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Genetics

Splice junction mutation in some Ashkenazi Jews with Tay–Sachs disease: Evidence against a single defect within this ethnic group

(β-*N*-acetylhexosaminidase/lysosomal enzyme/polymerase chain reaction)

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ABSTRACT **Tay–Sachs disease is an inherited disorder in which the α chain of the lysosomal enzyme β-*N*-acetylhexosaminidase A bears the mutation. Ashkenazi Jews are found to be carriers for a severe type of Tay–Sachs disease, the classic form, 10 times more frequently than the general population. Ashkenazi Jewish patients with classic Tay–Sachs disease have appeared to be clinically and biochemically identical, and the usual assumption has been that they harbor the same α-chain mutation. In this study I have isolated the α-chain gene from an Ashkenazi Jewish patient, GM2968, with classic Tay–Sachs disease and compared its nucleotide sequences with that of the normal α-chain gene in the promoter region, exon and splice junction regions, and polyadenylylation signal area. Only one difference was observed between these sequences: at the 5′ boundary of intron 12, a guanosine in the conserved splice junction dinucleotide sequence G-T had been altered to a cytidine. The alteration is presumed to be functionally significant and to result in aberrant mRNA splicing. Utilizing the polymerase chain reaction to amplify the region encompassing the mutation, I developed an assay to screen patients and heterozygote carriers for this mutation. Surprisingly, in each of two Ashkenazi patients, only one α-chain allele harbored the splice junction mutation. Only one parent of each of these patients was positive for the defect. Another Ashkenazi patient did not bear this mutation at all nor did either of the subject's parents. In addition, 30% of obligate heterozygotes tested carried the splice junction mutation, whereas 20 Ashkenazi Jews designated noncarriers by enzymatic assay were negative for this alteration. The data are consistent with the presence of more than one mutation underlying the classic form of Tay–Sachs disease in the Ashkenazi Jewish population.**

Tay–Sachs disease is an autosomal recessive disorder caused by a deficiency of the lysosomal enzyme, β-*N*-acetylhexosaminidase A, a heteropolymer composed of two polypeptides called α and β (1, 2). In this disorder, mutations in the α-chain gene render the enzyme defective, resulting in the accumulation of GM2 monosialoganglioside in the nervous system. Tay–Sachs disease is really a group of disorders, ranging in clinical severity from mild to fatal (1–3) and differing in biochemical parameters, including (*i*) residual enzyme activity (4), (*ii*) immunoprecipitable α-chain polypeptide (5), and (*iii*) detectable α-chain mRNA (6, 7). Such variations reflect the consequences of different α-chain genetic lesions. Although the disease is rare, Jews of Eastern European descent, Ashkenazi Jews, have a 10-fold higher gene frequency than does the general population for a severe form of the disorder known as classic Tay–Sachs disease. In contrast to the heterogeneity described above, Ashkenazi Jews with classic Tay–Sachs disease have one clinical course leading to death in early childhood and identical biochemical profiles—i.e., lack of residual enzyme activity, absence of immunoprecipitable α-chain polypeptide, and inability to detect α-chain mRNA (1, 8–10). Southern blot analysis of genomic DNA from Ashkenazi patients revealed an intact gene (11) that was transcriptionally active (12). It has been assumed that only one mutation gives rise to the severe form of Tay–Sachs disease in this ethnic group (1).

This report describes the isolation of the α-chain gene from an Ashkenazi Jewish patient with classic Tay–Sachs disease and the identification of a mutation in a splice junction within that gene. Surprisingly, analysis of other affected individuals as well as obligate heterozygotes revealed that the mutation causing classic Tay–Sachs disease is not homogeneous within this ethnic group.

MATERIALS AND METHODS

Materials. Restriction endonucleases were purchased from New England Biolabs. T4 ligase, calf intestinal alkaline phosphatase, the Klenow fragment of DNA polymerase, and pTZ-18 vector were obtained from Pharmacia Biotechnology. *Taq* polymerase was from Perkin–Elmer Cetus. λEMBL3, a phage λ *in vitro* packaging system and LamdaSorb were from Promega Biotec (Madison, WI). Nytran was purchased from Schleicher & Schuell, and Biotrans nylon membranes were from ICN. GeneScreen*Plus* and radioisotopes were from DuPont–New England Nuclear. All oligonucleotides were prepared by the Midland Certified Reagent Company by phosphoramidite methods (13). Blood samples of Ashkenazi patients with classic Tay–Sachs disease and of their parents were obtained through the Kingsbrook Jewish Medical Center, Brooklyn, NY. Pellets of leukocytes from Ashkenazi Jewish obligate heterozygotes and Ashkenazi Jews assigned noncarrier status by enzymatic screening were kindly provided by Eugene Grebner (Thomas Jefferson University, Philadelphia, PA).

General Methods. Plasmid DNA was isolated by alkaline lysis (14) followed by centrifugation with a cesium chloride/ethidium bromide step gradient (15). Phage DNA was isolated in milligram quantities by the method of Kaslow (16). Rapid purification of approximately 20 μg of phage DNA was achieved by adsorption of lysates obtained from two 90-mm culture dishes to a conjugate of fixed *Staphylococcus aureus* and rabbit polyclonal antibodies directed against λ bacteriophage particles (LamdaSorb) as described by the manufacturer. Double-stranded DNA fragments were labeled with [α-^{32}P]dCTP (6000 Ci/mmol; 1 Ci = 37 GBq) by random primer method (17), and oligonucleotides were end-labeled with [γ-^{32}P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase.

Cell Culture. Fibroblast cultures GM2968, GM3051, GM3052, GM515, and IMR90 were obtained from the Na-

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Abbreviation: PCR, polymerase chain reaction.

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tional Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, NJ). Cell strains WG103 and WG107 were obtained from the Repository of Mutant Cell Strains (Montreal, Canada). Conditions for culture were as described (18).

Construction of Genomic Library and Isolation of Clones. High molecular weight DNA was isolated from cultured fibroblasts of an Ashkenazi Jewish patient with classic Tay–Sachs disease, GM2968. The DNA was partially digested with *Mbo* I and treated with phosphatase; 2.1 μg was used to construct a genomic library in vector λEMBL3 as described (19). Recombinant clones generated in this manner were screened directly without amplification with a [^{32}P]-cDNA clone that coded for the entire α chain of β-hexosaminidase, ρβHα-5 (9). Positive clones were isolated after two additional cycles of screening. When required, α-chain inserts of λEMBL3 or portions thereof were subcloned into pTZ-18 vectors by standard methods (20).

Analysis of the Clones. Assignment of clones to their appropriate locations on the α-chain gene map (21) was achieved by Southern analysis. After digestion with *Sal* I, *Sal* I and *Eco*RI, and *Sal* I and *Bam*HI, DNA was subjected to electrophoresis in a 0.5% agarose gel, transferred to GeneScreen*Plus* under alkaline conditions, and hybridized sequentially with a series of ^{32}P-labeled probes. The probes included (*i*) a 312-base-pair (bp) cDNA fragment obtained from the 5′ end of ρβHα-5(11), (*ii*) a 356-bp cDNA fragment obtained from the 3′ end of ρβHα-5(11), and (*iii*) appropriate α-chain exon-specific oligomers (unpurified).

DNA Sequencing. Plasmid DNA was sequenced by the Sanger dideoxy chain termination method (22) utilizing ^{35}S-substituted dATP (23) and either reverse transcriptase or the Klenow fragment of DNA polymerase I. α-Chain exon-specific oligomers (18 or 19 bases) were utilized as primers without purification.

Assay for α-Chain Mutation. DNA for analysis was isolated from cultured fibroblasts (20) or whole-blood leukocytes (24). Two 23-base oligonucleotides (PCA_1 and PCA_2) complementary to sequences flanking the α-chain mutation were used as primers in the polymerase chain reaction (PCR) (25) to amplify a 135-bp sequence of genomic DNA encompassing the mutation (Fig. 1*A*). Genomic DNA (1 μg) served as the initial template for primers PCA_1 and PCA_2, which were present in the 100-μl reaction mixture at a concentration of 100 mM. The chain reaction was carried out by using the Klenow fragment of DNA polymerase I as described (25). After 22 cycles of replication, half of the reaction mixture was incubated with *Dde* I overnight at 37°C. After extraction with phenol/chloroform, 1:1 (vol/vol), the reaction products were precipitated with ethanol, applied to an 8% polyacrylamide (19:1 methylenebisacrylamide) gel and electrophoresed in a Tris acetate/EDTA buffer, pH 8.0, at constant voltage until the bromophenol blue dye reached the bottom of the gel. Samples were transferred electrophoretically in the same buffer from the gel to a nylon membrane (Nytran, 0.2 μm) at a constant amperage of (200 mA) overnight at 4°C. DNA was denatured by soaking the membrane in 1 M NaCl/0.5 M NaOH for 30 min. After neutralization in 0.5 M Tris, pH 7.4/1.5 M NaCl, the membrane was baked for 1 hr at 60°C to bind the DNA. Membrane-bound DNA was hybridized (manufacturers conditions) at 65°C to a ^{32}P-labeled 41-base oligonucleotide specific for a section of exon 12 (Fig. 1*B*). Membranes were washed as described by the manufacturer and exposed to x-ray film in the presence of a Cronex Hi Plus intensifying screen (DuPont).

In an alternate assay the polymerase chain reaction was carried out by using *Taq* I polymerase as described by the manufacturer. Without prior digestion with *Dde* I, duplicate sets of samples (7-μl aliquots) were applied, denatured, and fixed to Biotrans membranes (0.2 μm) according to the DNA dot-blot protocol described by the manufacturer. Both sets of samples were prehybridized for 1 hr at 37°C in 0.1% polyvinylpyrrolidone/0.1% Ficoll/0.1% bovine serum albumin/0.9 M sodium chloride, 0.05 M sodium phosphate, pH 8.3/0.005 M EDTA/500 μg of denatured salmon sperm DNA per ml. One set of samples was hybridized with a ^{32}P-labeled 19-base oligomer complementary to the sense strand of the normal allele (5′ C-A-G-G-C-T-C-T-G-G-T-A-A-G-G-G-T-T-T 3′), and the other set was hybridized with a 19-base oligomer complementary to the sense strand of the mutant allele (5′ C-A-G-G-C-T-C-T-G-C-T-A-A-G-G-G-T-T-T 3′). Filters were washed in 0.36 M sodium chloride/0.02 M sodium phosphate, pH 8.3/0.002 M EDTA for 15 min at 4°C, then for 30 min at ambient temperature, and finally for 2 min at 53°C. The ambient-temperature wash solution was 0.1% in $NaDodSO_4$. Filters were exposed to x-ray film in the presence of a Cronex Hi Plus intensifying screen for 2 hr.

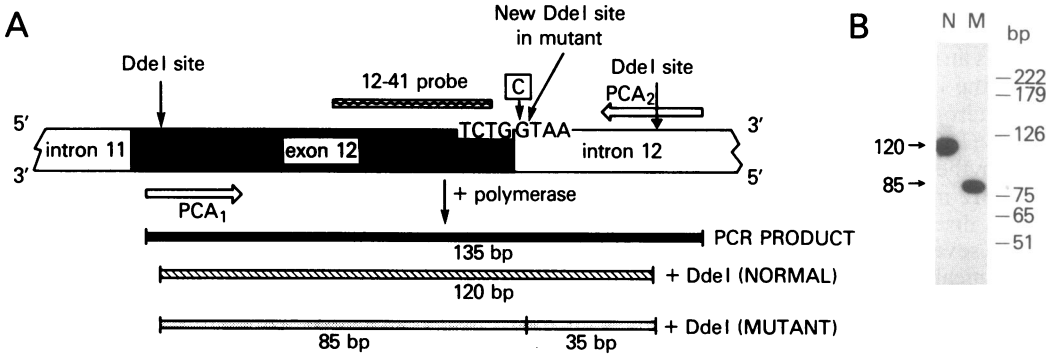

FIG. 1. Assay for the splice-junction mutation in Ashkenazi Jews with classic Tay–Sachs disease. (*A*) The schematic shows the two strands of a section of the α-chain gene, including part of intron 11 and 12 (open boxes) and all of exon 12 (solid box). *Dde* I restriction sites are as indicated, including the additional *Dde* I site found in the Ashkenazi mutant DNA at the 5′ boundary of intron 12, where a cytidine had replaced a guanosine. PCA_1 = 5′ C-C-C-C-T-G-A-G-C-A-G-A-A-G-G-C-T-C-T-G-G-T-G 3′ and PCA_2 = 5′ T-C-C-T-G-C-T-C-T-C-A-G-G-C-C-C-A-A-C-C-C-T-C 3′ (shown by open arrows) are the primers complementary to opposite strands of DNA used in the PCR to amplify a 135-bp DNA fragment (solid bar) inclusive of the mutation. The hatched line shows the 120-bp DNA fragment obtained after digestion of the PCR product from a normal allele of genomic DNA with *Dde* I, and the stippled lines show the two fragments (85 bp and 35 bp) obtained upon *Dde* I digestion of the PCR product from a mutant allele. (*B*) Polyacrylamide gel electrophoresis of *Dde* I-digested DNA (either PCR product from genomic DNA or cloned DNA; cloned DNA was used in this example) followed by transfer to Nytran and hybridization with a ^{32}P-labeled oligomer, 12-41, [bar (enclosing wavy line) above schematic of exon 12] yields a 120-bp fragment for the normal DNA (lane N) and an 85-bp fragment for the mutant DNA (lane M). Probe 12-41 is specific for exon 12 and has the sequence 5′ G-G-G-G-A-G-A-A-T-A-T-G-T-G-G-A-C-A-A-C-A-C-A-A-A-C-C-T-G-G-T-C-C-C-C-A-G-G-C-C-T-C 3′.

RESULTS AND DISCUSSION

Identification of the α-Chain Mutation. Southern analysis has shown (11) that the restriction enzyme cleavage patterns of the α-chain gene of Ashkenazi Jewish patients with classic Tay–Sachs disease were identical to those observed in normal controls, indicating that the genetic lesion was either a small deletion or a single-base-pair change. α-Chain mRNA was lacking in mRNA preparations from cultures of classic Tay–Sachs patients from this ethnic group, suggesting faulty transcription or incorrect RNA processing that had resulted in an unstable α-chain message (6). These observations prompted the search for a single-base-pair change or small deletion by sequence analysis of the promoter region, splice junction regions, and polyadenylylation signal area of the α-chain gene. The first step in this study required isolation of the mutant α-chain gene harboring the mutation. Toward that end a genomic library was constructed in λEMBL3 with DNA obtained from fibroblasts of an Ashkenazi Jewish patient with classic Tay–Sachs disease (GM2968). The library contained 2×10^6 independent recombinant phage particles and was screened for the α-chain gene by using the α-chain cDNA, pBHα-5 (9), as probe. With the exception of a 1500-bp piece of DNA inclusive of exon 8, clones were obtained that spanned the entire mutant α-chain gene. [The presence of exon 8 and its flanking introns in GM2968 was demonstrated by Southern analysis of an *Eco*RI digest of genomic DNA from that cell strain utilizing a 35-base oligonucleotide specific for exon 8 as the probe (data not shown).] Employing 17- and 18-base exon-specific oligonucleotides as primers, I sequenced the promoter region, all of the exons except exon 8 including their 5′ and 3′ splice junction regions, and the polyadenylylation signal region.

These sequences from GM2968 exactly matched those in corresponding areas of the normal α-chain gene (21) with the single exception of a cytidine residue that substituted for a guanosine as the first nucleotide at the 5′ boundary of intron 12 (Figs. 1 and 2). In higher eukaryotes almost all intron sequences adhere to the "G-T/A-G rule" (26)—i.e., they begin at the 5′ end with dinucleotide G-T and terminate with dinucleotide A-G at the 3′ end. Substitutions at these invariant regions can result in mRNA splicing errors (27, 28). The replacement of G-T at the 5′ boundary of exon 12 with dinucleotide C-T is likely to cause aberrant splicing in the α-chain mutant gene. An unstable α-chain message is presumably produced, explaining our inability to detect this message in Ashkenazi Tay–Sachs patients.

Assay for the Splice Junction Mutation. Identification of a splice junction alteration in one patient allowed testing for its presence in other Ashkenazi patients and obligate heterozygote carriers. To rapidly screen patients and carriers, I developed an assay for the splice junction mutation based on the observation that change of a guanosine to a cytidine at the 5′ boundary of intron 12 created a new *Dde* I restriction site in the mutant. Amplification of the region encompassing the mutation by PCR followed by digestion of the PCR product with *Dde* I yielded a 120-bp fragment of DNA in a normal α-chain allele but two fragments (due to the new *Dde* I site) 85 bp and 35 bp long in the mutant allele (Fig. 1*A*). In practice only the 120-bp fragment in the normal and the 85-bp fragment in the mutant is observed because the 41-base ^{32}P-labeled probe utilized in Southern analysis of the samples does not hybridize to the 35-bp fragment (Fig. 1*B*). The alternate assay using allele-specific probes is rapid and, therefore, useful for screening large numbers of heterozygote carriers.

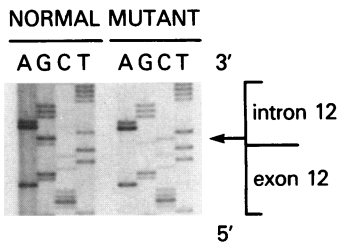

FIG. 2. Nucleotide sequence analysis of the α-chain genes from a normal subject and from an Ashkenazi Jewish patient with classic Tay–Sachs disease in the region surrounding the 5′ boundary of exon 12. DNA sequence analysis was performed with plasmids containing exon 12 of genomic DNA from a normal subject (21) and from patient GM2968 as templates for the sequencing reaction. The primer used was an exon-12-specific oligomer with the sequence 5′ G-T-A-C-C-C-C-T-G-A-G-C-A-G-A-A-G-G 3′. The change of a guanosine to a cytidine at the 5′ border of intron 12 in GM2968 is indicated by the arrow.

Analysis of Patients and Heterozygote Carriers and Noncarriers for Splice Junction Mutation. As expected, the DNA isolated from two normal subjects, an Ashkenazi Jew (D.B.) and a non-Jewish donor (IMR90), was negative for the splice junction mutation (Fig. 3*A*). DNA from a non-Jewish French Canadian patient with classic Tay–Sachs disease (WG107) in which 7.6 kilobases at the 5′ end of the α-chain gene are missing (19) gave a similar result (Fig. 3*A*). Assuming that one mutation gives rise to the classic form of Tay–Sachs disease and knowing that this disease is inherited in an autosomal recessive manner (i.e., both alleles must be mutated for expression of the disorder), I expected DNA from GM2968 and GM515, two cell strains of Ashkenazi patients with classic Tay–Sachs, to be homozygous for the splice junction mutation. However, GM2968, the cell strain originally used to clone the mutant α chain, had only one allele with the splice

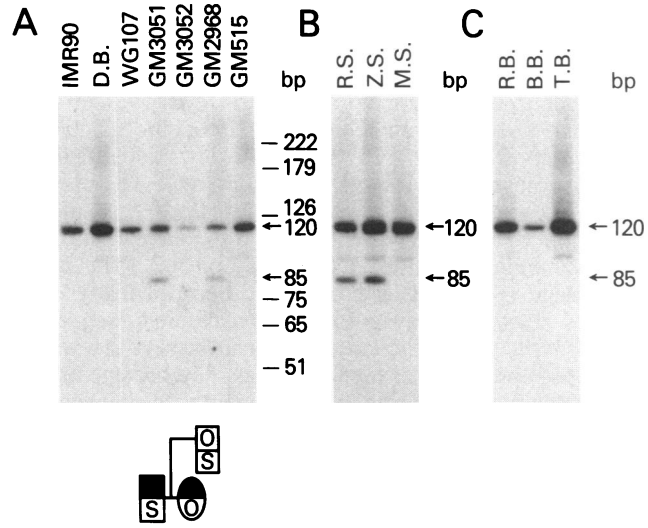

FIG. 3. Assay for the splice junction mutation in various α-chain genotypes. Genomic DNA samples (1 μg) were assayed for the replacement of a guanosine with a cytidine at the 5′ boundary of exon 12 of the α chain of β-hexosaminidase as described in *Materials and Methods* and Fig. 1. (*A*) Lanes: IMR90 (normal), D.B. (Ashkenazi normal), WG107 (non-Jewish French Canadian with classic Tay–Sachs), GM3051 (Ashkenazi obligate heterozygote; father of GM2968), GM3052 (Ashkenazi obligate heterozygote; mother of GM2968), GM2968 (Ashkenazi with classic Tay–Sachs), GM515 (Ashkenazi with classic Tay–Sachs). (*B*) Lanes: R.S. (Ashkenazi with classic Tay–Sachs), Z.S. (Ashkenazi obligate heterozygote; mother of R.S.), M.S. (Ashkenazi obligate heterozygote; father of R.S.). (*C*) Lanes: R.B. (Ashkenazi with classic Tay–Sachs), B.B. (Ashkenazi obligate heterozygote; father of R.B.), T.B. (Ashkenazi obligate heterozygote; mother of R.B.). The schematic below *A* shows the genotypes of GM2968 (affected child), GM3052 (mother), and GM3051 (father). The shaded areas signify the normal α-chain allele. S, splice-junction mutation; O, another mutation.

junction mutation, and GM515 lacked the mutation (Fig. 3A). The simplest explanation for these results is that at least two mutations rather than a single mutation underlie the classic form of Tay–Sachs disease in the Ashkenazi group. GM515 must bear a different α-chain mutation in both alleles, whereas GM2968 bears one allele with the splice junction mutation; the second allele harbors a different mutation. Since the splice junction assay is specific for only that mutation, GM515 appears to have a normal genotype and GM2968 appears to have that of a heterozygote carrier, but both must carry two defective α-chain alleles.

Since GM2968 must inherit the splice junction mutation from one parent and another mutant allele from the second parent, it would be anticipated that only one parent would carry the splice junction mutation. In fact, the genotypes of the parents are consistent with this prediction. GM3051, the father of GM2968, has one allele with the splice junction mutation, whereas GM3052, the mother, does not (Fig. 3A). GM3052 must carry a different mutation not detected by our assay. Fig. 3B demonstrates another family in which the affected child (R.S.) and the mother (Z.S.) exhibit the splice junction mutation, each in one allele; therefore, the father (M.S.) must carry a different mutation. In a third family (Fig. 3C), neither parent carried the splice junction mutation (B.B. and T.B.). Consistent with these findings, the affected child (R.B.) was negative for the splice junction mutation.

In both affected patients and heterozygote carriers (Fig. 3 A and B) the intensity of the band representing the allele bearing the splice junction mutation is less than that of the normal allele. Theoretically, the intensities should be equivalent because each allele contributes 50% of the α-chain gene. This may result from the fact that the band representing the mutant is 35 bp smaller than that of the normal and, therefore, is more easily washed off the nylon membrane in the steps preceding binding to the probe.

To obtain some measure of the frequency of this mutation and its presumed role as a causative agent for classic Tay–Sachs disease among Ashkenazi Jews, we screened DNA isolated from leukocytes of (*i*) 14 additional obligate heterozygotes and (*ii*) 20 Ashkenazi Jews identified via enzymatic assay to be noncarriers for Tay–Sachs disease. None of the noncarriers tested showed the splice-junction alteration. Six of the 20 carriers tested to date exhibited the splice junction mutation, a frequency of 30%. The actual percentage is viewed as preliminary because many more carriers must be assayed to obtain a credible frequency. Assay for the splice junction mutation and assays that will be developed for the other mutation(s) as they became known will be of practical importance for carrier testing in instances in which the enzymatic assay currently used is inconclusive. The dot-blot assay described is rapid and useful for screening large numbers of samples.

This discussion has assumed that the splice junction mutation at the 5′ boundary of intron 12 is a functionally significant defect resulting in aberrant α-chain mRNA and not merely a neutral polymorphism. Examples of β-thalassemias resulting from nucleotide changes at the conserved splice site sequences argues against the latter possibility (28). Substitution of an adenosine for guanosine at the conserved 5′ dinucleotide G-T in the first intron of the β-globin gene (29) and in the second intron of the β-globin gene (30) resulted in abnormal RNA splicing. In addition, mutagenic conversion of the conserved splice junction G-T sequence to A-T in the rabbit globin gene led to aberrant gene transcripts (31).

On the other hand, exceptions to the G-T/A-G rule have been reported and argue for the possibility that the splice junction mutation observed in Ashkenazi Jewish Tay–Sachs patients and heterozygote carriers has no functional consequences. The genes for normal human prothrombin (32) and human adenine phosphoribosyltransferase (33) each contain one G-C rather than G-T donor splice site, and the gene encoding the largest subunit of mouse RNA polymerase II contains two G-C donor splice sites (34). However, if the G-T → C-T change we observe were a neutral polymorphism one would have expected to find it in the Ashkenazi noncarriers, unless the alteration arose on a background of the classical Tay–Sachs mutation and is tightly linked to it. All of the results presented in this study are consistent with this latter possibility. In that case, it would be erroneous to conclude that more than one mutation causes the classic form of Tay–Sachs disease in Ashkenazi Jews from the results of this study. Conclusive data are not available to distinguish between the possible interpretations of the data. Development of an assay for the functional effects of the α-chain splice junction mutation found in Ashkenazi Tay–Sachs patients is necessary to firmly establish its significance.

This study is dedicated to the memory of my father, Calmun Myerowitz. I am grateful to the patients at Long Island Jewish Hospital, Brooklyn, NY and to their families for donating blood and to Mr. Lyndon Badal, who so kindly facilitated transport of those samples to my laboratory. Their cooperation made this study possible. I thank Eugene Grebner for providing leukocyte samples of obligate heterozygote carriers and noncarriers. I thank Richard L. Proia for providing normal α-chain genomic clones, Susan Lontkowski for her assistance in screening the genomic library, and Christina Costigan for her expert technical assistance. I am grateful to April Robbins and William B. Jakoby for careful reading of this manuscript. I thank Valerie King for typing this manuscript with care. This work was supported in part by March of Dimes Birth Defects Foundation Grant 1-1071.

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