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A Point Mutation in the 5′ Splice Region of Intron 7 Causes a Deletion of Exon 7 in Adenosine Deaminase mRNA

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Abstract An adenosine deaminase (ADA;EC 3.5.4.4)-deficient B lymphoblastoid cell line BAD05 derived from a Japanese patient with severe combined immunodeficiency was characterized. As previously reported, one allele of BAD05 expresses undetectable ADA mRNA, and the other allele produces an aberrant mRNA without exon 7. Genomic ADA DNA of BAD05 spanning from a portion of exon 6 to a portion of exon 8 was amplified by PCR. The amplified fragments were cloned into a vector, and 8 clones were isolated and sequenced. The analytical result showed a single base change of G to A at the invariant 5′ GT of intron 7 of ADA gene in one allele of BAD05, which accounts for the elimination of exon 7 during splicing. © 1993 Wiley-Liss, Inc.

Key words: adenosine deaminase, severe combined immunodeficiency, polymerase chain reaction, splicing, deletion

Adenosine deaminase (ADA;EC 3.5.4.4) is an enzyme in the purine salvage pathway which catalyzes the deamination of adenosine and deoxyadenosine into inosine and deoxyinosine. The ADA gene on chromosome 20 consists of 12 exons and 11 introns, spanning a total of 32 kbp. The absence of ADA activity causes severe combined immunodeficiency (SCID) and is recognized in about 15–20% of patients with the disease [1]. Patients with complete ADA deficiency lack both B and T cell function [2–5]. Several lymphoblastoid cell lines derived from patients with ADA deficiency have been analyzed to identify the defects of the gene at a molecular level. Most of the reported abnormalities of ADA deficiency are point mutations which alter the amino acid sequences [see 6–10 for review]. One cell line has a point mutation at the 3′ splice region of intron 3 of the ADA gene, which causes a deletion of exon 4 during splicing [11]. Two cell lines express no ADA-specific mRNA as a result of the large deletion of the gene including the entire ADA promoter region and exon 1 [12,13]. An ADA-deficient B lymphoblastoid cell line BAD05, derived from a Japanese patient with SCID [14], and two B lymphoblastoid cell lines, BAM05 from his mother and BAF05 from his father, were characterized. The ADA activity of BAD05 was 0.6% of the control value. Northern blotting analysis showed that the amount of BAD05 mRNA was approximately 20% of the control amount (S. Monden et al., unpublished results). Sequence analysis of one of the BAD05 ADA cDNA clones revealed a deletion of exon 7 resulting from a point mutation of base 629 from G to A [15]. A ribonuclease protection assay showed that the BAD05 ADA genes resulted from one allele expressing the undetectable ADA mRNA and the other allele producing an aberrant mRNA without exon 7 [15]. The present study was performed to identify the defect of the BAD05 ADA gene which results in the production of the aberrant mRNA without exon 7.

MATERIALS AND METHODS

Cell Line

The B lymphoblastoid cell line BAD05 was established through the Epstein-Barr virus

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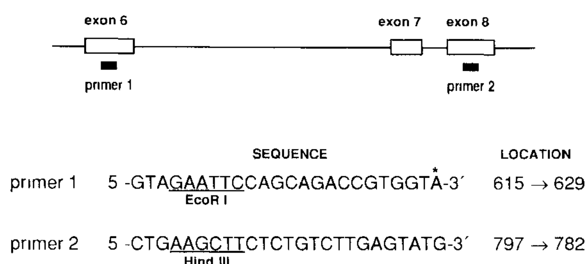

Fig. 1. Oligonucleotide primers for PCR amplification. Base numbers refer to the cDNA sequence with number 1 as the first base of the major initiation start site [17]. An asterisk denotes 629 A which is a point mutation in BAD05 ADA cDNA without exon 7 [15]. Primer 1 was synthesized to secure this mutation at its 3′ end so as to amplify the gene of the target allele selectively. The desired restriction sites, Eco RI for primer 1 and Hind III for primer 2, were added to the 5′ end of the primers. The length of the amplified product should be 1414 bp.

transformation of peripheral mononuclear cells which were purified by Ficol-Hypaque (Pharmasia LKB Biotechnology Inc.) from a patient with SCID associated with ADA deficiency [14]. The cell line was cultured in RPMI-1640 medium containing 10% fetal bovine serum.

DNA Purification

DNA obtained from the interface of the cesium chloride gradient after ultracentrifugation was dialyzed and purified by phenol extraction.

Amplification of Genomic DNA by PCR

Genomic DNA from BAD05 was amplified by PCR. Oligonucleotide primers were synthesized on an Applied Biosystems 393 DNA synthesizer. Figure 1 shows the location of two primers. Primer 1 was designed to secure the base A at its 3′ end which was a point mutation conserving amino acid sequence in the BAD05 cDNA without exon 7 [15], so that it can be completely homologous to the sequence of the gene of the target allele. Primers 1 and 2 correspond to a portion of exon 6 and exon 8, respectively, so the amplification covers all splice sites and branch sites that may be involved in the missplicing of exon 7. The desired restriction sites, Eco RI for primer 1 and Hind III for primer 2, were added to the 5′ ends of the primers. The reaction comprised (1) genomic DNA template (1 μg); (2) each primer (0.8 μg); (3) 5 mM potassium chloride, 10 mM Tris, pH 8.3, 1.5 mM magnesium chloride, 0.01% (W/V) gelatin; (4) 200 μM of each dNTP; and (5) Thermus aquaticus (Taq) DNA polymerase (2.5 U) in a total volume of 100 μl. The first cycle of PCR was subjected to denaturing at 92°C for 5 min, anealing at 55°C for 2 min, and extension at 72°C for 3 min. The following cycles were subjected to denaturing for 1 min, anealing for 1 min, and extension for 3 min. During the final cycle the extension time was increased to 13 min. Amplification was carried out for 27 cycles and gave a 1414 bp fragment which included a portion of exon 6, the entire length of intron 6, exon 7, and intron 8, and a portion of exon 8.

Cloning and Sequencing of the Amplified DNA

The amplified fragments were cloned into pB–sII (Stratagene) and were transfected into *E. coli* competent cells JM109 (Takara). Twenty-four colonies containing plasmids with inserts were obtained through blue/white color selection on LB plates with 50 μg/ml ampicillin, 80 μg/ml of fresh X-gal, and 20 mM IPTG. From 24 independent plasmid clones, 14 clones containing full-length amplified fragments were isolated through restriction mapping. To avoid misreading ascribed to possible misincorporation by Taq polymerase used in PCR, 8 out of the 14 clones were analyzed. Sequencing was carried out by a modified dideoxy-chain termination method [16]. Approximately 300 bp of the 5′ and 3′ ends of each cloned fragment were sequenced. These sequenced regions included all splice sites and branch sites of intron 6 and intron 7.

RESULTS

The use of primer I with the base 629 A at its 3′ end did not give a selective amplification of the target gene which produces ADA mRNA without exon 7, because this mutation is a commonly observed polymorphism in normal ADA genes which is reported to exist in over one-half of the ADA alleles [10]. Sequence analysis of 8 clones showed that 6 of them (clones 1–6) had a single base change of G to A at the position +1 in the invariant bases of 5′ splice region of intron 7, and the other 2 (clones 7 and 8) had a deletion of two bases 634–635 TT in exon 6. Within the approximately 600 bp of sequence analyzed for each clone which included all splice sites and branch sites, there were no other base differences from the normal ADA gene [17]. The sequence of clones 1–6 represents one allele (allele 1), and that of clones 7 and 8 the other allele (allele 2) (Fig. 2).

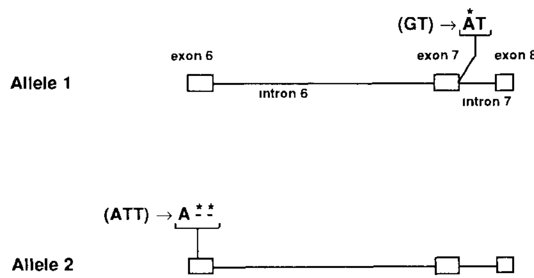

Fig. 2. Sequence analysis of the amplified genomic clones of BAD05 ADA gene spanning from exon 6 to exon 8 Exons are shown as open boxes, and introns as lines Of the 8 clones analyzed, 6 demonstrated a sequence representing one allele (allele 1), and 2 the other allele (allele 2) Allele 1 had a single base change (*) of G to A at position +1 in the 5′ splice region of intron 7 Allele 2 had a deletion of two bases 634–635 TT (**) in exon 6

DISCUSSION

A number of natural point mutations in 5′ and 3′ splice sites have been described in higher eukaryotes. In the case of the ADA gene, only one allele with a mutation that affects mRNA splicing has been reported [11]. In this allele of GM2825A, a point mutation AG to GG in the 3′ splice region leads to exclusion of exon 4 during splicing. Point mutations of 5′ splice regions of ADA gene have not been reported so far.

There is a consensus sequence around the 5′ and 3′ splice sites and around the branch site [18,19]. G at position +1 in intron is absolutely conserved, while the nucleotide at position +2 is T [20] in most cases and C in a few cases [21–25]. Point mutations in the 5′ splice regions, in particular conversion of the invariant GT to another dinucleotide such as AT [26–28] or GG [29], abolish or impair splicing in vivo. In an in vitro study of β-globin gene consisting of three exons and two introns, the 5′ GT to AT mutation of intron 2 abolishes correct splicing and promotes the formation of an aberrant joining of exon 1 and exon 3, while in vivo the same mutation leads to the use of a cryptic site in either exon or intron which is a relatively good match with the 5′ splice region consensus sequence. The use of cryptic site yields various other kinds of aberrant mRNAs containing a portion of intron 2 or lacking a portion of exon 2, but does not yield the exon 1–exon 3 splice product [30]. In another case of a natural 5′ GT to AT mutation of human β-globin gene, the exon 1–exon 3 splice product has been found in vivo at a low level [26].

S1 nuclease mapping has demonstrated that 10–15% of the total ADA mRNA in normal and ADA deficient cell lines lacks exon 7 or contains intron 7 as a result of inefficient splicing of intron 7 [31]. This inefficient splicing which occurs even in normal cells is probably due to the small size of intron 7 which contains only 76 bases close to the minimum intron size of 60 bases which appear to be required for accurate splicing [32], and the two noncanonical bases of the 5′ splice region which may affect the efficiency of RNA splicing [30]. A ribonuclease protection assay with BAD05 ADA mRNA showed that all ADA mRNA species of BAD05 lacked the entire exon 7 [15]. Sequence analysis of ADA gene in this study revealed that one allele of BAD05 had a single point mutation of G to A at the 5′ splice region of intron 7 of the ADA gene. This mutation impairs the correct splicing at the 5′ splice site of intron 7, whereas cryptic sites were not used as determined from the ribonuclease protection assay.

In the situation where a single 5′ splice site can react with two different 3′ splice sites, a distal pair of sites is used in some cases [33] and a proximal pair of sites in others [34]. In the case of BAD05 allele 1 in which the 5′ splice site of intron 6 could react with the two 3′ splice sites of intron 6 and intron 7, the distal pair of sites is used, resulting in the aberrant joining of exon 6 to exon 8 and the elimination of exon 7.

In addition, sequence analysis showed that the other allele of BAD05 had a deletion of two bases TT in exon 6 of the ADA gene. This allele, however, expresses undetectable mRNA [15], so some other genomic defect, such as an abnormality of the promoter region, might account for the ADA deficiency of BAD05. Two cases of the large deletion including the whole ADA promoter region and exon 1 have been reported [12,13].

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