Identification and Expression of a Common Missense Mutation (L302P) in the Acid Sphingomyelinase Gene of Ashkenazi Jewish Type A Niemann-Pick Disease Patients

By Orna Levran, Robert J. Desnick, and Edward H. Schuchman

Types A and B Niemann-Pick disease (NPD) result from the deficient activity of acid sphingomyelinase (ASM; EC 3.1.4.12) and the resultant lysosomal accumulation of sphingomyelin. Type A disease is a fatal, neurodegenerative disorder of infancy, whereas type B disease has no neurologic manifestations and is characterized primarily by reticuloendothelial involvement and survival into adulthood. Both disorders occur more frequently among individuals of Ashkenazi Jewish ancestry than in the general population. Recently, a missense mutation in the ASM gene (designated R496L) was detected in more than 30% of the ASM alleles from Ashkenazi Jewish type A NPD patients. We report a second, common mutation that resulted from a T to C transition at nucleotide 905 and predicted a leucine to proline substitution at ASM codon 302 (designated L302P). Notably, the L302P mutation occurred in 23.5% (8 of 34) of the Ashkenazi Jewish type A NPD alleles studied. In contrast, it was not found in any of the ASM alleles from non-Jewish type A patients, in 36 alleles from type B patients, or in 100 ASM alleles from normal Ashkenazi Jewish individuals. To confirm the authenticities of the L302P and R496L mutations, each nucleotide change was separately introduced into the full-length ASM cDNA by site-directed mutagenesis and transiently expressed in COS-1 cells. Neither mutation expressed ASM catalytic activity, consistent with the type A phenotype of homoallelic patients. The identification of the L302P mutation should further facilitate molecular carrier detection for NPD in the Ashkenazi Jewish population, particularly because the L302P mutation can be easily detected using the restriction enzyme, AlwNI.

Niemann-Pick Disease (NPD) is an inborn error of sphingomyelin catabolism that results from the deficient activity of the lysosomal hydrolase, acid sphingomyelinase (ASM; sphingomyelin phosphodiesterase; EC 3.1.4.12). Two allelic forms of this autosomal recessive disorder have been delineated based on their distinct phenotypes. Type A NPD is a severe, neurodegenerative disorder of infancy characterized by progressive psychomotor retardation, hepatosplenomegaly, and death by 3 years of age. In contrast, type B NPD is a nonneuronopathic disorder characterized by hepatosplenomegaly, respiratory involvement, and survival into adulthood. The clinical course of patients with type B NPD is quite variable, and some mildly affected patients may survive into the sixth decade of life. Both types occur more frequently among individuals of Ashkenazi Jewish descent (1/40,000) than in the general population. However, mass screening for carrier detection in this population has been precluded because of the marked overlap of ASM enzymatic activities in leukocytes and cultured cells from obligate heterozygotes and normal individuals.

To date, biochemical investigations into the genetic heterogeneity underlying the neuronopathic type A and nonneuronopathic type B phenotypes have been limited. In general, type A NPD patients tend to have slightly lower residual activities in crude cell and tissue homogenates than type B patients using various substrates and analogues for assay. However, the residual activities have not been well characterized, nor have the physiokinetic properties from type A and B patients been compared. Limited immunologic studies showed that patients with both subtypes have cross-reactive immunologic material in crude cell homogenates, although the amounts of enzyme protein have not been determined.

Recently, investigations into the molecular nature of the genetic heterogeneity in NPD have been facilitated by the isolation and characterization of the full-length cDNA encoding human ASM. The 2,347-bp cDNA contained an open reading frame of 1,890 bp encoding a polypeptide of 629 amino acid residues. The availability of this cDNA permitted the localization of the ASM gene to the narrow chromosomal region 11p15.1 to 15.4 by the use of somatic cell hybrids and in situ hybridization techniques. Studies have also been initiated to determine the nature of the genetic lesions causing types A and B NPD. By sequencing polymerase chain reaction (PCR)-amplified ASM cDNAs from a type A patient of Ashkenazi Jewish descent, a single G to T transversion was identified that predicted an arginine to leucine substitution in residue 496 of the ASM polypeptide (designated R496L). The R496L mutation was found in about one third (ie, ~32%) of the Ashkenazi Jewish type A NPD alleles studied. In contrast, only about 5% of the alleles from non-Jewish type A patients had this mutation. Some type A patients were homoallelic for the R496L mutation, whereas others were heteroallelic, indicating the occurrence of other ASM mutations that caused the type A phenotype. Interestingly, the R496L mutation also was detected in one of the two Ashkenazi Jewish type B patients studied. The other ASM allele in this mildly affected patient had a 3-base deletion that resulted in the

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absence of an arginine residue at position 608 of the ASM polypeptide (designated ΔR608).19

We report the identification of a second, common point mutation that occurred in more than 20% of the ASM alleles studied from Ashkenazi Jewish type A NPD patients. This T to C transition, which predicted a leucine to proline substitution at residue 302 of the ASM polypeptide (designated L302P), was not identified in any of the non-Jewish type A NPD alleles studied. In addition, it was not identified in any of the 36 alleles studied from Jewish or non-Jewish type B patients. Transient expression studies showed that neither the L302P nor the R496L mutation expressed catalytically active ASM, consistent with the fact that homoallelic expression of either of these mutations results in the neuronopathic type A NPD phenotype.

MATERIALS AND METHODS

Cell lines. Primary cultures of fibroblasts and lymphoblasts were established from skin biopsies and peripheral blood samples obtained with informed consent from NPD patients and family members, and from normal individuals. NPD lines GM00165, GM00370, GM00406, GM00559, GM02895, and GM03252 were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Cell lines 444X.F01, 534R.F03, 556X.F01, 888V.F01, 2789X.F01, 4293Q.E02, 5113C.L01, 5115E.F01, and 6791M.F01 were obtained from the Service de Biochimie, Hospice de Lyon (Lyon, France). Cell lines DMN 83.126, DMN 84.135, DMN 84.87, DMN 86.49, DMN 87.71, DMN 87.99, DMN 88.12, DMN 88.9, and RNS were provided by Dr Peter Penchev (Developmental and Metabolic Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, MD). Cell lines AR (proband 4; GM00112A) and SL (proband 5) were provided by Dr Elaine Zakai (Children's Hospital, Philadelphia, PA) and Dr Gideon Bach (Hebrew University-Hadassah Medical School, Jerusalem, Israel), respectively. The cells were grown in RPMI 1640 media supplemented with 10% fetal bovine serum, 1% penicillin, and 1 mg/mL streptomycin by standard procedures. The diagnosis of type A or B NPD was based on clinical criteria (eg, age at onset, presence of neurologic involvement) and by the demonstration of markedly deficient ASM activity in cultured cells.8

Identification of the L302P mutation. To identify the ASM mutation(s) in an Ashkenazi Jewish type A NPD patient, proband 4, PCR amplification20 was performed on genomic DNA isolated from cultured skin fibroblasts.21 The complete ASM coding region, including the intron/exon boundaries, was amplified in four fragments using the PCR primer pairs shown in Table 1 (primers P1 to P8). PCR amplifications were performed for 30 cycles with Taq polymerase (Promega, Madison, WI). Each amplification cycle consisted of denaturation for 1 minute at 94°C, annealing for 1 to 2 minutes at temperatures varying from 60°C to 72°C, and extension for 1 to 2 minutes at 72°C. For PCR product 1, which included exon 1 and 632 bp of exon 2, a PCR “boost” procedure was required and a slightly modified PCR buffer was used.17 After PCR amplification, the PCR products were precipitated by isopropanol in the presence of ammonium acetate (final concentration, 2.5 mol/L), and subcloned into the PCR-1000 vector (Invitrogen, San Diego, CA). For each ligation, from four to six independent subclones were sequenced by the dyeodeoxy nucleotide chain termination method.22

Confirmation of the L302P mutation by dot blot hybridization and restriction enzyme analyses. For detection of the L302P mutation in other NPD patients, their family members, and normal individuals, dot blot hybridization analyses23 were performed on a 570-bp genomic fragment amplified from cell lysates using PCR primers P3 and P4 (Table 1). PCR amplification was performed for 30 cycles, each consisting of denaturation for 1 minute at 94°C, annealing for 1.5 minutes at 62°C, and extension for 1.5 minutes at 72°C. Dot blot hybridization was performed using Zetabind nylon membranes (AMF-Cuno, Richmond, CA) and a BioRad dot-blot apparatus (Richmond, CA). Hybridizations were performed by standard techniques using the normal and mutant allele-specific oligonucleotides (ASOs), (5'-GTCACAGACCTGCTGAG-3') and (5'-GTCACAGACCTGCTGAG-3'), respectively, with the mutated nucleotide underlined. After hybridization, the blots were

<table>
<thead>
<tr>
<th>Primer</th>
<th>Amplified Region</th>
<th>Fragment (bp)</th>
<th>Sequence (5'-3')</th>
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<tr>
<td>P1 (sense)</td>
<td>Exons 1 and 2</td>
<td>1,443</td>
<td>TACATCTCGAGACGGCGAGCACCAGCAACCA</td>
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<td>Exon 2</td>
<td>570</td>
<td>AGTAGCTGAGACCCAGGCTACCTGAGCTTG</td>
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<tr>
<td>P3 (antisense)</td>
<td>Exons 3-5</td>
<td>969</td>
<td>AGTAGTGACCGCTACTGAGCAATCAG</td>
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<tr>
<td>P5 (sense)</td>
<td>Exon 6</td>
<td>569</td>
<td>AGTAGTGACCGCTACTGAGCAATCAG</td>
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<tr>
<td>P6 (antisense)</td>
<td>Exon 1</td>
<td>320</td>
<td>TACATCTCGAGACGGCGAGCACCAGCAACCA</td>
</tr>
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<td>P7 (sense)</td>
<td>nt 611-913</td>
<td>432</td>
<td>AGAGCTCACAGAGCTGAGGCTACCTGAG</td>
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<td>P9 (sense)</td>
<td>nt 898-1330</td>
<td>356</td>
<td>CAATATGAATTmGTT</td>
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<tr>
<td>P10 (antisense)</td>
<td>nt 1139-1495</td>
<td>496</td>
<td>GGTACACAAGGTAACCAGGATTAAG</td>
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<tr>
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<td>569</td>
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<tr>
<td>P12 (sense)</td>
<td>nt 1478-1974</td>
<td>496</td>
<td>AGTAGTGACCGCTACTGAGCAATCAG</td>
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</tbody>
</table>

Underlining indicates mutated nucleotides.
washed at room temperature for 15 minutes in 6× SSC containing
0.1% sodium dodecylsulfate (SDS), and then in the same solution
for 1 hour at 50°C.

The L302P mutation also could be detected by restriction
enzyme analysis because the T to C transition introduced an AlwNI
site into the ASM gene. The 570-bp ASM genomic fragment was
amplified using PCR primers P3 and P4. After amplification, the
fragment was digested with AlwNI and electrophoresed on a 1.5%
agarose gel. Only the mutant L302P allele was digested, resulting in
cleavage of the 570-bp PCR product into two fragments of 280 and
290 bp.

Site-directed mutagenesis. To evaluate the effects of the L302P
and R496L mutations on ASM catalytic activity, each mutation was
introduced into the full-length ASM cDNA using the PCR-overlap
mutagenesis technique.23,24 As shown in Fig 1 for the L302P
mutation, two different PCR amplifications were performed. In the
first reaction, two overlapping fragments were independently
amplified from the full-length ASM cDNA, pASM-3,16 using PCR
primer sets P9 and P10 and P11 and P12, respectively. After PCR
amplification, the fragments were purified from agarose gels and
combined for use as the template in a second PCR amplification
using sense and antisense PCR primers P9 and P12, respectively.
Note that primers P9 and P12 overlapped unique Sfi I and Bcl I
sites in the pASM-3 cDNA, respectively. PCR amplifications were
performed for 20 to 25 cycles, each consisting of denaturation for
1 minute at 94°C, annealing for 1 to 2 minutes at temperatures
varying from 45°C to 65°C, and extension for 1 to 2 minutes at 72°C.
After amplification, the 734-bp PCR product was subcloned into
the PCR-1000 vector and sequenced to confirm the presence of the
L302P mutation and to determine that no other mutations had
been introduced into the ASM cDNA fragment. To construct the
full-length ASM cDNA containing the L302P mutation, the mu-
tated Sfi I-BcI I fragment was isolated from the PCR-1000 subclone
and inserted into the pASM-3 cDNA by ligation exchange. The
mutated full-length cDNA was then resequenced to ensure that the
only mutation was L302P. To perform the methylation sensitive Bcl

![Fig 1. Construction of the p91023(B) expression vector containing the L302P mutation. The methods used for introduction of the L302P mutation into the full-length pASM-3 cDNA are described in the text. The positions of the EcoRI, Sfi I, and Bcl I restriction sites are indicated, as are the locations and orientations of the PCR primers (P9-P12).](image-url)
were electroporated in the presence of buffer alone. The trans-
were inserted into the eukaryotic expression vector p91023(B).25*26
496-bp products, respectively. Primers P13 and P16, which over-
sequencing are described in the text. A small area of the genomic
sequence obtained from
on ASM catalytic activity, the mutated full-length ASM cDNAs
were introduced into COS-1 cells by
denegative control for the transient expression studies, COS-1 cells
fected cells were harvested after 72 hours and ASM activities were
fected, Ashkenazi Jewish type A NPD patient, proband
4, did not have an R496L allele,16 this Ashkenazi Jewish type A patient was selected as a candidate for identifying new type A NPD mutations. The ASM coding sequence was PCR-amplified from genomic DNA obtained from proband 4, and the four fragments containing the entire ASM coding region, including the intron/exon boundaries, were subcloned and sequenced. Figure 2A shows the DNA sequence obtained from a region of exon 2; note that proband 4 had a T to C transition at nucleotide 905 in each of the six sequenced subclones. This single-base change predicted a leucine to proline substitution in codon 302 of the ASM polypeptide (designated L302P).

To confirm the authenticity of the L302P missense mutation, dot blot hybridization analysis was performed on genomic DNA obtained from proband 4 and his family members (Fig 2B). These studies showed that proband 4 was homoallelic for the L302P mutation and that his phenotypically normal brother, sister, and maternal grandfather were heterozygous.

I digest, the PCR-1000 subclone containing the mutated fragment, and a Bluescript (Stratagene, La Jolla, CA) subclone containing the pASM-3 cDNA were grown in the dam− Escherichia coli strain GM2163.

The full-length ASM cDNA containing the R496L mutation was constructed by essentially the same method with the following modifications. For the first PCR amplification, primer sets P13 and P14 and P15 and P16 (Table 1) were used to amplify 356- and 496-bp products, respectively. Primers P13 and P16, which overlapped unique BclI and SphI sites, respectively, were then used for the second PCR amplification.

Transient expression of the L302P and R496L mutations in COS-1 cells. To evaluate the effects of the L302P and R496L mutations on ASM catalytic activity, the mutated full-length ASM cDNAs were inserted into the eukaryotic expression vector p91023(B).25,26 DNA (15 to 20 μg) from each construct, as well as the wild-type, full-length pASM-3 cDNA, were introduced into COS-1 cells by electroporation using a BioRad electroporation apparatus. As a negative control for the transient expression studies, COS-1 cells were electroporated in the presence of buffer alone. The transfected cells were harvested after 72 hours and ASM activities were
determined using the fluorogenic natural substrate, [N-11(1-
pyrenesulfonyl)[amido decanoyl]sphingomyelin (PSA11-sphingo-
myelin], as previously described.9 One unit of activity equals that amount of enzyme that hydrolyzes one nanomole of substrate per hour. Protein determinations were performed by a modified fluorescamine assay.27

RESULTS

Identification of the L302P mutation in proband 4. Because previous dot blot hybridization studies had shown that proband 4 did not have an R496L allele,16 this Ashkenazi Jewish type A patient was selected as a candidate for identifying new type A NPD mutations. The ASM coding sequence was PCR-amplified from genomic DNA obtained from proband 4, and the four fragments containing the entire ASM coding region, including the intron/exon boundaries, were subcloned and sequenced. Figure 2A shows the DNA sequence obtained from a region of exon 2; note that proband 4 had a T to C transition at nucleotide 905 in each of the six sequenced subclones. This single-base change predicted a leucine to proline substitution in codon 302 of the ASM polypeptide (designated L302P).

To confirm the authenticity of the L302P missense mutation, dot blot hybridization analysis was performed on genomic DNA obtained from proband 4 and his family members (Fig 2B). These studies showed that proband 4 was homoallelic for the L302P mutation and that both of his parents, a phenotypically normal sister and brother, as well as the maternal grandfather were heterozygous. The L302P mutation, which introduced a unique AlwNI restriction site into the ASM gene, was not identified in more than 100 ASM alleles from normal Ashkenazi Jewish individuals, indicating that this nucleotide change was not a polymorphism in this population. Further confirmation of the authenticity of the L302P mutation was obtained by sequencing the PCR-amplified ASM coding region from an unrelated, Ashkenazi Jewish type A NPD patient, proband 5 (not shown). This patient also was homoallelic for the L302P mutation.

Occurrence of the L302P mutation in types A and B NPD. Table 2 shows the frequency of the L302P mutation in the ASM alleles of other NPD patients and obligate heterozygotes, as determined by dot blot or AlwNI restriction enzyme analysis of PCR-amplified genomic DNA. Notably, this mutation occurred in about 23.5% (8 of 34) of the ASM

<table>
<thead>
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<th>Source</th>
<th>Mutant Alleles Studied</th>
<th>% of NPD Alleles</th>
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<tbody>
<tr>
<td></td>
<td>L302P</td>
<td>R496L</td>
</tr>
<tr>
<td>NPD type A</td>
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<td>Ashkenazi Jewish</td>
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<tr>
<td>Non-Jewish</td>
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<tr>
<td>NPD type B</td>
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<td>Ashkenazi Jewish</td>
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</tr>
<tr>
<td>Non-Jewish</td>
<td>32</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table 2. Frequency of the L302P and R496L Mutations in Ashkenazi Jewish and Non-Jewish Families With Types A and B Niemann-Pick Disease
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alleles studied from Ashkenazi Jewish type A NPD patients, whereas it was not found in 43 alleles studied from non-Jewish type A patients. In addition, the L302P mutation was not identified in 36 alleles studied from Jewish and non-Jewish type B NPD patients.

**Transient expression of the L302P and R496L mutations.** To confirm the authenticities of the L302P and R496L mutations, each base substitution was introduced into the full-length ASM cDNA by the PCR-overlap method and transiently expressed. Table 3 shows the ASM activities expressed in COS-1 cells using the fluorogenic natural substrate, PSA11-sphingomyelin.8 Untransfected COS-1 cells had a mean activity of 7.8 nmol/hr/mg. In comparison, COS-1 cells transfected with the full-length ASM cDNA, pASM-3, had a mean activity of 23.6 nmol/hr/mg. Notably, neither mutant construct expressed catalytically active ASM, consistent with the fact that A NPD patients homoallelic for the L302P or R496L mutations had less than 1% residual ASM activity in cultured cells.18,38

**DISCUSSION**

Previously, a missense mutation, R496L, was reported as the first lesion in the ASM gene causing type A NPD.18 This mutation occurred in more than 30% of the mutant alleles from Ashkenazi Jewish type A NPD patients. In this report, a second, common mutation (L302P) was identified in Ashkenazi Jewish type A NPD patients. Transient expression in COS-1 cells showed that the L302P and R496L amino acid substitutions altered the ASM polypeptide such that the residual enzymes had no detectable catalytic activity, consistent with the fact that homoaallelism for either of these mutations leads to the severe type A NPD phenotype. These amino acid substitutions may result in the production of unstable residual enzymes that are rapidly degraded within the cell or inefficiently targeted to the lysosomes. In fact, it is likely that the L302P substitution causes incorrect folding of the ASM polypeptide because it occurs within an α-helix region, and the introduction of proline residues often leads to the formation of β-turns.29 Alternatively, the residual enzymes may be stable and properly targeted to the lysosomes but remain catalytically inactive because of an altered active and/or substrate binding site. Further characterization of the proteins expressed from these mutant alleles will require immunologic studies using monospecific antihuman ASM antibodies that are not currently available.

The L302P and R496L mutations are responsible for more than 50% of the alleles causing type A NPD in the Ashkenazi Jewish population. Whether one additional major mutation or a series of less frequent mutations will account for the remainder of the lesions causing this disorder must await further analyses. However, evidence from three other disorders that occur in the Ashkenazi Jewish population with a frequency of 1 in 2,500 or greater suggest that one or more common (15% to 50%) alleles is likely. For example, two common mutations, a 4-bp insertion30 and a splice-site mutation,31 account for more than 95% of the mutant β-hexosaminidase α-chain alleles in Ashkenazi Jewish patients with infantile Tay-Sachs disease. Ashkenazi Jewish patients who have the milder, adult-onset form of the disease have one of these alleles and the S269G missense mutation in the other α-chain allele.32 In type 1 Gaucher’s disease, three mutations in the acid β-glucosidase gene, N370S,33 L444P,34 and 84GG,35 account for about 90% of the Ashkenazi Jewish patients. Similarly, in cystic fibrosis, three mutations, AF508,36 W1282X,37 and G542X38 in the cystic fibrosis transmembrane regulatory gene are responsible for about 95% of the disease alleles in Ashkenazi Jewish individuals.

Based on these findings, it might be expected that perhaps one or two remaining common mutations causing type A NPD will be identified in the Ashkenazi Jewish population. However, it should be noted that type A NPD is 20 to 30 times less frequent than Tay-Sachs disease, Gaucher’s disease, or cystic fibrosis in this population, so the occurrence of multiple rare alleles causing the remainder of type A NPD would not be surprising. Clearly, the continued elucidation of the mutations causing type A NPD should provide additional insights into the molecular epidemiology of the diseases that occur frequently among the Ashkenazim.

Previously, the genetic mechanism(s) underlying the higher gene frequencies of Tay-Sachs (q = ~0.02), Gaucher’s (q = ~0.02), and NPD (q = ~0.005) among Ashkenazi Jews was the subject of debate.39-41 However, the recent molecular evidence showing that two or more mutations occur in each disease argues that heterozygotes for these alleles have a selective advantage. Although a variety of hypotheses have been proposed, a likely basis for this advantage could have been increased resistance to certain infections that were endemic in European cities during the past 2 millennia. That three lysosomal lipid storage diseases are frequent among the Ashkenazim suggests a common mechanism for this selective advantage. The recent finding that certain membrane sphingolipids are involved in the recognition and binding of various bacteria and bacterial toxins42,43 may be relevant to such a hypothesis. It is tempting to speculate that the slight accumulation of these membrane lipids in heterozygous individuals and the slightly higher levels of these lipids in the circulation could lead to an increased ability to bind, endocytose, and degrade these toxic agents, thereby providing a selective advantage for carriers of these mutant alleles. Alternatively, the slight accumulation of these lipids may have offered a protective effect. Clearly, further insights into the nature of this selective advantage may be gained by the

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Intracellular ASM Activity (nmol/hr/mg)</th>
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</thead>
<tbody>
<tr>
<td>COS-1 cells</td>
<td>7.8 ± 3.42</td>
</tr>
<tr>
<td>ASM constructs</td>
<td></td>
</tr>
<tr>
<td>pASM-3 (sense)</td>
<td>23.6 ± 8.12</td>
</tr>
<tr>
<td>L302P</td>
<td>7.5 ± 2.44</td>
</tr>
<tr>
<td>R496L</td>
<td>6.6 ± 2.29</td>
</tr>
</tbody>
</table>

Values represent the mean ± SD of four independent transfections.
future delineation of the function of these substrates and enzymes in health and disease.

In summary, a common missense mutation, L302P, has been identified in Ashkenazi Jewish type A NPD patients and shown to be authentic by transient expression in COS-1 cells. This lesion accounts for over 20% of the mutant ASM alleles in this population and should facilitate carrier testing for this debilitating disease, because analysis of this and the R496L mutation account for about 55% of the type A NPD alleles among Ashkenazi Jewish individuals.

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Identification and expression of a common missense mutation (L302P) in the acid sphingomyelinase gene of Ashkenazi Jewish type A Niemann-Pick disease patients

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