Juvenile G\textsubscript{M}2 gangliosidosis is a rare neurodegenerative disorder closely related to Tay-Sachs disease but of later onset and more protracted course. The biochemical defect lies in the \(\alpha\)-subunit of the lysosomal enzyme \(\beta\)-hexosaminidase. Cultured fibroblasts derived from patient A synthesized an \(\alpha\)-subunit which could acquire mannose 6-phosphate and be secreted, but which failed to associate with the \(\beta\)-subunit to form the enzymatically active heterodimer. By contrast, fibroblasts from patient B synthesized an \(\alpha\)-subunit that was retained in the endoplasmic reticulum. To identify the molecular basis of the disorder, DNA from fibroblasts of these two patients was reverse-transcribed, and the cDNA encoding the \(\alpha\)-subunit of \(\beta\)-hexosaminidase was amplified by the polymerase chain reaction (PCR) in four overlapping fragments. The PCR fragments were subcloned and shown by sequence analysis to contain a G to A transition corresponding to substitution of arginine at position 499 or 504 in the \(\alpha\)-subunit of \(\beta\)-hexosaminidase.

The \(\alpha\)-subunit gene. The ArgSo4 + His mutation was found in fragments of DNA corresponding to exon 13 of the \(\alpha\)-subunit gene. The ArgSo4 + His mutation was found in the DNA of a fourth patient. The Arg -\(\rightarrow\) His mutation was found on both alleles of patient A as well as of another unrelated patient; the homozygosity of this mutant allele is attributable to consanguinity in the two families. The Arg\(100\rightarrow\)His mutation was found in patient B in compound heterozygosity with a common infantile Tay-Sachs allele. There is additional heterogeneity in juvenile G\textsubscript{M}2 gangliosidosis, as neither mutation was found in the DNA of a fourth patient. The Arg\(\rightarrow\)His mutations at positions 499 and 504 are located at CpG dinucleotides, which are known to be mutagenic "hot spots."

Recent studies have shown molecular heterogeneity within the \(\beta\)-hexosaminidase A deficiency diseases, the consequence of mutations in the \(\alpha\)-subunit gene (reviewed in Ref. 2). An insertion of 4 nucleotides in exon 11 (3) is the most common mutation among Ashkenazi Jews, accounting for over two-thirds of the carriers (3, 4) or about 2% of that population as a whole. The same population also harbors a splice site mutation in intron 12, but at a lower frequency (5-7). Deletion of exon 1 and its flanking sequences has been found among some French-Canadians (8, 9). These are null mutations, resulting in absence of mature \(\alpha\)-subunit mRNA and lack of synthesis of \(\alpha\)-subunit polypeptide and cause the infantile form of Tay-Sachs disease. Other less common allelic mutations that cause infantile Tay-Sachs disease encode defective polypeptides; they are not associated with any particular demographic group. These mutations include a substitution of lysine for glutamic acid at position 482 (10) and a deletion of cytosine in codon 504 resulting in premature termination (11); in both cases, the \(\alpha\)-subunit is defective in its intracellular transport from the endoplasmic reticulum to the Golgi (11-13). In contrast, the "B1" mutation allows normal processing and transport of the \(\alpha\)-subunit and association into the heterodimeric \(\alpha\) isoenzyme; however, a substitution of histi-
rine for arginine at position 178 (14) abolishes its catalytic activity toward the Gm₂, gangliosidosis (15). Finally, a substitution of serine for glycine at position 269 (16, 17) causes the synthesis of a defective α-subunit that can be transported through the biosynthetic organelles and be secreted, but which fails to associate with the β-subunit (18); this mutation has been found in patients with adult-onset/chronic Gm₂ gangliosidosis, usually in compound heterozygosity with one of the common Ashkenazi Jewish mutations (16, 17), but occasionally in homozygous form (19).

In the present study, we have characterized at the level of polypeptide, mRNA, and DNA the mutations of two patients, Juvenile A and Juvenile B, with enzymatically diagnosed β-hexosaminidase A deficiency and clinically diagnosed Gm₂ gangliosidosis of the juvenile type; for two additional patients, the mutations were examined at the level of DNA only. Data on the biosynthesis of the Juvenile B α-subunit were included in a previous study (18) where her fibroblasts were identified as "California Juvenile." Identification of the molecular defects was achieved by amplification of the entire α-subunit cDNA (after reverse transcription) by the polymerase chain reaction (20) and sequence analysis of the subcloned PCR fragments. Hybridization with allele-specific oligonucleotide probes confirmed the respective mutations in the oligonucleotide probes.

MATERIALS AND METHODS

Clinical Summary—The patient identified here as Juvenile A is of Assyrian origin, and her parents are related as first cousins. She had already developed progressive ataxia, spastic paraplegia, dysarthria, and cherry-red maculae at the time of referral at age 10. Juvenile B, who is of mixed Jewish and Scottish-Irish origin, manifested a progressively severe neurologic deterioration similar to that of her affected brother (21), that began at age 3-5 and progressed until her death at age 26. A third patient, a 12-year-old male, for whom only genomic DNA was examined, is of Armenian extraction. His parents are first cousins; symptoms began at age 4 with clumsiness of hands, followed by progressive ataxia, spasticity, and dementia. A fourth patient is a 4-year-old non-Jewish Caucasian female, with onset of tremors and psychomotor retardation beginning at age 2.

Reagents—Restriction and modifying enzymes were purchased from Promega Biotec, Stratagene, Thermus aquaticus (Taq) and modifi ed T7 (Sequenase) DNA polymerases were from United States Biochemical, and avian myeloblastosis virus reverse transcriptase was from Glycogen (Schleicher and Schuell). N-ethylmaleimide was from Pharmacia LKB Biotechnology Inc. [γ-32P]ATP (1000 Ci/mmol), [γ-32P]dCTP (6000 Ci/mmol), [32P]phosphate (carrier-free), and L-[3,4,5-3H]leucine (150 Ci/mmol) were obtained from Amersham Corp., [α-32P]dATP (1000 Ci/mmol), [α-32P]dCTP (6000 Ci/mmol), and [γ-32P]ATP (700 Ci/mmol) were from ICN. Nitrocellulose membranes were from Schleicher and Schuell and Hybond nylon membranes were from Amersham Corp. The plasmid pGEMZ was from Promega Biotec. Oligonucleotide primers were synthesized by Dr. D. Glitz (UCLA) on a Du Pont/Vega Coder 300 oligonucleotide synthesizer.

Antisera against the β-hexosaminidase A and B isoenzymes and against the normal α-subunit had been raised (22) and characterized (23) previously. Fixed protein A-bearing Staphylococcus aureus cells (Pansorbin) and goat antisera against the polypeptide were purchased from Calbiochem. Fetal bovine serum was from Irvine Scientific and other cell culture reagents were from GIBCO.

Cells and Cell Culture—The strain of normal human fetal lung fibroblasts, IMR 90, was obtained from the Cornell Institute for Medical Research, Camden, NJ. The strain of skin fibroblasts from a patient with juvenile Gm₂ gangliosidosis, designated Juvenile A, was kindly provided by Dr. R. Gatti (UCLA). The strain of fibroblasts from the patient designated Juvenile B was initiated in the laboratory of one of the authors (M. M. K.). Fibroblasts were cultured at 35 °C in 5% CO₂ in Eagle's minimal essential medium supplemented with 105 mM fetal bovine serum, pyruvate, nonessential amino acids, and antibiotics.

Peripheral blood leukocytes from two additional juvenile Gm₂ gangliosidosis patients and their parents, as well as from enzymatically proven noncarriers, were prepared by standard methods (24).

Biosynthesis Studies—Radiolabeling of fibroblasts, immunoprecipitation of β-hexosaminidase and its subunits, electrophoresis, and fluorographic visualization of the polypeptides were carried out as described (23) except for a change in the labeling medium. The medium used was Eagle's minimal essential medium with Earle salts, prepared from a Gibco Selectamine kit to be free of either leucine or phosphate for labeling with [3H]leucine or [32P]phosphate, respectively; it was supplemented with antibiotics, pyruvate, nonessential amino acids, and 5% dialyzed fetal bovine serum.

RNA Preparation and Northern Blot Analysis—Total RNA was isolated from cultured fibroblasts by a published procedure (25). About 20 µg of total RNA was electrophoresed in a 1% agarose, 0.6 M formaldehyde gel and transferred onto a nitrocellulose filter. Radiolabeling (26), blot hybridization (27, 28) with a full-length α-subunit cDNA probe and washing (29) were performed according to standard procedures.

Reverse Transcription and PCR Amplification of cDNA—The sequencing was reverse-transcribed and amplified in four overlapping pieces, using the four sets of oligonucleotide primers identified in group 1, Table 1. About 20 µg of total RNA was incubated for 1 h at 42 °C with 40 units of reverse transcriptase and one set of primers (250 pmol each) at 50 µl of 50 mM Tris, pH 8.3, 50 mM KCl, 10 mM MgCl₂, 1 µM dithiothreitol, 10 ng/mL bovine serum albumin, 0.2 µM each of dNTP. The RNA template was digested with 1.5 µg of RNase A at 37 °C for 30 min. Taq polymerase (1 µl) was added in 50 µl of PCR amplification buffer (42 mM Tris, pH 8.7, 3.7 mM MgCl₂, 17 mM (NH₄)SO₄, 10 mM 2-mercaptoethanol, 0.17 µg/µl bovine serum albumin, 10% dimethyl sulfoxide (v/v)). PCR amplification of the cDNA was performed in a Perkin Elmer-Cetus DNA Thermal Cycler using the following profile: 1-min denaturation at 94 °C, 2-min annealing at 48 °C, 4-min extension at 72 °C for 40 cycles.

Subcloning and Plasmid Sequencing—The products of PCR amplification were purified by electrophoresis in a 5% nondenaturing polyacrylamide gel. Eluted DNA fragments were sequentially 5'-phosphorylated with T4 polynucleotide kinase, treated with T4 DNA polymerase, and blunt-end-ligated into the dephosphorylated Smal restriction site of pGEM3Z. The plasmid DNA was sequenced by the dideoxy method (31) with Sequenase as recommended by the manufacturer.

Isolation and PCR Amplification of Genomic DNA—High molecular weight genomic DNA was isolated from fibroblasts and leukocytes as described (39). The DNA from relatives of Juvenile A was provided by Dr. R. Gatti. Genomic DNA (1 µg) was annealed to 500 pmol each of sense and antisense primers (Table I, group 2) and amplified with 2.5 units of Taq polymerase in 100 µl of 67 mM Tris, pH 8.1, 6.1 mM MgCl₂, 17 mM (NH₄)SO₄, 10 mM 2-mercaptoethanol, 0.17 µg/µl bovine serum albumin, 10% dimethyl sulfoxide, 0.2 mM each dNTP. Amplification was carried out for 35 cycles as follows: 2-min denaturation at 94 °C, 2-min annealing at 48 °C, and 3-min extension at 72 °C. The PCR products were purified by phenol/chloroform extraction and ethanol precipitation.

Allele-Specific Oligonucleotide Hybridization—A minor modification of a published procedure (35) was used. About 10 ng of genomic PCR products were dotted onto Hybond nylon membrane in a Schleicher and Schuell dot blot manifold and hybridized with oligonucleotide probes listed in Table I, group 3. Filters were washed as described (33) at 54 °C and subjected to autoradiography.

RESULTS

Defective α-Subunits Identified by Biosynthetic Studies—As shown in Fig. 1, fibroblasts of Juvenile A synthesized as much α-subunit in a 3-h labeling period as did IMR 90 control fibroblasts (panel A, "pulse" lanes). But in contrast to the normal α-subunit, the patient's α-subunit was not processed proteolytically to the mature form and disappeared almost entirely from the cell during overnight incubation (panel A, "chase" lanes); it was not found extracellularly (not shown) and was presumed to have been degraded. Normal synthesis and maturation of the β-subunit served as internal control in these experiments. The Juvenile A α-subunit could undergo a late posttranslational event, acquisition of the mannose 6-phosphate marker, in normal fashion (panel B, 32P lanes). Secretion of the Juvenile A α-subunit could be induced by the presence of 10 mM NH₄Cl in the medium; as shown by use of
TABLE I

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<th>Synthetic oligonucleotides</th>
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<tr>
<td>Segments covered</td>
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<tr>
<td>Group 1: primers for reverse transcription of mRNA and PCR amplification of cDNA</td>
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<tr>
<td>Exons 1–5</td>
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<tr>
<td>Exons 5–9</td>
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<td>Exons 8–11</td>
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<td>Group 2: primers for PCR amplification of genomic DNA</td>
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<td>Introns 12–13</td>
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<td>Exon 11–Intron 12</td>
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<tr>
<td>Group 3: allele-specific oligonucleotides</td>
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<td>Arg506 → His (Juvenile A)</td>
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<tr>
<td>Arg509 → His (Juvenile B)</td>
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<tr>
<td>Exon 11 Insertion (Tay–Sachs)</td>
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Fig. 1. Synthesis of the α-subunit by fibroblasts derived from juvenile GM2 gangliosidosis patient A. Normal (IMR 90) and Juvenile A confluent fibroblast cultures in 100-mm Petri dishes were labeled with 0.25 mCi of [3H]leucine/dish for a 3-h pulse, followed by an 18-h chase (panel A) and for the same period of pulse and chase in the presence of 10 mM NH4Cl (panel C). In panel B, the cells were labeled for 3 h with 0.25 mCi of [3H]leucine or 1.0 mCi of [32P]phosphate, as indicated. Antiserum raised against β-hexosaminidase A was used for immunoprecipitation from cell extracts, shown in panels A and B. Panel C shows immunoprecipitation from NH4Cl-induced secretions using three different antisera, anti-A, anti-B, and anti-α, as indicated. The antisera precipitate: A, all forms of the α- and β-subunits; B, α-subunit that is associated with β-α, as well as all forms of the β-subunit; α, only monomeric α-subunit. Abbreviations: αp, βp = precursor α-, β-subunit; αm, βm = mature α-, β-subunit.

Specific antisera, the secreted Juvenile A α-subunit was a monomer, in contrast to the normal α-subunit which was associated with the β-subunit (panel C).

The fibroblasts of Juvenile B had been shown previously to synthesize an abnormal α-subunit that did not associate with the β-subunit (18); this finding has been confirmed (not shown). Fig. 2 shows that this abnormal α-subunit was not processed to the mature form and disappeared during an overnight chase (panel A, chase lanes). Unlike its counterpart in Juvenile A, the α-subunit of Juvenile B did not acquire the mannose 6-phosphate marker (panel A, 32P lanes), nor was it secreted from the cells in the presence of NH4Cl (panel B).

Identification of the Mutations in α-Subunit cDNA—The α-subunit mRNA from fibroblasts of the two index patients was found by Northern analysis to be of normal size (Fig. 3, left panel). The amount of α-subunit mRNA was found to be normal in fibroblasts of Juvenile A but reduced in fibroblasts of Juvenile B. The β-subunit mRNA served as an internal control (Fig. 3, right panel).

The entire α-subunit mRNA was reverse-transcribed and amplified by PCR in four overlapping fragments. Sequence analysis of the subcloned PCR fragments from the mRNA of Juvenile A revealed a single change from the normal: a G to A transition at nucleotide position 1511, resulting in substitution of histidine (CAC) for arginine (CGC) at codon 504
amplified DNA was analyzed by allele-specific oligonucleotide hybridization. As shown in Fig. 5, Juvenile A, whose parents are related to each other, had inherited the Arg499 → His mutation in homozygous fashion. The identical mutation was found in an unrelated juvenile Ga, gangliosidosis patient, whose parents are also consanguinous. The amplified DNA from the parents of both patients hybridized to both normal and mutant probes as expected for obligate heterozygotes (Fig. 5). Amplified DNA from 63 enzymatically proven noncarriers of β-hexosaminidase deficiency hybridized solely to the normal oligonucleotide probe, confirming that this mutation was not a neutral polymorphism (data not shown).

The PCR fragment from the DNA of Juvenile B hybridized to both the normal and mutant oligonucleotides for codon 499, showing her to be a compound heterozygote for the Arg499 → His mutation (Fig. 5). This mutation was inherited from her mother; the other allele, inherited from her Jewish father, carried the more common 4-base pair insertion in exon 11 that underlies infantile Tay-Sachs disease. The reduced amount of α-subunit mRNA (Fig. 3) is consistent with compound heterozygosity with a null allele. Amplified DNA from 63 noncarriers did not hybridize to the Arg499 → His oligonucleotide probe, indicating that this change likewise is not a polymorphism (not shown). The genomic DNA of Juvenile B was also tested for base changes observed in some of the subcloned PCR fragments (see above), using oligonucleotides designed specifically for that purpose. In all instances, the genomic DNA was found to hybridize only to the normal oligonucleotide, confirming that the changes which had been observed in occasional clones were the result of Taq polymerase errors (not shown). Amplified DNA from the fourth juvenile Ga gangliosidosis patient failed to hybridize to mutant probes at codon 499 or 504 (data not shown).

Identification of the Mutations in Genomic DNA—To demonstrate the nucleotide substitutions in genomic DNA isolated from the probands and their relatives, the exon 13 region encompassing the mutations was amplified by PCR, and the amplified DNA was analyzed by allele-specific oligonucleotide hybridization. As shown in Fig. 5, Juvenile A, whose parents are related to each other, had inherited the Arg499 → His mutation in homozygous fashion. The identical mutation was found in an unrelated juvenile Ga, gangliosidosis patient, whose parents are also consanguinous. The amplified DNA from the parents of both patients hybridized to both normal and mutant probes as expected for obligate heterozygotes (Fig. 5). Amplified DNA from 63 enzymatically proven noncarriers of β-hexosaminidase deficiency hybridized solely to the normal oligonucleotide probe, confirming that this mutation was not a neutral polymorphism (data not shown).

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Fig. 4. Identification of the juvenile Ga gangliosidosis mutations in cDNA. Total RNA (20 µg) from the normal (IMR 90) and patients' fibroblasts was subjected to Northern blot analysis. The RNA blot was first hybridized with an α-subunit cDNA probe (left) and then stripped and rehybridized with a β-subunit cDNA probe (right). (Fig. 4, upper panel). This mutation was found in five independently isolated clones. The PCR products derived from mRNA of Juvenile B had a G to A transition at nucleotide position 1496, resulting in substitution of histidine (CAT) for arginine (CGT) at codon 499 (Fig. 4, lower panel). This mutation was found in three independently isolated clones. The nucleotide changes were confirmed by sequencing the opposite strand (not shown). Additional base changes were observed in some of the clones derived from Juvenile B, but since each one was found in a solitary clone, they were attributed to errors introduced during amplification by Taq polymerase; this was later confirmed by oligonucleotide hybridization (see below).

Identification of the Mutations in Genomic DNA—To demonstrate the nucleotide substitutions in genomic DNA isolated from the probands and their relatives, the exon 13 region encompassing the mutations was amplified by PCR, and the amplified DNA was analyzed by allele-specific oligonucleotide hybridization. As shown in Fig. 5, Juvenile A, whose parents are related to each other, had inherited the Arg499 → His mutation in homozygous fashion. The identical mutation was found in an unrelated juvenile Ga, gangliosidosis patient, whose parents are also consanguinous. The amplified DNA from the parents of both patients hybridized to both normal and mutant probes as expected for obligate heterozygotes (Fig. 5). Amplified DNA from 63 enzymatically proven noncarriers of β-hexosaminidase deficiency hybridized solely to the normal oligonucleotide probe, confirming that this mutation was not a neutral polymorphism (data not shown).

The PCR fragment from the DNA of Juvenile B hybridized to both the normal and mutant oligonucleotides for codon 499, showing her to be a compound heterozygote for the Arg499 → His mutation (Fig. 5). This mutation was inherited from her mother; the other allele, inherited from her Jewish father, carried the more common 4-base pair insertion in exon 11 that underlies infantile Tay-Sachs disease. The reduced amount of α-subunit mRNA (Fig. 3) is consistent with compound heterozygosity with a null allele. Amplified DNA from 63 noncarriers did not hybridize to the Arg499 → His oligonucleotide probe, indicating that this change likewise is not a polymorphism (not shown). The genomic DNA of Juvenile B was also tested for base changes observed in some of the subcloned PCR fragments (see above), using oligonucleotides designed specifically for that purpose. In all instances, the genomic DNA was found to hybridize only to the normal oligonucleotide, confirming that the changes which had been observed in occasional clones were the result of Taq polymerase errors (not shown). Amplified DNA from the fourth juvenile Ga gangliosidosis patient failed to hybridize to mutant probes at codon 499 or 504 (data not shown).
Molecular Basis of Juvenile G\textsubscript{M2} Gangliosidosi

Discussion

We have identified mutations of the \( \alpha \)-subunit gene of \( \beta \)-hexosaminidase, guanine to adenine transitions in exon 13, that are present on one or both alleles of patients with juvenile G\textsubscript{M2} gangliosidosis. Two unrelated patients were shown to be homozygous for the Arg\textsuperscript{504} \( \rightarrow \) His mutation. In both families, the parents are consanguinous. Another patient was shown to be a compound heterozygote for the Arg\textsuperscript{499} \( \rightarrow \) His allele with the exon 11 infantile Tay-Sachs allele that is common in the Ashkenazi Jewish population. The absence of either substitution in a fourth patient shows that yet other mutations may give rise to juvenile G\textsubscript{M2} gangliosidosis.

The defective \( \alpha \)-subunit of Juvenile A (Arg\textsuperscript{504} \( \rightarrow \) His) acquires the mannose 6-phosphate recognition marker but fails to associate with the \( \beta \)-subunit and is not processed to the mature lysosomal form (presumably because in the absence of association, it is not transported to lysosomes (25)). In the presence of NH\(_4\), which diverts newly made hydrolysases from the lysosomal to the secretory pathway (22, 24), the monomeric \( \alpha \)-subunit of Juvenile A is secreted. This shows that it can be transported out of the endoplasmic reticulum and Golgi complex. Overall, the abnormal \( \alpha \)-subunit of Juvenile A resembles the association-defective \( \alpha \)-subunit (18) caused by the Glu\textsuperscript{492} \( \rightarrow \) Lys mutation in the \( \beta \)-subunit gene and in intron 12 of the \( \beta \)-subunit gene (39). Cpg sites are known to be mutated “hot spots,” accounting for a disproportionate number of human polymorphisms (40) as well as of disease-producing mutations (e.g., of hemophilia due to deficiency of Factor VIII (41–43) or Factor IX (44)). Such mutations reflect the tendency of 5-methylcytosine, frequently found at Cpg sites, to deaminate spontaneously to thymidine (45). Depending on the DNA strand on which the deamination occurs, the result is either a C to T or a G to A transition on the sense strand. Such mutations occur sporadically, and the same base change in different individuals can be the result of separate mutational events, as has been observed in Factor VIII (43); thus the Arg\textsuperscript{504} \( \rightarrow \) His mutation might have occurred independently in the two kindreds in which it was observed. A two-step repair system that excises the mismatched TMP and fills the gap with CMP has recently been described in mammalian cells (46); the deletion of cytosine at the Cpg site in codon 504 (11) could have resulted from an error in the second phase of this repair process. Since the coding sequences of the two subunits of \( \beta \)-hexosaminidase each contain about three dozen Cpg dinucleotide sites, additional mutations can be anticipated, even though not all the sites would be methylated and many of the mutations would be silent.

Acknowledgments—We thank Dr. Richard Gatti (University of California, Los Angeles) for the fibroblast culture of patient Juvenile A and DNA samples from the family, Dr. Elena Boder (University of California, Los Angeles) for information about that patient, Dr. Robert Carrel (Santa Barbara Regional Center) for referral of patient Juvenile B and her family, Dr. Ira Lott (University of California, Irvine) for referral of the Armenian family, Drs. Rachel Myerowitz and Richard Proia (National Institutes of Health) for recombinant plasmids containing \( \beta \)-hexosaminidase \( \alpha \)-subunit and \( \beta \)-subunit cDNA, Dr. Dohm Giltz (University of California, Los Angeles) for oligonucleotide synthesis, Doris Quon for help with some of the biosynthetic studies, and Larry Tabata for illustrations.

Note Added in Proof—The Arg\textsuperscript{504} \( \rightarrow \) His mutation was independently discovered by R. M. Boustany and K. Suzuki (personal communication) in a Juvenile G\textsubscript{M2} gangliosidosis patient from a consanguinous Lebanese Christian family.

References

Molecular Basis of Juvenile GM2 Gangliosidosis

Juvenile GM2 gangliosidosis caused by substitution of histidine for arginine at position 499 or 504 of the alpha-subunit of beta-hexosaminidase.
B H Paw, S M Moskowitz, N Uhrhammer, N Wright, M M Kaback and E F Neufeld