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Adenosine Deaminase Deficiency Due to Heterozygous Abnormality Consisting of a Deletion of Exon 7 and the Absence of Enzyme mRNA

Saburo Kashii, Kazuhiko Ito, Sumie Monden, Yoshiki Sasai, Kunihiro Tsuchida, Masahiro Fujita, Hiroshi Kawamoto, Mihoko Norioka, and Minoru Okuma

First Division, Department of Internal Medicine, Faculty of Medicine, Kyoto University, Kyoto 606 (S.K., S.M., M.F., H.K., M.O.); Department of Transfusion Medicine, Kyoto University Hospital, Kyoto 606 (K.I., M.N.); Institute for Immunology, Faculty of Medicine, Kyoto University, Kyoto 606 (Y.S., K.T.), Japan

Abstract An adenosine deaminase (ADA;EC 3.5.4.4)-deficient B lymphoblastoid cell line BADO5 derived from a Japanese patient with severe combined immunodeficiency disease and two B lymphoblastoid cell lines, BAMO5 from his mother and BAFO5 from his father, were characterized. To identify mutations affecting ADA activity, we prepared cDNAs to ADA mRNAs of the BADO5 cell line for nucleotide sequencing. Sequence analysis of one of the BADO5 ADA cDNA clones revealed deletion of exon 7, and one point mutation of base 629 from G to A that did not affect the amino acid sequence. All clones of the BADO5 cell line so far examined showed the absence of exon 7 by Southern blotting analysis. Ribonuclease protection assay with an RNA probe spanning from exon 5 to exon 11 showed that the BADO5 ADA mRNA had a deletion of exon 7, the BAMO5 mRNA had normal length, and the BAFO5 mRNA had two species with a deletion of exon 7 and with normal length. Consequently, the patient's ADA genes resulted from one allele of the BAMO5 ADA gene that did not produce a detectable mRNA, and the other allele of the BAFO5 ADA gene producing an aberrant mRNA without exon 7.

Key words: severe combined immunodeficiency, point mutation, ribonuclease protection assay, splicing, cDNA sequence

Adenosine deaminase (ADA;EC 3.5.4.4) is an enzyme in the purine salvage pathway which catalyzes the deamination of adenosine and deoxyadenosine to inosine and deoxyinosine. Absence of ADA activity in humans causes severe combined immunodeficiency disease (SCID) which is characterized by the failure of differentiation and function of both T and B lymphocytes [1–4]. About 15 to 20% of infants with an autosomal recessive form of SCID are ADA-deficient [5]. Several lymphoblastoid cell lines have been established from ADA-deficient patients and studied to identify the defects of the gene at a molecular level. Most of the abnormalities of ADA deficiency are point mutations which alter the ADA amino acid sequence and structure of the ADA protein [6–10 for review]. One cell line has been found that contains a mutation in which exon 4 is lost during splicing [11]. In two patients no ADA mRNA was transcribed because of the deletion of the ADA promoter regions, including exon 1 [12,13]. A B lymphoblastoid cell line (BADO5) was established from a Japanese patient [14]. The ADA activity of BADO5 was 0.5% of the control value. Northern blotting analysis showed that the amount of BADO5 ADA mRNA was approximately 20% of the control amount (S. Monden et al., submitted for publication). The present study was performed to identify the precise molecular defects of the BADO5 ADA cDNA clones.

MATERIALS AND METHODS

Cell Lines

B lymphoblastoid cell lines were established by Epstein-Barr virus transformation of peripheral mononuclear cells purified with Ficol-Hypaque (Pharmacia LKB Biotechnology Inc.) from a patient (BADO5) with SCID associated with ADA deficiency, his father (BAFO5), his mother (BAMO5), and a healthy volunteer (B-ITO) [14].

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Address reprint requests to Saburo Kashii, Department of Transfusion Medicine, Kyoto University Hospital, 54 Shogoin-Kawahara-cyo, Sakyo-ku, Kyoto 606, Japan.

They were cultured in RPMI-1640 medium containing 10% fetal bovine serum.

RNA and DNA Purification

Total cellular RNA was prepared by the technique of Chirgwin et al. [15] using guanidine isothiocyanate extraction and cesium chloride gradient centrifugation. Poly(A)$^+$ RNA was isolated with oligo(dT)$_{30}$-Latex (Nippon Roche, Kyoto, Japan) [16]. The DNA was obtained from the interface of the cesium chloride gradient after ultracentrifugation. The DNA was dialyzed and purified by phenol extraction.

cDNA Libraries and Screening

BADO5 cDNA was synthesized by the method of Gubler and Hoffman [17]. After synthesis of double-stranded cDNAs, the EcoRI adaptors were added and the cDNA mixture was inserted into the EcoRI site of Lambda ZAPII vector (Stratagene). The synthesis was conducted by using a cDNA synthesis kit (Pharmacia LKB Biotechnology Inc.). The libraries were obtained via in vitro packaging reactions and subsequent transfection to host *Escherichia coli* XL1-Blue (Stratagene).

Three hundred thousand clones derived from the cDNA library were transferred to a nylon membrane (Du Pont–New England Nuclear) and screened by the plaque hybridization method [18] with a 1.5 kb ADA cDNA probe which was derived from pSV2 ADA (presented by Dr. S.H. Orkin, The Children's Hospital, Boston) [19] by HindIII digestion and labeled by the random primer method [20]. Forty independent positive clones were identified and isolated by the repeated plate lysate method [21]. Each of the Lambda ZAPII phages containing the cDNA insert was converted to the cDNA of plasmid Bluescript II (Stratagene) by rescue excision, and analyzed by restriction endonuclease mapping. Nine of these clones contained cDNA insert consisting of a single and nearly full-length ADA cDNA.

cDNA Sequencing

One representative clone (BADO53b) of nine clones containing BADO5 cDNA insert was subjected to sequence determination of both strands of the cDNA by a modified dideoxy-chain termination method [22] in the presence of [α-^{32}P]-dCTP (Du Pont–New England Nuclear). To resolve compressions, sequencing reactions were carried out with 7-deaza-dGTP (Takara Shuzo, Kyoto, Japan) [23].

Ribonuclease Protection Assay

A 664 bases PstI restriction fragment of the cDNA spanning from exon 5 to exon 11 derived from the pSV2 ADA was subcloned for the template of the riboprobe into a multicloning site downstream of a bacteriophage T7 promoter of the plasmid Bluescript II. The template was inserted into the plasmid in an orientation to produce complementary RNA. The plasmid including the template DNA was linearized at a BamHI site downstream from it, and transcribed with use of T7 RNA polymerase (Stratagene) in the presence of [α-^{32}P]CTP (Du Pont–New England Nuclear). The RNA probe was prepared by removing the template DNA with ribonuclease free deoxyribonuclease I, the protein with phenol/chloroform extraction, and the free [α-^{32}P]CTP by ethanol precipitation in the presence of 2 M ammonium acetate. The RNA probe was hybridized with 10 or 8 μg of total mRNAs prepared from each cell line and with 10 μg of yeast tRNA. The hybridization products were digested with 2 μg/ml of ribonuclease T1 (Sigma) at 30°C for 1 hr. The hybridization and ribonuclease digestion were performed as described by Melton et al. [24]. The ribonuclease-resistant products were analyzed by electrophoresis on a 5% polyacrylamide gel containing 7 M urea.

RESULTS

The consensus ADA cDNA sequence [10] is shown schematically in Figure 1A. Five small fragments of the BADO53b ADA cDNA digested with RsaI and PstI were purified electrophoretically with low melting agarose (Sigma). These fragments were subcloned into the plasmid BluescriptII for the following nucleotide sequencing. The full sequences of two strands of each subclone were analyzed from both 5′ ends.

The clone BADO53b was a slightly shorter fragment of cDNA with 1,372 bases of the total sequence which begins at base 55. In the open reading frame, 72 bases of exon 7 were deleted and there was only one point mutation at base 629 from G to A in exon 6 without alteration of amino acid (Fig. 1B). There was neither deletion nor point mutations in the 5′ and 3′ untranslated sequence.

Ribonuclease protection assay was carried out to confirm that the deletion of exon 7 was univer-

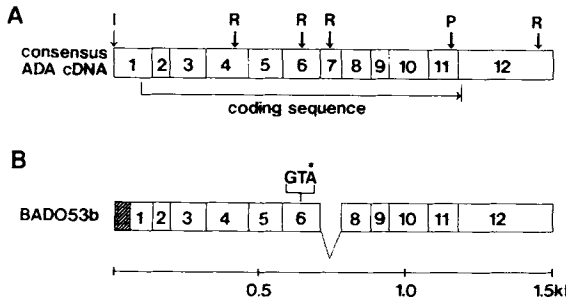

Fig. 1. Sequence analysis of an ADA cDNA clone of the ADA-deficient cell line BADO5. **A:** Linear map of consensus ADA cDNA. Full-length ADA cDNA is 1,498 bp with 12 exons. The coding sequence of 1,089 bases indicated by the lower line begins 96 bases 3′ of the primary transcription start site (I). The 3′ untranslated sequence containing 314 bases included a polyadenylation signal (AATAAA) 23 bases from the 3′ end. The Rsa I sites (R) and Pst I site (P) were used for subcloning of BADO53b. **B:** Sequence analysis of BADO53b indicated the deletion of exon 7 and conservative base change (*) from G to A at base 629 in exon 6. The hatched area designates no detectable sequence of the additional 5′ noncoding region.

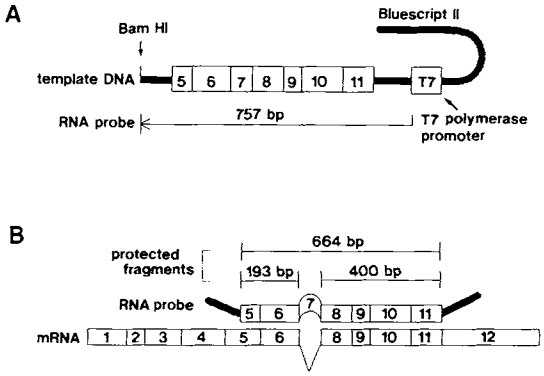

Fig. 2. Structure of the RNA probe. **A:** The template including intact ADA cDNA spanning exons 5 to 11 is linearized at Bam HI site in the plasmid Bluescriptll. The complementary RNA probe produced from T7 promoter is 757 base length including partial vector sequences (bold line). **B:** The expected fragments of the probe protected by the mRNAs. The deletion of exon 7 results in producing two protected fragments of 193 bases and 400 bases. The 664 bases are protected by the mRNA with no deletion.

sal in the mRNA of BADO5 and to analyze the abnormal genes of the patient's parents. The probe shown in Figure 2A was hybridized with total mRNA prepared from the cell lines. Normal ADA mRNA would protect 664 bases for the length of the probe. However, two fragments of 193 bases and 400 bases would be protected by the mRNA lacking exon 7 (Fig. 2B).

After digestion with ribonuclease T1, the protected probes were analyzed by electrophoresis on a polyacrylamide gel under denaturing conditions (Fig. 3). Undigested residues of the probe

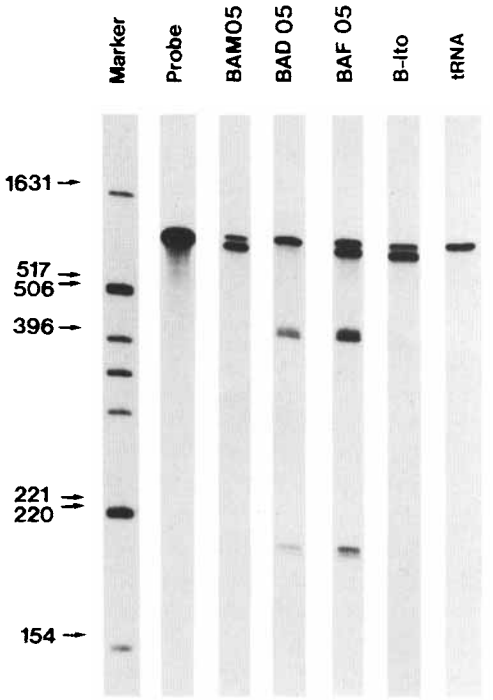

Fig. 3. Ribonuclease protection mapping for human ADA mRNAs of BADO5, BAMO5, BAFO5, and B-ITO. The ribonuclease protection assay was carried out with 10 μg of total RNA of BADO5, BAFO5, B-ITO, and yeast tRNA, and 8 μg of total RNA of BAMO5. Size markers consisted of plasmid pBR322 digested with HinfI. Marker sizes (nucleotides) are indicated on the left.

were seen as a band of 757 bases at the top of each lane. Only one band of 664 bases was seen in each BAMO5 and B-ITO lane. Two expected bands of 193 bases and 400 bases were seen in the BADO5 lane which had no band of 664 bases. Both the band of 664 bases and the two expected bands were seen in the BAFO5 lane. Non-specific self-hybridization of the probe was not detectable in the tRNA lane. These results indicated that the patient was heterozygous for one allele producing exon 7–deleted mRNA transmitted from his father and the other allele producing no detectable mRNA transmitted from his mother.

If BADO5 had the ADA mRNAs transcribed from the two alleles, two species of ADA cDNA clones would be obtained. Six clones (3, 3′, 4, 12, 14, 14′) of the nine appropriate clones were analyzed by Southern blotting. Since there was one RsaI restriction site in exon 6 and in exon 7, a 119 bases fragment was produced in the digestion of normal ADA cDNA with the RsaI restriction endonuclease. None of the six clones revealed a RsaI band of 119 bases, although a normal cDNA revealed the band (data not

shown). This finding indicated that these clones consisted of one species of ADA cDNA lacking exon 7.

It was previously reported that Northern blots of total RNA from BADO5 had a single band of slightly short and low abundance of ADA mRNA (Monden et al., submitted for publication). These experimental results confirmed that the allele inherited from his mother did not produce a detectable ADA mRNA.

DISCUSSION

Recently, the complete sequence of the human ADA gene was described [25] and several ADA-deficient cell lines have been investigated [10]. Several point mutations have been noted to alter the amino acid sequence affecting the enzymatic activities. A mutation at base 1,081 from C to T was found in a high proportion of patients with ADA deficiency [26]. Other point mutations affecting the amino acid sequence were found in exon 4, exon 7, exon 10, or exon 11 in eight alleles of five ADA-deficient cell lines. Conservative point mutations not affecting the amino acid sequence were also found. A base change at base 629 from G to A occurred in over one-half the normal and mutant ADA alleles so far examined [10].

In the ADA-deficient cell line GM2825A, exon 4 was deleted during splicing because of a single base change from A to G in the 3′ splice site of intron 3 [11]. In two patients, there was no expression of ADA mRNA due to the deletion of the first exon and promoter sequence of the ADA gene. This deletion occurred in the homologous recombination event between two Alu repeats located 1,494 bases 5′ to the ADA mRNA cap site and 1,757 bases 3′ to the cap site in the first intron [27].

This is the first report of the deletion of exon 7 detectable in all of the ADA mRNA of a cell line leading to a deficiency of ADA activity. It is not known whether this abnormality of the ADA mRNA is caused by abnormal splicing of exon 7 or by deletion of exon 7 in the ADA gene.

On the other hand, S1 nuclease mapping has shown that 10–15% inefficiently spliced mRNA without exon 7 or including intron 7 exists in the total ADA mRNA from healthy persons and also in abnormal mRNAs from GM2756 and GM2825A which have other mutations affecting the ADA activities [29]. This occasional inefficient splicing of exon 7 in the ADA mRNA is due either to a sequence different from the consensus one of the 5′ splice site of intron 7 or the small size of intron 7 containing only 76 bases. A minimum intron size of 60 bases is required for accurate splicing [28,30]. Our ribonuclease protection assay also demonstrated, after prolonged exposure, that the small amounts of mRNA without exon 7 were present in the control B-ITO.

Southern blotting analysis of the genomic DNA in BADO5 demonstrated no particular abnormalities such as a large deletion (Monden et al., submitted for publication). The elimination of exon 7 was constant in the ADA mRNA of BADO5, probably because of the small deletion in the ADA gene, including exon 7, or some different mechanism during splicing, such as a base change of the 3′ splice site of intron 6.

The allele inherited from his mother did not produce a detectable mRNA. Many causes are possible; for example, the gene may not be transcribed or the mRNA may not be stable.

Six Japanese patients with ADA-deficient SCID have been known, but no genetic studies were carried out. This report of BADO5 is the first analysis of a Japanese patient with an ADA deficiency.

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