

Exon skipping associated with A→G transition at +4 of the IVS33 splice donor site of the neurofibromatosis type 1 (NF1) gene

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Received January 4, 1994, Revised and Accepted February 11, 1994

Von Recklinghausen neurofibromatosis (neurofibromatosis type 1, NF1, McKusick no. 162200) is one of the most common human genetic disorders, with an incidence of approximately 1 in 3500 in all ethnic groups (1). The mutation rate is close to 1 in 10^4 meioses, and about half the cases result from new mutations (2). The clinical hallmarks of the disease are cutaneous or subcutaneous neurofibromas and hyperpigmented café-au-lait skin patches, but the disorder presents a remarkable phenotypic variability, with many other clinical signs such as axillary freckling, Lisch nodules, bone deformities, or learning disabilities. In addition, NF1 patients show an increased frequency of nervous system tumors, such as malignant neurofibrosarcomas, optic nerve gliomas, and pheochromocytomas. The NF1 locus was mapped by linkage analysis to chromosome 17q11.2 (3–5), and subsequently the NF1 gene was identified by positional cloning (6–8). The gene, which consists of 51 exons extending over a genomic distance of approximately 350 kilobases, produces a ubiquitously-expressed mRNA of 13 kilobases (kb) (6–8). The gene codes for the protein neurofibromin, which contains a domain related to GAP (GTPase-activating protein) (9), and is capable of down-regulating *ras* by stimulating its intrinsic GTPase activity (10, 11). Many germline mutations have been identified in patients with NF1 (see ref. 12 for review). The observation of loss of heterozygosity in at least one patient (13) supports a tumor suppressor gene hypothesis for NF1.

In screening for mutations in 42 NF1 patients by RT-PCR, we used a set of 7 primer pairs to amplify the coding region of the NF1 cDNA. We found a 59 year-old Swiss patient (C34-1) with NF1 where RT-PCR revealed one abnormally small amplification product, of about 1 kb instead of the expected 1.3 kb (Figure 1). The amplification product extends from the 3' end of exon 28 to the 5' end of exon 34. Although no formal quantification was performed, a consistent difference in the intensity of the two bands suggested that slightly less mutant than normal mRNA was present.

Sequence analysis revealed that the abnormal cDNA lacked the 280 nucleotides of exon 33 and had a perfect juxtaposition of exons 32 and 34. Genomic DNA sequences from nucleotide 47773 (exon 32) to nucleotide 49075 (IVS 34) were determined from uncloned PCR products (see Figure 2). A single nucleotide change was found, an A→G transition at nucleotide +4 of the IVS33 splice donor site (GTG/gtaagt to GTG/gtaggt; Figure 2). The relative frequencies of the four nucleotides at the +4 position of mammalian splice donor sites are: A – 0.71; G – 0.12; C – 0.08; T – 0.09 (14).

Although G occurs at position +4 in 12% of splice donor sites, we hypothesized that, in the context of this intron, the IVS33 +4 A→G transition was responsible for the splicing error and

was disease-causing. In order to exclude the possibility that this alteration was merely a polymorphism, we used a mismatch PCR assay to detect the A→G transition. By introducing a single mismatch in the sequence of one primer, the presence of G (mutant) rather than A (normal) at the mutation site creates a *Sau96I* restriction site in the amplification product, easily detected by subsequent digestion (Figure 3). The +4 A→G transition was not present in 170 non-NF1 chromosomes from unrelated Swiss individuals (data not shown), excluding the possibility of it being

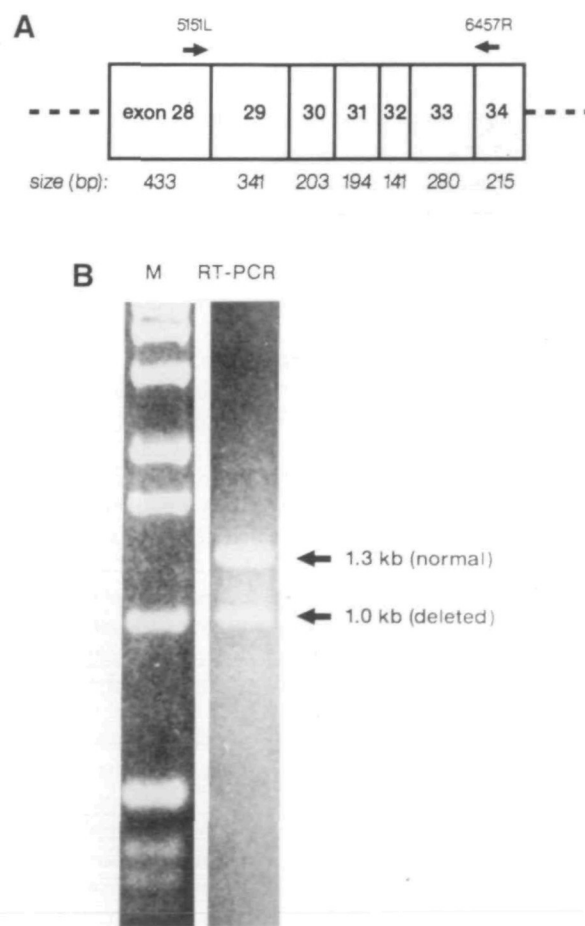


Figure 1. RT-PCR of NF1 cDNA exons 28 to 34. **A.** The specific primers for this PCR were 5151L (5'-GGTATTCCACAATGCTCTCAAG-3') and 6457R (5'-CTGACTTGACTTTGCTAATGCC-3'), numbered according to EMBL database, accession no. M89914. **B.** The lower band of 1.0 kb corresponds to the abnormal RT-PCR product which lacks exon 33.

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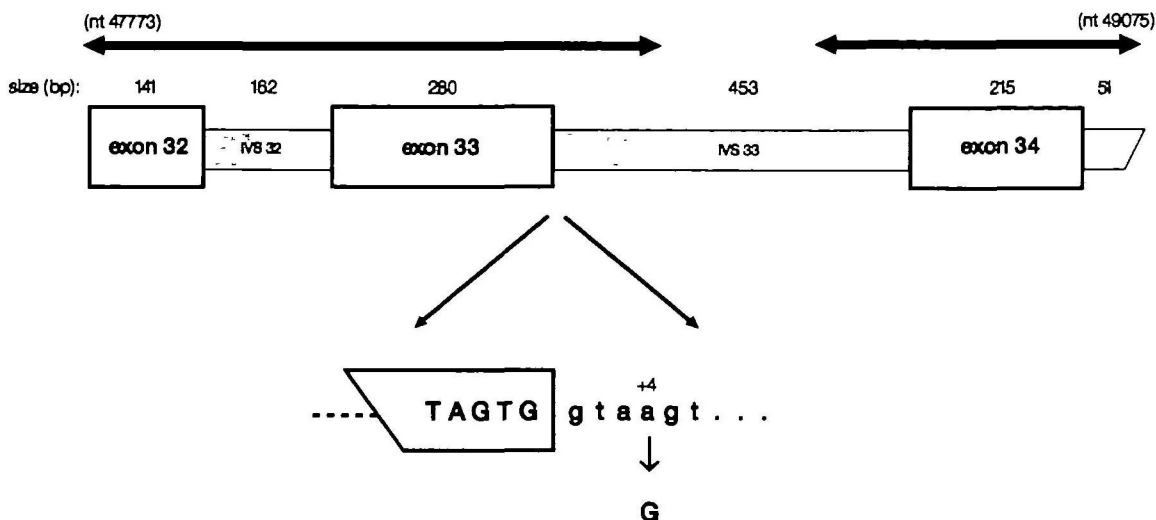


Figure 2. Structure of the genomic region surrounding the IVS33 splice donor site. The mutation is shown at the bottom. The heavy lines above represent the areas for which the genomic sequence was determined. Nucleotide numbers are according to the EMBL database, accession nos L05367 and L03723.

a common polymorphism. In addition, the aberrant splicing of exon 33 is predicted to result in a change of the reading frame and a loss of 777 of the 2811 amino acids of the mature protein. Finally, the mutation correlated with the disease in the patient's family: the mildly-affected son but not the asymptomatic daughter (28 year-old dizygotic twins), had inherited the mutation (Figure 3). We are thus led to conclude that mutations at nucleotide +4 of the splice donor site may lead to abnormal splicing and human disease.

The mutation reported here is the second independent case of NF1 with skipping of exon 33, the first being due to the insertion of an Alu retrotransposon in the 3' end of IVS32 (15). The 31 year-old patient of ref. 15 had no café-au-lait spots but one cutaneous neurofibroma, axillary freckling, Lisch nodules, cervical root tumors and macrocephaly. Our patient (C34-1) had typical but relatively mild dermatological features (multiple small neurofibromas, and three café-au-lait spots), and from the age of 48 showed progressive sensorimotor impairment due to multiple radicular neurofibromas, mostly in the intervertebral foramen. His affected son, who has five café-au-lait spots and three neurofibromas, had a history of excision of minor cysts in his childhood.

Three other +4 mutations at splice donor sites have been reported in human disorders. The first (A→G) was detected by genomic sequencing in the IVS19 of the CFTR gene in a patient with CF (16); however, the possibility of exon skipping was not investigated. The second +4 mutation (also A→G) was in the IVS33 of the COL1A2 gene, and led to the use of an alternative splice site within the intron in a patient with a non-lethal form of osteogenesis imperfecta (17). Finally, an IVS4 +4 A→T transversion has been reported in the FACC gene in Ashkenazi Jewish patients with Fanconi anemia, where the mutation apparently results in either complete or partial excision of exon 4 during splicing (18).

ACKNOWLEDGMENTS

This work was supported by a grant from the Carlos et Elsie de Reuter Foundation to P.H. and a grant from the Fondation Suisse pour l'Encouragement de la

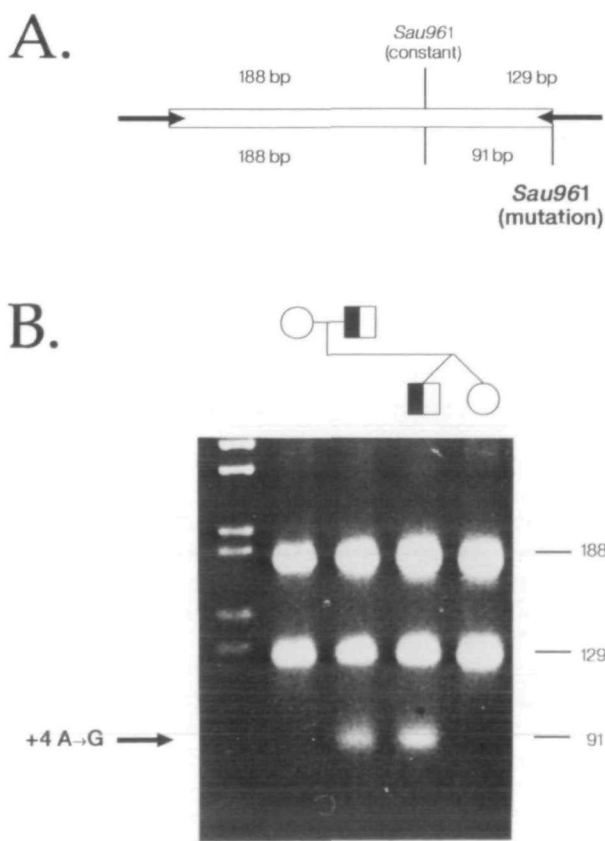


Figure 3. Diagnostic mismatch PCR analysis for the +4 IVS33 splice donor site mutation in the NF1 gene. The sequence of the two primers were NFx33L 5'-TAATTGGGAGTGTATTCGAATTCACAAGACATGCTTATC-3' and NF133Sau96R 5'-AATACGACTCACTATAGCACAAATTCCTTCCTAGGAC-3' (the mismatched base is underlined). PCR amplification from mutant DNA produces a novel *Sau96I* restriction site (GGNCC). A. The expected fragments from normal and mutant DNAs after *Sau96I* digestion. B. The proband and his son are heterozygous for the +4 A→G mutation as shown by the presence of the 91 bp fragment.

Recherche Scientifique sur l'Arriération mentale (FERAM) to M.M. We thank Dr P.Cirafici for his clinical contribution and Dr A.Schönbörner for technical help.

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