al. (1997) have published two mutations in intron 3 of the human growth hormone (GH) gene that lead to exon 3 skipping. Both mutations are located 28 bp downstream from the 5′ splice site, within a XGGG repeat similar to the repetitions found in the chicken β-tropomyosin gene. Cogan et al. (1997) suggest that the repeats may regulate alternative splicing in the human GH gene. This sequence motif does not occur in the known 210 bp of the 5′ end of intron 7, whereas intron 37 contains a number of scattered single copies of the motif at greater distances from the 5′ splice site.

With regard to an influence of alterations of the secondary structure on the splicing process, Steingrimsdottir et al. (1992) have published interesting data. For exon 8 of the HPRT mRNA, a potential stem-loop structure could be shown. A total of seven mutations have been identified in this exon, all of which are localized at different positions within this structure. Three of these mutations do affect splicing of exon 8 to varying degrees. It could be shown that the mutations at paired C residues lead to much more aberrant splicing than the alteration of the non–base-paired G residue. Maintenance of this stem-loop structure may be essential for correct splicing—for example, for exposure of the splice sites. The disruption of such a structure by specific mutations may be an explanation of splicing abnormalities (Steingrimsdottir et al. 1992). We have calculated the minimum-free-energy structures for the direct and the indirect stop mutations that we have found in exons 7 and 37 of the NF1 gene. Such computations demonstrate, at the exon-intron junctions and the branch-point regions, characteristic secondary structures that were surprisingly invariant to the sizes of the flanking sequences taken into account (W. Leistner, unpublished data). These calculations have revealed a secondary structure for the mutation (C910T) in exon 7, which is completely different from the calculated structures of the wild-type sequence and of the mutant sequence (918delT) (fig. 4) (the latter two sequences have indistinguishable secondary structures). We therefore suggest that, in the case of the direct stop mutation in exon 7, the altered secondary structure might in fact cause the anomalous splice phenomenon. We are aware of the hypothetical nature of the calculated secondary structures, whose relationship to the in vivo situation remains to be elucidated. These structures do, however, provide a feasible starting point for the comparison of the possible structural consequences of various sequence alterations.

In conclusion, mutations generating PTCs may result in exon skipping, by various mechanisms. The NF1 gene stop mutations C910T and C6792A trigger the skipping of the affected exon in different ways but apparently independently of any reading -frame surveillance mechanism that might have evolved for either the maintenance of an open reading frame (Dietz and Kendzior 1994) and/or nuclear down-regulation of aberrant transcripts bearing PTCs (Carter et al. 1996). The direct stop mutation (C910T) in exon 7 possibly interferes with the normal splicing process, because of the specifically altered secondary structure that was not observed with the unskipped stop mutation found nearby in exon 7. It is conceivable that the altered secondary structure in the respective area of the pre-mRNA leads to a covering of the respective splice sites, including the upstream branch point, as has been speculated for some mutations in exon 8 of the HPRT gene (Steingrimsdottir et al. 1992). With regard to exon 37, the direct stop mutation C6792A seems to generate a new branch-point sequence in an inappropriate position that might interfere with the initial splice-site recognition and spliceosomal complex assembly (Reed 1996), whereas the other two stop mutations found in the immediate vicinity do not affect the splicing process. What the skipping phenomena observed in both exons seem to have in common is an

impaired exon definition, caused by a particular stop mutation. In our view, exon definition is the immediate consequence of the interaction between the spliceosomal complex (and its auxiliary factors) and the appropriate sequence signals. This process is dependent on more or less stringent structural constraints, so that alterations of the secondary structure of the sequences involved may or may not perturb the splicing process, depending on the kind and the extent of the affected sequences. The generation and the abrogation of splicing-signal sequences with their characteristic secondary structures will be important factors to consider in this process.

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