Genetic Cause of a Juvenile Form of Sandhoff Disease

ABNORMAL SPlicing OF \( \beta \)-HExOSAMINIDASE \( \beta \) CHAIN GENE TRANSCRIPT DUE TO A POINT MUTATION WITHIN INTRON 12*

(Received for publication, September 19, 1988)

Takeshi Nakano and Kunihiko Suzuki†
From the Biological Sciences Research Center, Departments of Neurology and Psychiatry, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

Abnormal \( \beta \)-hexosaminidase \( \beta \) chain cDNA clones were isolated from a library constructed from cultured fibroblasts of a patient with a juvenile form of Sandhoff disease (genetic \( \beta \)-hexosaminidase A and B deficiency). Sequence analysis of a cDNA clone isolated from these fibroblasts contained an extra 24-base segment between exons 12 and 13. This segment was identified as the 3' terminus of intron 12. The remainder of the coding sequence was completely normal. The same 24-base insertion was found in four additional clones by sequencing. Restriction mapping analysis of seven other clones was consistent with the presence of the same 24-base intron 12 segment. This insertion is in frame and adds 5 amino acids between amino acids 491 and 492 of the primary sequence of the normal enzyme protein. It is located only 5 amino acids away from a possible glycosylation site. The finding is consistent with the slightly larger than normal size of the \( \beta \) subunit precursor protein observed by immunoprecipitation. No normally spliced mRNA was detected. Gene amplification by the polymerase chain reaction and subsequent sequencing of genomic DNA indicated that the patient was a compound heterozygote.

\( \beta \)-Hexosaminidase (\( \beta \)-N-acetyl-\( \beta \)-hexosaminidase, EC 3.2.1.52) is a lysosomal hydrolase consisting of two subunits, \( \alpha \) and \( \beta \) (1). Of the two catalytically active isozymes present in tissues, \( \beta \)-hexosaminidase A is a heterodimer (\( \alpha \beta \)) and \( \beta \)-hexosaminidase B is a homodimer (\( \beta \beta \)) (1). The complete sequences of the cDNAs coding for the normal human \( \beta \)-hexosaminidase \( \alpha \) and \( \beta \) chains and the structural organization of the genes are now known (2-5). Genetic deficiency of the \( \beta \) subunit abolishes catalytic activity of both of the isozymes, resulting in a category of GM2 gangliosidoses in which both A and B isozyme activities are deficient (Sandhoff disease, total hexosaminidase deficiency) (1). The classical form of the disease is a rapidly fatal and predominantly neurological disorder of infants. However, various clinical variants exist including rare late-onset or juvenile forms (1). Precise diagnosis of genetic defects on the gene level is now feasible with the availability of the cDNA and genomic clones. A recent survey indicated complex genetic heterogeneity of Sandhoff disease (6). In this report, we describe a specific genetic abnormality in one of the earliest known patients with a juvenile form of Sandhoff disease (7).

MATERIALS AND METHODS

The fibroblast cell line was from the patient with the juvenile form of Sandhoff disease originally reported by Wood and MacDougall (7). It was obtained from the Human Genetic Mutant Cell Depository, Coriell Institute for Medical Research, Camden, NJ (GM2094A, the same patient as GM2144). The cells were maintained under our standard culture conditions before use. Control cell lines were selected from our collection. Commercial materials were purchased from standard suppliers, including Bethesda Research Laboratories, Boehringer-Mannheim, International Biotechnologies Inc. (New Haven, CT), Pharmacia LKB Biotechnologies, Inc., and New England Biolab. Radioisotopes were obtained from ICN Radiochemicals (Irvine, CA). Sources for non-standard materials will be indicated below as appropriate.

Northern Analysis—Poly(A)* RNA was prepared from fibroblasts from 100-150-ml flasks (8). For the Northern analysis, approximately 1 \( \mu \)g of poly(A)* RNA sample was electrophoresed in 1% agarose/formaldehyde denaturing gel (9). Transfer of RNA to Biotrans-Nylon membrane (1.2 \( \mu \)m) (ICN, Irvine, CA), hybridization with a full-length normal \( \beta \)-hexosaminidase \( \beta \) cDNA (5) labeled with \( [\alpha-\text{P}] \)dATP by nick-translation (10), and subsequent washing were carried out as described by the manufacturer. Blots were exposed to Kodak x-ray film at \(-70^\circ \text{C}\) using a Cronex Hi-Plus intensifying screen.

Isolation of cDNA Clones—A cDNA library was constructed from 5 \( \mu \)g of the mRNA fraction essentially according to the procedure of Gubler and Hoffman (11) but using a commercial cDNA-synthesizing kit (Amersham Corp.) and ligated into the A gt11 phage through the EcoRI linker. The library was screened, without amplification, with the \( 32^\text{P} \)-labeled normal full-length \( \beta \)-hexosaminidase \( \beta \) cDNA.

DNA Sequence Analysis—DNA sequence analysis was carried out by the Sanger dideoxy chain-termination method (12) with appropriate M13 vectors, the 17-mer sequencing primer, and \( 32^\text{P} \)-labeled dATP (13). The DNA polymerase used was a commercial modified T7 polymerase (Sequenase®, U.S. Biochemical Corp., Cleveland, OH).

Southern Analysis—cDNA clones were evaluated by the Southern blotting analysis. They were digested simultaneously with \( H\text{haIII} \), EcoRI, and PsI and electrophoresed in 3% Nu-Sieve agarose (FMC BioProducts, Rockland, ME), blotted, and hybridized to a 754-bp* DNA fragment.

*This investigation was supported in part by Research Grant R01 NS-24289 and Mental Retardation Research Center Core Grant P30 HD-03110 from the United States Public Health Service. The content of this article was presented in part at the International Seminar on Molecular Basis of Inherited Diseases, held in Tokyo, Japan, September 29-30, 1988. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence and reprint requests should be addressed: Biological Sciences Research Center, CB#7250, University of North Carolina School of Medicine, Chapel Hill, NC 27599.

1 The abbreviation used is: bp, base pair(s).
probe prepared from the normal $\beta$-hexosaminidase $\beta$ cDNA by double digestion with EcoRI and PstI and labeled with $^{32}$P as above.

DNA Polymerase Chain Reaction and DNA Sequence Analysis—A segment of the $\beta$-hexosaminidase $\beta$ gene including exons 12, 13, and the entire intron 12 were amplified by the polymerase chain reaction procedure with agarose primer pairs (14–17). The two primers were at the 5' terminus of exon 12 and the 3' terminus of exon 13, respectively, and thus flanked the HindIII site in exon 12 and the PstI site in exon 13. The protocol for the reaction was as follows. The target sequence was amplified in a 100-μl reaction volume containing 50 mM Tris-HCl, pH 8.3, 1.5 mM MgCl$_2$, 0.2 mM each of dNTPs, and 1 μM of each primer in a siliconized microcentrifuge tube. Samples were heated at 94°C for 6 min, centrifuged momentarily, and annealed at 55°C for 3 min. Two and one-half units of Taq polymerase was added, and the samples were incubated at 72°C for 3.5 min for extension with an overlay of 100 μl of mineral oil. The manual cycling profile was 55°C for 2.5 min (annealing), 72°C for 3.5 min (extension), and 94°C for 1.5 min (denaturation). Usually, the extension reaction was repeated 40 times. Taq polymerase, 2.5 units, and the primers, 30 pmol each, were added at the 20th cycle, and Taq polymerase only, 2.5 units, at the 30th cycle. The procedure was terminated after the 41st extension reaction which was run for 7 min. Taq polymerase was purchased from Cetus/Perkin-Elmer Corp. or Stratagene (La Jolla, CA). After amplification, a portion of the reaction mixture was subjected to agarose gel electrophoresis to assess the size of the amplified sequence. The amplified segment in the remaining portion of the sample was purified after low melting point agarose gel electrophoresis, digested with HindIII and PstI, and subcloned into appropriate M13 vectors. The sequence analysis was carried out as described above. Multiple subclones were sequenced for statistical reliability and to exclude artifacts due to polymerase errors during the gene amplification.

RESULTS

A preliminary Northern blotting analysis of the poly(A$^+$) RNA fraction from cultured fibroblasts of this patient indicated the presence of $\beta$-hexosaminidase $\beta$ mRNA of apparently normal size and quantity (data not shown), in agreement with the previous report (6). A total of 18 cDNA clones were isolated and purified from the unamplified library. Restriction mapping and comparison with the published normal $\beta$-hexosaminidase $\beta$ cDNA sequence indicated that at least six of them were sufficiently long to include the entire coding sequence. One of these clones was sequenced in its entirety. The cDNA was 1845-bp long and contained a 65-bp 5'-untranslated region, a 1659-bp open frame, and a 121-bp 3'-untranslated region terminated by polyadenylation. The sequence was identical with the normal cDNA except that there was a 24-base insertion between exons 12 and 13 (Figs. 1 and 2). The 24-base insertion was identified as the 3' end of intron 12 by comparison with a published portion of intron 12 (5). The insertion kept the reading frame intact and contained no termination codon. Thus, it would add 8 amino acids, -Gly-Asp-Val-Leu-Asp-Arg-Glu-, between amino acids 491 (tryptophan) and 492 (proline) of the normal enzyme protein. This insertion is located at only 5 amino acids downstream of one of the possible glycosylation sites (Fig. 2).

Four additional cDNA clones which included the region of exons 12 and 13 were also sequenced. All of them contained the identical 24-base insertion. Seven more clones were then examined for the presence of the extra AhaIII site located within the insertion. All of them not only contained the extra AhaIII site but all of the AhaIII-Aha111 and AhaIII-PstI fragments appeared to be of the same size (Fig. 3). While the exact sequence of the insertion was determined in only five clones, these findings were consistent that all 12 clones contained the same 24-base insertion.

Gene amplification by the polymerase chain reaction with primers flanking the HindIII and PstI sites in exons 12 and 13 (Fig. 1) gave amplified segments of the same size from the normal and mutant genomic DNA. Double digestion of the amplified segments with HindIII and PstI, subcloning into the M13 vectors and subsequent sequencing gave two distinct sets of sequences (Figs. 4 and 5). One was identical with that of the normal control sequence and the other had a single nucleotide transition within intron 12. This finding indicated that the patient was a compound heterozygote and that intron 12 was abnormal in one allele but normal in the other allele. The abnormality was a single nucleotide transition from the normal G to A at 26 bases from the 3' terminus of intron 12. This transition generated a sequence, -CAG/GG-, at this site. This is a consensus sequence for the 3' splicing site for an intron (18). Thus, the retention in mRNA of the 24 bases of intron 12 downstream of this mutation can be readily explained.

DISCUSSION

Three genetically distinct forms of GM$_2$ gangliosidoses are known, each caused by mutations in different genes; Tay-Sachs disease ($\beta$-hexosaminidase $\alpha$ chain mutations),
can be identified by the additional cDNA in this region are shown at the bottom. The mutant sequence cDNAs which give three positive bands, 448, 230, and 100 bp. Five of positive bands, 524 and 230 bp, respectively. All others are mutant of the normal cDNA. The restriction maps of the normal and mutant cDNAs were digested with EcoRI, electrophoresed, blotted, and probed with the EcoRI-PstI fragment insertion. The sample in the middle is the normal cDNA, giving two positive bands, 448-bp bands are believed to be artifacts due to overexposure.

The entire normal intron 12 sequence is shown in Fig. 4. The same single nucleotide transition from G to A is indicated with the newly generated consensus sequence for the 3' intron splice site. The sequences of the region of the 3' terminus of intron 12 and the mutant cDNA all appear to be of the same sizes. The minor signals just above the 448-bp bands are believed to be of the normal enzyme by 8 amino acids. This is consistent with the observation of the slightly larger than normal β-hexosaminidase β subunit precursor, immunoprecipitated from fibroblasts of this patient and visualized on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1).

The polymerase chain reaction and subsequent sequencing of the region in question showed that the gene abnormality underlying the extra 24 bases in the mRNA sequence was a single nucleotide transition within intron 12. The transition results in a mutant sequence, CAGGG-. According to a recent survey of over 1400 exon-intron junctional sequences (18), the last three nucleotides just prior to the 3' splice site of introns were C (72%), A (100%), and G (100%) and the first nucleotide of exons was G in the majority of the cases. Thus, this mutation generates the most frequent consensus sequence for 3' splicing sites of introns. The normal intron 12-exon 13-splice site of this gene has a sequence of TAGG. The splicing mechanism appears to utilize the newly generated mutant splice site preferentially over the normal splice site located 24 bases downstream. If the normal splice site is used at all, it must be used infrequently, because all 12 independent cDNA clones examined retained the same 24-base segment of intron 12. If one assumes certain percentages of normally spliced mRNA, the probabilities of not finding it in the first 12 mRNA molecules are 0.28 at 10%, 0.54 at 5%, and 0.78 at 2%. Thus, it is entirely possible that we did not find a normally spliced cDNA in our 12 clones, if it constituted 5% or less of the total β chain message.

The 8-amino acid insertion occurs in a region of the enzyme protein highly conserved between the β-hexosaminidase α and β subunits (4, 5). Particularly, out of 31 amino acids in exon 12, 24 are identical between the two subunits. The exon 13 sequence is less well conserved (18 identity out of 35). Besides the abnormally large size of the nascent polypeptide, functional consequences of this mutation are not known in any detail, except that the end result is the defective catalytic

---

**FIG. 4.** Mutation in the juvenile Sandhoff disease patient. The entire normal intron 12 sequence is shown in **bold face**. The single nucleotide transition from G to A is indicated with the newly generated consensus sequence for the 3' intron splice site.

**FIG. 5.** Mutation in the juvenile Sandhoff disease patient. The sequences of the region of the 3' terminus of intron 12 and the 5' end of exon 13 in the normal (A) and the mutant (B) genes are shown. Nucleotides are for the non-coding strand. Methodological details of the polymerase chain reaction and subsequent procedures are given under "Materials and Methods." The arrows indicate the position of the mutation. Note the identity of the sequences between the genes in this Figure and the mutant cDNA in Fig. 2 from the bottom of the figures up to the transition.
activity of \( \beta \)-hexosaminidase. A point mutation within exon 13 of \( \beta \)-hexosaminidase \( \alpha \) chain causes defective processing/routing of the synthesized enzyme protein (25). Considering the high homology between the subunits, the insertion of 8 amino acids between the exon 12 and exon 13 is also likely to prevent the newly synthesized \( \beta \) subunit precursor from being processed normally. Furthermore, one of the possible glycosylation sites is only 5 amino acids away from the insertion. If this site is normally glycosylated and particularly if the carbohydrate chain at this site is the one to be phosphorylated to form the mannose 6-phosphate recognition marker, the insertion may well interfere with these critical steps for the processing and intracellular routing of this enzyme.

The gene amplification and sequence analyses showed clearly that the patient was a compound heterozygote. The other allele was normal with respect to the intron 12 and the flanking exon sequences. This allele, however, must also be abnormal, because the patient would have been clinically asymptomatic otherwise. Southern analysis with full-length cDNA did not show any obvious abnormality in the \( \beta \)-hexosaminidase \( \beta \) gene in this patient (6). Thus, the nature of the abnormality in the other allele cannot be assessed with the available data but it is likely to be the type that generates no or highly unstable mRNA, because all of the 12 independent mRNAs isolated and examined derived from the allele with the mutation in intron 12.

A few other cases of \( \beta \)-hexosaminidase \( \beta \) and \( \beta \) deficiency in older age groups are known. The patient reported by Goldie et al. (27) was symptomatic with clinical features somewhat similar to the patient in this study. The fibroblast line from this patient was lost in our laboratory and no further information is available. A father and a daughter with nearly complete deficiency of \( \beta \)-hexosaminidase \( \beta \) but with significant residual activity of the A isozyme were described by Dreyfus et al. ("hexosaminidase Paris") (28, 29). These individuals with the hexosaminidase Paris, however, are asymptomatic. Nevertheless, the hexosaminidase Paris gave rise to a \( \beta \)-hexosaminidase \( \beta \) chain precursor larger than normal (1). In view of the differences in the clinical phenotypes, the mutation underlying hexosaminidase Paris may or may not be the same as the one described here. It is tempting to speculate that hexosaminidase Paris may be due to a mutation similar to ours. In our case, we cannot exclude the possibility of a small percentage of normally spliced mRNA, as discussed above. Such normally spliced mRNA will produce normal \( \beta \)-hexosaminidase \( \beta \) subunit that can associate with the \( \alpha \) subunit to form "residual" \( \beta \)-hexosaminidase \( \alpha \). Relatively small residual activity appears to be sufficient to maintain the clinically normal phenotype (30). If the mutation in the hexosaminidase Paris turns out to be the same as in the juvenile Sandhoff disease patient, the major difference in the clinical pictures could then be due to the nature of the second allele or some epigenetic reasons.

While this manuscript was under editorial review, Dlott et al. (31) presented results of a detailed study on processing of the \( \beta \)-hexosaminidase \( \beta \) subunit in two juvenile Sandhoff disease patients, including the one we studied, and two asymptomatic individuals. All exhibited an abnormally large \( \beta \) subunit precursor. They found that the \( \beta \) subunit was gycosylated but did not acquire the mannose 6-phosphate recognition marker, did not associate with the \( \alpha \) subunit, and was degraded rapidly. Residual \( \beta \)-hexosaminidase \( \alpha \) activity was higher in the asymptomatic individuals (7–10% versus 2–5%). The same group of investigators then found the same 8-amino acid insertion and the same base transition in intron 12 in one of the asymptomatic individuals and in the other juvenile Sandhoff patient.

Acknowledgments—We thank Dr. Richard L. Proia for kindly providing us with the normal \( \beta \)-hexosaminidase \( \beta \) cDNA. Dr. Proia also indicated that the patient's cells make larger-than-normal enzyme protein. Oligonucleotide primers for the polymerase chain reaction were synthesized in the Nucleotide Synthesis Laboratory of the Program in Molecular Biology and Biotechnology of the University of North Carolina under the supervision of Dr. Dana Fowlkes.

REFERENCES

1. Sandhoff, K., Conselainm, E., Neufeld, E. F., Kaback, M. M., and Suzuki, K. (1989) in The Metabolic Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) Sixth Ed, McGraw-Hill Book Co., New York, in press.
2. Myerowitz, R., Piekarsz, R., Neufeld, E. F., Shows, T. B., and Suzuki, K. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7830–7834.
3. Proia, R. L., and Savarir, E. (1987) J. Biol. Chem. 262, 5677–5681.
4. Korneluk, R. G., Mahuran, D. J., Neote, K., Klevins, M. H., O'Dowd, B. F., Troppman, M., Willard, H. F., Anderson, M. J., Lowden, J. A., and Gravel, R. A. (1986) J. Biol. Chem. 261, 5407–5413.
5. Proia, R. L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1883–1887.
6. O'Dowd, B. F., Klevins, M. H., Willard, H. F., Gravel, R. L., and Mahuran, D. J. (1986) J. Biol. Chem. 261, 12880–12885.
7. Wood, S., and MacDougall, B. G. (1976) Am. J. Hum. Genet. 28, 490–495.
8. Aviv, H., and Leder, P. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1408–1412.
9. Goldberg, D. A. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5704–5708.
10. Rigby, P. W. J., Diekmann, M., Rhodes, C., and Berg, P. (1977) J. Mol. Biol. 115, 237–251.
11. Gubler, U., and Hoffman, B. J. (1983) Gene (Amst.) 25, 265–269.
12. Sanger, F., Nollen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467.
13. Biggin, M. D., Gibson, J. J., and Hong, G. F. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 3953–3955.
14. Kogak, S. C., Debary, M., and Gitschier, J. (1987) Engl. J. Med. 317, 985–990.
15. Wong, C., Dowling, C. E., Sakai, R. K., Higuchi, R. G., Erlich, H. A., and Kizuka, H., Jr. (1987) Nature 330, 384–386.
16. Oste, C. (1988) Biotechniques 4, 162–167.
17. Stoffet, E., Koeberl, D. D., Sarker, G., and Sonnmez, S. S. (1988) Science 239, 491–494.
18. Shapiro, M. B., and Sensapathy, P. (1987) Nucleic Acids Res. 15, 7135–7174.
19. Myerowitz, R., and Hogkyzai, N. D. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 15396–15400.
20. Myerowitz, R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3955–3958.
21. Arpaia, E., Dumbrille-Row, A., Matal, T., Neeke, K., Troppman, M., Troxel, C., Virling, J. L., Pita, J. S., Basset, B., Lashenahaw, A. M., Mahuran, D. J., Schuster, S. M., Clarke, T. J. R., Lowden, J. A., and Gravel, R. A. (1988) Nature 333, 85–86.
22. Ohno, K., and Suzuki, K. (1988) Biochem. Biophys. Res. Commun. 153, 463–469.
23. Ohno, K., and Suzuki, K. (1988) J. Biol. Chem. 263, 18552–18567.
24. Ohno, K., and Suzuki, K. (1988) J. Neurochem. 50, 316–318.
25. Nakano, T., Muscillo, M., Ohno, K., Hoffmann, A. J., and Suzuki, K. (1988) J. Neurochem. 54, 984–987.
26. Goldie, W. D., Holtzman, D., and Suzuki, K. (1977) Ann. Neurol. 2, 156–158.
27. Dreyfus, J. C., Poeau, L., and Swanover-Hoffman, L. (1975) Engl. J. Med. 292, 61–63.
28. Dreyfus, J. C., Poeau, L., Vibert, M., Ravisé, N., and Boué, J. (1977) Am. J. Hum. Genet. 29, 287–293.
29. Conselainm, E., and Sandhoff, K. (1983/84) Dev. Neurosci. 6, 58–71.
30. Dlott, B., and Neufeld, E. F. (1986) Am. J. Hum. Genet. 43, 82.

* B. Dlott and E. F. Neufeld, personal communication.