Insertion of a T Next to the Donor Splice Site of Intron 1 Causes Aberrantly Spliced mRNA in a Case of Infantile GM1-Gangliosidosis.

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RESEARCH ARTICLE

Insertion of a T Next to the Donor Splice Site of Intron 1 Causes Aberrantly Spliced mRNA in a Case of Infantile G_{M1}-Gangliosidosis

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The lysosomal storage disorders G_{M1}-gangliosidosis and Morquio B syndrome are caused by a complete or partial deficiency of acid β-galactosidase. Here, we have characterized the mutation segregating in a family with two siblings affected by the severe infantile form of G_{M1}-gangliosidosis. In total mRNA preparations derived from the patients' fibroblasts at least two aberrantly spliced β-galactosidase transcripts (1 and 2) have been identified. Both transcripts contain a 20 nucleotide (nt) insertion derived from the 5′ end of intron 1 of the β-galactosidase gene. Furthermore, in transcript 2 sequences encoded by exon II are deleted during the splicing process. Comparison of the 20-nt insertion with wild-type intronic sequences indicated that in the genomic DNA of the patients an extra T nucleotide is present immediately downstream of the conserved GT splice donor dinucleotide of intron 1. Both patients are homozygous for the T nucleotide insertion. We propose that this single base insertion is the mutation responsible for aberrant splicing of β-galactosidase pre-mRNA, giving rise to transcripts that cannot encode a normal protein.

KEY WORDS: β-Galactosidase, Pre-mRNA splicing, T nucleotide insertion, G_{M1}-Gangliosidosis

INTRODUCTION

The lysosomal enzyme β-D-galactosidase is required for removal of β-D-galactosyl residues from glycolipids, glycoproteins, glycosaminoglycans, and glycopeptides (Conzelmann and Sandhoff, 1987; O'Brien, 1989). Mutations in the human β-galactosidase locus on chromosome 3 (Shows et al., 1979) lead to absence or abrogation of the normal function of β-galactosidase, which results in lysosomal accummulation of undegraded substrates, particularly in the central nervous system (O'Brien, 1989; Suzuki, 1993). In tissues and cultured cells the majority of the active enzyme is found in complex with lysosomal neuraminidase (sialidase) and the protective protein/cathepsin A (Verheijen et al., 1982; 1985; Yamamoto et al., 1982; Yamamoto and Nishimura, 1987). It has been demonstrated that the latter is essential for intralysosomal activation and stabilization of the two glycosidases (d'Azzo et al., 1982; Hoogeveen et al., 1983; Verheijen et al., 1985; Van der Horst et al., 1989).

Absence or reduction of β-galactosidase activity results in two clinically and biochemically distinct metabolic storage disorders: G_{M1}-gangliosidosis, a lipidosis, and Morquio-B syndrome, a mucopolysaccharidosis (Okada and O'Brien, 1968; O'Brien et al., 1976; Groebe et al., 1980; for reviews see O'Brien, 1989; Neufeld and Muenzer, 1989; Suzuki, 1993). G_{M1}-Gangliosidosis patients suffer from a severe neurodegenerative disorder and, depending on the clinical symptoms, they are classified as having either the infantile or the mild

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juvenile/adult type of the disease. Patients with the infantile form have extensive CNS involvement, dysmorphic features, and hepatosplenomegaly, leading to early death. The β-galactosidase activity in these cases is reduced to less than 1% of normal levels. Pathologic examination indicates excessive accumulation of G_{M1}-ganglioside, particularly in neuronal tissues, as well as glycosaminoglycans and glycopeptides in visceral organs and other tissues (Suzuki, 1993 and references therein). The juvenile and adult variants of this disease have milder clinical manifestations, a prolonged life expectancy, and the residual β-galactosidase activity is 10–15% of normal levels (Suzuki et al., 1977; Wenger et al., 1980; Suzuki, 1993).

In human cultured fibroblasts the first immunoprecipitable form of β-galactosidase is a glycosylated precursor of 85 kDa that is processed, through a series of intermediates, into a 64-kDa mature enzyme (d'Azzo et al., 1982). The cDNAs and genes encoding human and mouse β-galactosidase have been isolated and characterized (Oshima et al., 1988; Morreau et al., 1989, 1991; Yamamoto et al., 1990; Nanba and Suzuki, 1990, 1991). It has been shown that the human gene can give rise to at least two alternatively spliced mRNAs: a major transcript of 2.5 kb encodes the classic, catalytically active β-galactosidase protein, and a minor species of about 2.0 kb gives rise to a nonlysosomal β-galactosidase-related protein which is inactive toward the artificial substrate used (Morreau et al., 1989; Yamamoto et al., 1990).

The characterization of the human β-galactosidase cDNA has enabled the identification of several mutations underlying distinct clinical forms of G_{M1}-gangliosidosis and Morquio-B syndrome (Yoshida et al, 1991; Nishimoto et al., 1991; Oshima et al., 1991, 1992; Mosna et al., 1992). Most G_{M1}-gangliosidosis patients, so far analyzed, are of Japanese origin. With the exception of two duplications found in either both or one of the alleles in two infantile Japanese patients, all other mutations are base substitutions, leading to single amino acid changes. They are different in clinically distinct patients, although in some cases only one allele has been identified. In this report we describe the genetic lesion present in two siblings affected with the severe infantile form of G_{M1}-gangliosidosis. We have found that the insertion of an extra T nucleotide at the donor splice site of intron 1 leads to aberrant splicing of β-galactosidase pre-mRNA. Both patients are homozygous for the mutation, which leads to a complete lack of β-galactosidase protein.

EXPERIMENTAL PROCEDURES

Cell Culture

Human skin fibroblasts from the index patient, the fetus, and their parents were obtained from the European Cell Repository, Rotterdam (Dr. W.J. Kleijer). The index patient was originally diagnosed by Dr. Pirkko Ammälä (University Central Hospital, Helsinki, Finland). Cultured fibroblasts from the index patient and the affected fetus were provided by Prof. Pertti Aula (University Central Hospital, Turku, Finland). Fibroblasts were cultured in Dulbecco's modified Eagles–Hams F10 medium (1:1 vol/vol) with antibiotics and 10% fetal bovine serum.

Oligonucleotides

For cDNA synthesis, PCR amplification, nucleotide sequencing, and hybridization studies several oligonucleotide primers were constructed on the basis of either human β-galactosidase cDNA (HβGaL) or genomic sequences (Morreau et al., 1989; 1991). The oligonucleotide primers were synthesized on an Applied Biosystems 381A oligonucleotide synthesizer and purified as recommended by the manufacturer. The specific primers used in the experiments described in the text are the following:

a. sense, 5′ CGAATTCATGCCGGGGTTCCTGGTTCGC 3′ (nt 49–71, exon I)
b. antisense, 5′ CGAATTCCTCCCATTTCCCACTCTGCACAG 3′ (nt 452–428, exon IV/exon III)
c. sense, 5′ GGCTTGCGCAATGCCACC 3′ (nt 117–134, exon I/II)
d. sense, 5′ CTTGCGCgttaagtctgc 3′ (nt 119–125, exon I/intron 1 + extra T)
e. sense, 5′ gggacccggGTATGTGCC 3′ (intron 1/nt 256–304, exon III)
f. antisense, 5′ ggttccccgccagcctgt 3′ (intron 1)
g. sense, 5′ CTTGCGCgtaagtctgc 3′ (nt 119–125, exon I/intron 1)

cDNA Synthesis

Total RNA was isolated from cultured fibroblasts as described by Auffray and Rougeon (1980). The entire coding sequence of the β-galactosidase mRNA was reverse transcribed into six overlapping cDNA fragments using specific antisense primers (Hermans et al. 1988). The primers were constructed on the basis of the sequence of the long β-galactosidase cDNA clone, HβGaL (see above).

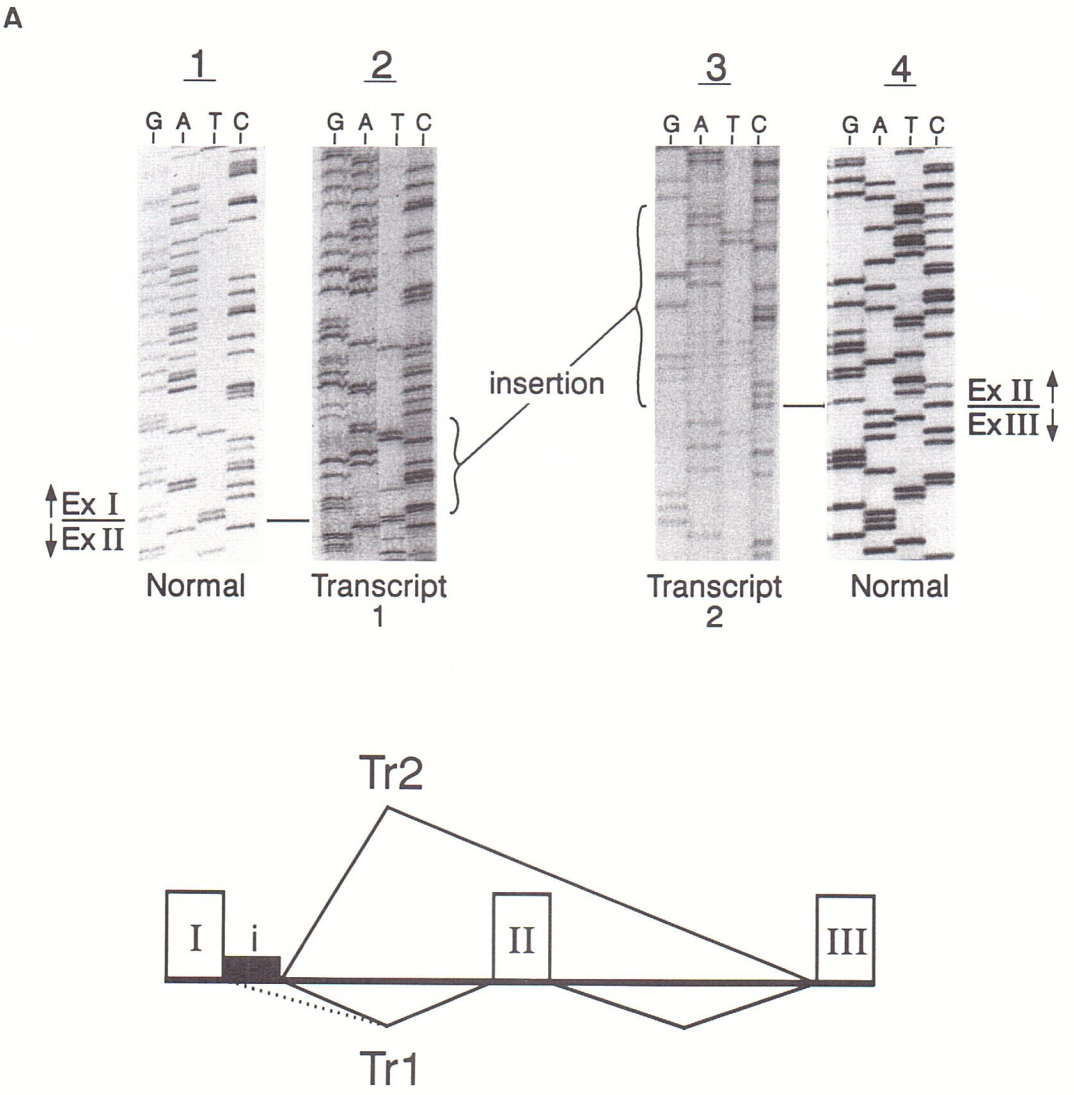

FIGURE 2. Partial nucleotide sequence of aberrant transcripts 1 and 2. (A) Total RNA was isolated from fibroblasts of the two patients, their parents, and an unaffected control, and reverse transcribed into cDNA with antisense primers located at different positions of β-galactosidase mRNA. These partial cDNA fragments were subsequently amplified, subcloned, and sequenced, or sequenced directly after an asymmetric polymerase chain reaction. A portion of the normal antisense sequence of β-galactosidase mRNA derived from ExII/ExI and ExIII/ExII is shown. Antisense sequences of aberrant transcripts 1 and 2 are given, which include the 20-nt insertion, marked with a bracket. (B) Schematic representation of the splicing mechanism leading to aberrant transcripts 1 and 2. Exon sequences are indicated by roman numbers I/II and III. The black bar represents the 20-nt intronic sequence (i) present in transcript 1 and 2. Normal splicing is indicated by the dotted line.

site and 3′ flanking sequences of intron 1 (Morreau et al., 1991). However, an additional T nucleotide is present in transcripts 1 and 2 of the patients, after the conventional GT dinucleotide of the splice donor site. The abnormal mRNAs must have arisen from aberrantly spliced pre-mRNA molecules, in a manner similar to that depicted schematically in Figure 2B. Apparently the normal splice donor site of intron 1 is no longer recognized, instead a cryptic site 21 nucleotides downstream is preferentially chosen. These transcripts were not amplified from mRNA preparations of both parents, indicating that they probably represent a minor pool. The 20-nt insertion causes a frameshift in the open reading frames (ORFs) of the aberrant mRNAs. The reading frame of transcript 1 terminates in an early stop codon. As a result, only a truncated peptide could be translated from transcript 1. In contrast, due to the deletion of exon II-encoded sequences in transcript 2, the

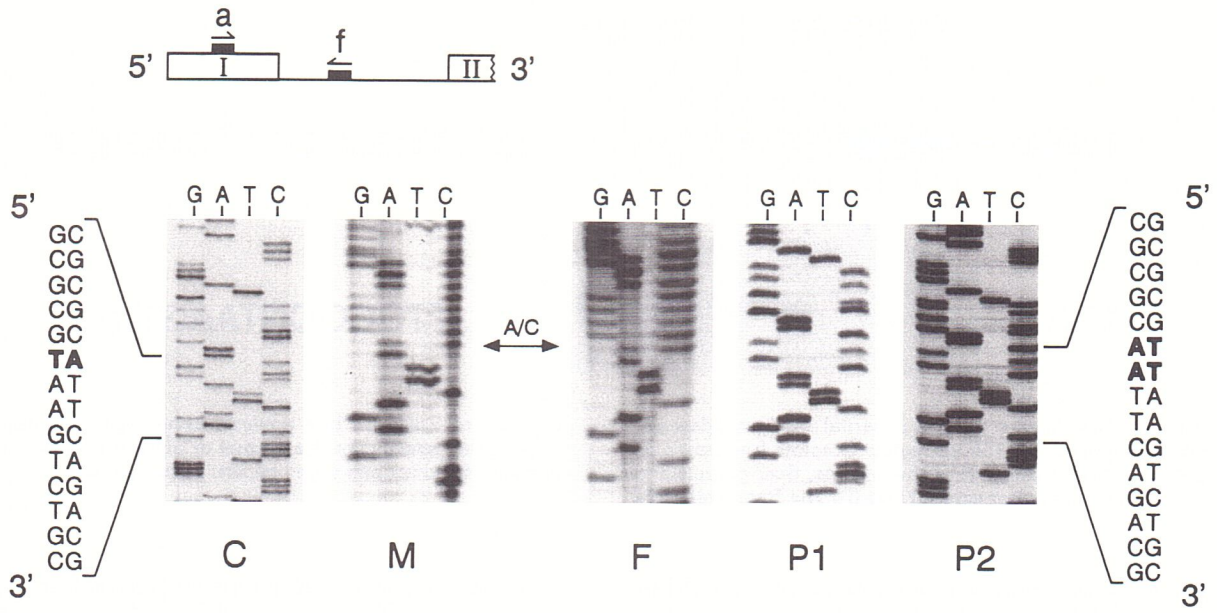

FIGURE 4. Partial nucleotide sequence of the β-galactosidase genes from two G_{M1}-gangliosidosis patients (P1, P2), their parents (M, F), and a control (C). Genomic DNA was isolated and subjected to asymmetric PCR in the region containing the mutation using the exonic primer (a) and the intronic primer (f). Portions of the antisense sequences of amplified fragments are shown.

are clearly homozygous for the T nucleotide insertion.

Finally, the 150-bp genomic fragment was again amplified, transferred onto nylon membranes, and hybridized with allelic-specific oligonucleotide probes either derived from the normal sequence (g) or carrying the T nucleotide insertion (d). As seen in Figure 5, this experiment convalidates that the two patients carry the same mutation in both alleles.

DISCUSSION

In this report we have described the genetic lesion present in both alleles of the β-galactosidase gene from two siblings affected with the severe infantile form of G_{M1}-gangliosidosis. A single T nucleotide insertion, immediately after the conserved GT dinucleotide of the splice donor site of intron 1, is likely to interfere with the normal splicing process, leading to a preferential use of a more downstream cryptic splice site. In total RNA preparations from fibroblasts of both patients we have identified two aberrant transcripts, however, we cannot rule out the possibility that more alternatively spliced or erroneously processed mRNA forms may exist. The 20-nt insertion found in patients' mRNAs cannot account for the presence of a slightly bigger transcript detected in Northern blots. The origin of this species, which is not visible in neither of the parents' preparations, is at present unknown. Interestingly, the same mutation has recently been found in two compound heterozygous siblings, also of Scandinavian ancestry, having an adult form of G_{M1}-gangliosidosis (S. Chakraborty and D.A. Wenger, personal communication). In this case only transcript 1 has been identified and represents a very small percentage of the total mRNA pool. The other allele carries a point mutation that is likely responsible for the mild clinical phenotype (Chakraborty et al., 1991).

The role of conserved structural elements in splicing of higher eukaryotic pre-mRNAs is well documented (for a review see Breathnach and Chambon, 1981). These elements consist of a 5′ splice donor site, a 3′ splice acceptor site, and a less conserved branch point sequence. Mutations at the GT/AG dinucleotides of 5′ and 3′ splice sites were shown to interfere with the normal splicing process both in site directed mutagenized genes and naturally occurring mutants (Padgett et al., 1986). It was empirically calculated that a T nucleotide in the third position of a normal splice donor site is found only in 5% of the cases, whereas an A nucleotide is most commonly present (70%; Padgett et al., 1986). Indeed an A is the base normally encountered in the third position of the

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