

Mucopolidosis II (I-Cell Disease) and Mucopolidosis IIIA (Classical Pseudo-Hurler Polydystrophy) Are Caused by Mutations in the GlcNAc-Phosphotransferase α/β -Subunits Precursor Gene

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Mucopolidosis II (MLII; I-cell disease) and mucopolidosis IIIA (MLIIIA; classical pseudo-Hurler polydystrophy) are diseases in which the activity of the uridine diphosphate (UDP)-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase) is absent or reduced, respectively. In the absence of mannose phosphorylation, trafficking of lysosomal hydrolases to the lysosome is impaired. In these diseases, mistargeted lysosomal hydrolases are secreted into the blood, resulting in lysosomal deficiency of many hydrolases and a storage-disease phenotype. To determine whether these diseases are caused by mutations in the GlcNAc-phosphotransferase α/β -subunits precursor gene (*GNPTAB*), we sequenced *GNPTAB* exons and flanking intronic sequences and measured GlcNAc-phosphotransferase activity in patient fibroblasts. We identified 15 different mutations in *GNPTAB* from 18 pedigrees with MLII or MLIIIA and demonstrated that these two diseases are allelic. Mutations in both alleles were identified in each case, which demonstrated that *GNPTAB* mutations are the cause of both diseases. Some pedigrees had identical mutations. One frameshift mutation (truncation at amino acid 1171) predominated and was found in both MLII and MLIIIA. This mutation was found in combination with severe mutations (i.e., mutations preventing the generation of active enzyme) in MLII and with mild mutations (i.e., mutations allowing the generation of active enzyme) in MLIIIA. Some cases of MLII and MLIIIA were the result of mutations that cause aberrant splicing. Substitutions were inside the invariant splice-site sequence in MLII and were outside it in MLIIIA. When the mutations were analyzed along with GlcNAc-phosphotransferase activity, it was possible to confidently distinguish these two clinically related but distinct diseases. We propose criteria for distinguishing these two disorders by a combination of mutation detection and GlcNAc-phosphotransferase activity determination.

Mucopolidosis II (MLII [MIM 252500]), mucopolidosis IIIA (MLIIIA [MIM 252600]), and mucopolidosis IIIC (MLIIIC [MIM 252605]) are three related genetic diseases of lysosomal enzyme trafficking. Alterations in the activity of the uridine diphosphate (UDP)-*N*-acetylglucosamine:lysosomal enzyme *N*-acetylglucosamine-1-phosphotransferase (GlcNAc-phosphotransferase [IUBMB accession number EC 2.7.8.17]) have been demonstrated in all three of these diseases. Alterations of GlcNAc-phosphotransferase are believed to be the primary genetic defect in all three diseases. GlcNAc-phosphotransferase activity is absent in MLII, reduced in MLIIIA, and altered in MLIIIC (Hasilik et al. 1981; Reitman et al. 1981; Varki et al. 1981, 1982; Waheed et al. 1982*b*).

GlcNAc-phosphotransferase is essential for the lysosomal trafficking of most lysosomal hydrolases. In higher eukaryotes, most lysosomal hydrolases are targeted to the lysosome via a mannose 6-phosphate (M6P)-dependent pathway. In this pathway, lysosomal

enzymes are modified by the phosphorylation of mannose in a two-step reaction. In the first step, GlcNAc-phosphotransferase catalyzes the transfer of GlcNAc 1-phosphate from UDP-GlcNAc to certain terminal or penultimate mannoses on high-mannose-type glycans (Reitman and Kornfeld 1981*a*, 1981*b*; Waheed et al. 1982*a*). In the second step, occurring in the *trans*-Golgi network, the covering GlcNAc is removed by *N*-acetylglucosamine-1-phosphodiester α -GlcNAcase (IUBMB accession number EC 3.1.4.45) which has the trivial name "uncovering enzyme" (Varki and Kornfeld 1981; Waheed et al. 1981; Do et al. 2002). The lysosomal enzymes, now modified with M6P, bind to M6P receptors in the *trans*-Golgi network and are translocated to the endosome and subsequently to the lysosome. The recognition of lysosomal hydrolases by GlcNAc-phosphotransferase is the determining step in lysosomal hydrolase trafficking. Lysosomal hydrolases that are substrates for GlcNAc-phosphotransferase exhibit low K_m

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values, whereas nonlysosomal glycoproteins bearing similar glycans have high K_m values (Reitman and Kornfeld 1981a; Waheed et al. 1982a; Bao et al. 1996b). This difference in K_m appears to explain the substrate-specific modification in lysosomal targeting.

GlcNAc-phosphotransferase has an $\alpha_2\beta_2\gamma_2$ -subunit structure with a molecular mass of 540 kDa (Bao et al. 1996a). Recently, we cloned the cDNA and genomic DNA encoding the α/β -subunits precursor gene (*GNPTAB*) (Kudo et al. 2005). With this cloning and the previous cloning of the γ -subunit gene (*GNPTG*) (Raas-Rothschild et al. 2000), we concluded that the enzyme is a product of two genes. As a result, GlcNAc-phosphotransferase is an uncommon exception to the one enzyme–one gene theory of Garrod (1902).

Interestingly, before the subunits and gene structures were determined, substantial evidence for heterogeneity in the mucopolidoses had been described. Varki (1981) identified a variant form of MLIII on the basis of substrate recognition, which was later designated MLIIC. A series of elegant genetic-complementation studies demonstrated heterogeneity and multiple complementation groups in MLIII (Honey et al. 1982; Shows et al. 1982; Mueller et al. 1983; Little et al. 1986). Three complementation groups were identified in MLIII and were designated A, B, and C, where A is classical pseudo-Hurler polydystrophy, C is the variant form described by Varki (1981), and B is represented by a single patient whose cell line has subsequently been lost. Additional studies demonstrated complementation between MLII and MLIIC (Mueller et al. 1983).

Total or near-total deficiency of GlcNAc-phosphotransferase results in the nearly complete absence of lysosomal targeting in many cell types and tissues and the secretion of most lysosomal enzymes. This defect is clinically recognized as MLII (or I-cell disease), a disease often resulting in death in the 1st decade of life (Kornfeld and Sly 2001). MLIIIA (pseudo-Hurler polydystrophy) is a milder disease in which GlcNAc-phosphotransferase activity is reduced rather than absent (Kornfeld and Sly 2001). MLIIC (variant pseudo-Hurler polydystrophy) is clinically indistinguishable from MLIIIA but is characterized by a normal level of GlcNAc 1-phosphate transfer to the small-molecule acceptor α -methylmannoside but a reduced level of transfer to a lysosomal hydrolase substrate (Varki et al. 1981). The link between the γ -subunit and the substrate recognition function was provided by our previous studies demonstrating that mutations in the γ -subunit are the molecular basis for MLIIC but not MLIIIA (Raas-Rothschild et al. 2000, 2004). This result suggested that the α -subunit and/or β -subunit of the enzyme might contain the catalytic portion of GlcNAc-phosphotransferase. Our recent cloning and expression of the α/β -subunits precursor cDNA also supported the localization of the catalytic

domain on these two subunits. Since the catalytic activity is absent or decreased in MLII and MLIIIA with all substrates, we speculated that these patients might have mutations in the α - and/or β -subunit. Our recent cloning of *GNPTAB* allowed us to analyze the mutations in patients with MLII or MLIIIA, to confirm this speculation. Tiede et al. (2005) also cloned the cDNA for *GNPTAB* and identified seven mutations from six patients with MLII. One of the patients in their study has a mutation identical to one found by us.

In this study, we sequenced all the exons of *GNPTAB* in 18 patients with MLII or MLIIIA and, when available, their relatives. Mutations in both alleles were identified in all patients, which demonstrates that both MLII and MLIIIA are caused by mutations in *GNPTAB*. In addition, GlcNAc-phosphotransferase activity was measured in patient fibroblasts, and selected mutant enzymes were expressed and characterized. The results of these studies allowed us to demonstrate that these diseases are allelic, to define the molecular basis of these diseases, and to propose molecular criteria to distinguish them. One of us (M.K.) recently reported a mutation in *GNPTAB* in a patient with MLIIIA (Steet et al. 2005) that is not included here. Part of the work of the present study was presented at the Joint Meeting of the Society for Glycobiology and the Japanese Society of Carbohydrate Research (Kudo et al. 2004).

Material and Methods

Cell Lines

Fibroblasts or lymphoblasts were derived from subjects with clinically diagnosed MLII and MLIIIA. Clinical information is shown in table 1. Cell lines from pedigrees GM03066, GM00080, GM00081, GM02013, GM02014, GM02274, GM02687, GM02660, GM01586, GM01589, GM01590, GM02046, GM02047, GM02273, GM02558, GM02559, GM01494, GM07255, GM07256, GM00113, GM01759, GM02065, GM02425, GM03112, GM03685, and GM03392 that have mucopolidosis, as well as normal human fibroblast line GM05565, were obtained from the National Institute of General Medical Sciences (NIGMS) Human Genetic Cells Repository at the Coriell Institute for Medical Research (Camden, NJ). Cell line F1515 was a kind gift from Dr. A. Raas-Rothschild (Hadassah University Hospital, Israel). Cell line F2954 was obtained from Dr. Miko Stewart (Children's Memorial Hospital, Chicago). Cell lines GM1006 (LT) and C.M. were obtained from Dr. Thomas B. Shows (Roswell Park Cancer Institute, Buffalo, NY). Cell line R.S. was obtained from Dr. Catherine M. Bollard (Texas Children's Hospital, Houston). Two of the MLII cell lines (GM02273 and GM03112) could not be recovered after freezing. It is well known that fibroblasts from pa-

Table 1

Summary of Clinical Background, GlcNAc-Phosphotransferase Activity, and Identified Mutations

Mucopolipidosis Classification and Individual ^a	Family	Relation to Proband	Age ^b	Sex	Race ^c	GlcNAc-Phosphotransferase Activity ^d (%)	Allele 1 ^c	Allele 2 ^c
MLII:								
GM03066	34	(Proband)	23 fwk	Female	White	1	FS288X	FS546X
GM00080	34	Mother	16 years	Female	White	66	WT	FS546X
GM00081	34	Father	23 years	Male	White	96	FS288X	WT
GM02013	36	(Proband)	2 wk	Male	White	<.1	FS1081X	FS1172X
GM02014	36	Mother	18 years	Female	White	58	FS1081X	WT
GM02273	38	(Proband)	2 years	Male	White
GM02274	38	Mother	...	Female	White	66	FS1172X	WT
469 Proband	469	(Proband)
GM02687	469	Father	26 years	Male	White	82	FS588X	WT
GM02660	469	Mother	23 years	Female	...	80	WT	FS1172X
GM01586	1908	(Proband)	5 wk	Male	White	<.1	FS1172X	FS1172X
GM01589	1908	Father	...	Male	White	94	FS1172X	WT
GM01590	1908	Mother	...	Female	White	78	FS1172X	WT
GM03112	1909	(Proband)	21 fwk	Female	White
GM02046	1909	Mother	25 years	Female	White	41	FS737X	WT
GM02047	1909	Father	26 years	Male	White	52	WT	FS1172X
F29543	*FS1085X (type 1)	FS1172X
C.M.	<.1	FS211X (type 1)	FS1172X
R.S.	2 years	Male0	FS211X (type 1)	FS1172X
MLIIIA:								
GM02558	501	Sister	9 years	Female	Black	3	Q278X	*FS1085X (type 2)
GM02559	501	(Proband)	7 years	Female	Black	3	Q278X	*FS1085X (type 2)
501 Brother	501	Brother	...	Male
GM01494	1012	(Proband)	9 years	Male	White	14	K4Q	FS1172X
GM07255	1012	Father	53 years	Male	White	78	K4Q	WT
GM07256	1012	Mother	51 years	Female	White	70	WT	FS1172X
GM00113	...	(Proband)	2 years	Female	White	12	K4Q	K4Q
GM01759	...	(Proband)	13 years	Male	White	.8	*FS211X (type 2)	FS1172X
GM02065	...	(Proband)	9 years	Male	White	2	FS745X	*FS1085X (type 2)
GM02425	...	(Proband)	15 years	Male	White	1	*FS211X (type 2)	FS1172X
GM03685	...	(Proband)	15 years	Male	White	1	*FS1085X (type 2)	FS1172X
Reclassified as MLIIIA ^f :								
GM1006 (LT)	5.1	FS1172X	*FS1202X
F1515	4.4	R1205X	R1205X
MLIIIC:								
GM03392	610	Sister	13 years	Female	White	122	WT	WT

^a Clinically affected individuals are listed in bold italics.

^b Age at time of tissue collection. In the case of fetal samples, an estimated gestational age is provided in fetal weeks (fwk) from conception.

^c Two of the cell lines were classified as Caucasian for race and Arab for ethnicity by the NIGMS Human Genetic Cells Repository, from which we obtained the cell lines.

^d GlcNAc-phosphotransferase activity was measured as specific activity (pmol/mg/h) in cell lysate and is shown as a percentage of the activity in normal fibroblasts (GM05565).

^e Mutations are named on the basis of the resulting protein products, which are described in table 5. An asterisk (*) before the mutation indicates that the frameshift is caused by aberrant splicing. WT = wild type.

^f Reclassified from MLII to MLIIIA.

tients with mucopolipidosis are frequently difficult to recover after freezing. This work was performed with the approval of the Institutional Research Board of the University of Oklahoma Health Sciences Center. Although it is not current standard practice, patient initials have been used in some cases, to allow comparison with previously published results.

Culture of Cell Lines

Fibroblasts were cultured in Dulbecco's modified Ea-

gle's medium (DMEM) with 20% defined fetal bovine serum (FBS) supplemented with 2 mM L-glutamine in 5% CO₂/95% air at 37°C until confluent and were harvested using trypsin. Lymphoblasts transformed with Epstein-Barr virus (GM02687 and GM02660) were cultured in DMEM with 15% heat-inactivated FBS supplemented with 2 mM L-glutamine in 5% CO₂/95% air at 37°C until the density of the cells was 1–1.4 × 10⁶ cells/mL and were harvested by centrifugation. Cell pellets were stored at –80°C until used for GlcNAc-phosphotransferase activity assay, genomic DNA preparation, or mRNA preparation.

GlcNAc-Phosphotransferase Enzymatic Activity in Patient Cells

Approximately 3×10^6 cells were incubated in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, and 2 mM $MgCl_2$) on ice for 45 min, were sonicated for 20 min in an ice-bath sonicator (Ultrasonics Quantrex 90H [L&R Manufacturing]), and then were clarified by centrifugation at 40,000 g for 20 min. GlcNAc-phosphotransferase activity was determined by measuring the transfer of GlcNAc-phosphate from [β - ^{32}P]UDP-GlcNAc to α -methylmannoside, as described elsewhere (Reitman et al. 1984). [β - ^{32}P]UDP-GlcNAc was synthesized using an improved procedure modified from that of Reitman et al. (1984). In brief, [^{32}P]glucosamine 6-phosphate was synthesized by incubating 200 nmol glucosamine, 100 nmol ATP, and 5.0 mCi [γ - ^{32}P]ATP (7,500 Ci/mmol from MP Biomedicals) with 2 U of yeast hexokinase (Sigma) in 200 mM Tris-HCl (pH 7.4) and 20 mM $MgCl_2$ for 20 min at 37°C. After inactivation of hexokinase by boiling for 5 min, 100 nmol glucose 1,6-diphosphate, 200 nmol acetyl coenzyme A, and 200 nmol uridine triphosphate were added to the reaction, together with 7.5 μ g recombinant *Escherichia coli* glmM (phosphoglucosamine mutase), 50 μ g recombinant *E. coli* glmU (pyrophosphorylase and acetyltransferase), and 5 U of inorganic pyrophosphatase from baker's yeast (Sigma), and were incubated for 30 min at 37°C. The [β - ^{32}P]UDP-GlcNAc thus synthesized was purified by chromatography on a Microsorb-MV amino high-performance liquid chromatography column (250 \times 4.6 mm [Varian]) with a linear gradient of 5 mM potassium phosphate buffer (pH 3.0) to 1 M potassium phosphate buffer (pH 4.0). The [β - ^{32}P]UDP-GlcNAc peak was identified by GlcNAc-phosphotransferase assay and cochromatography with authentic UDP-GlcNAc. Purity of the [β - ^{32}P]UDP-GlcNAc thus synthesized and purified is >95%, and a very low non-specific background is exhibited in the GlcNAc-phosphotransferase assay. This low background is helpful in assaying MLII fibroblasts. One unit of GlcNAc-phosphotransferase activity is defined as 1 pmol of GlcNAc-phosphate transferred to α -methylmannoside per h in a reaction containing 150 μ M UDP-GlcNAc and 100 mM α -methylmannoside. Protein concentration was determined by Bradford assay (Pierce).

PCR Amplification of Exons in GNPTAB

PCR primers to amplify the exons in *GNPTAB* and sequencing primers were designed on the basis of the genomic sequence of *GNPTAB* in BAC 14951 (GenBank accession number AC005409) and are listed in table 2. Genomic DNA was prepared from cells by use of DNeasy Tissue Kits (Qiagen). The individual exons or groups of exons, along with the flanking intronic se-

quences, were amplified by PCR. Primers and PCR conditions are provided in table 3. PCR was performed in a GeneAmp PCR system 2400 thermocycler (Perkin Elmer) by use of the Expand High Fidelity PCR system (Roche), except for exon 1, for which the GC-rich PCR system (Roche) was used. PCR products were separated from the primers with a QIAquick PCR Purification Kit (Qiagen) and were sequenced using the primers listed in table 3.

RT-PCR Amplification of mRNA

RT-PCR was used to amplify mRNA from selected cell lines. Total RNA was prepared from fibroblasts by using an RNeasy mini kit and QIA shredder (Qiagen). First-strand cDNA was synthesized from $\sim 3 \mu$ g of total RNA with oligo(dT) or random hexamer primers by use of a First-Strand cDNA synthesis kit (Amersham Pharmacia). cDNA corresponding to exons 3–7 was amplified with primers 5'-cacctgggtgaatggcacagatc-3' and 5'-gcattagcactaatccaggac-3'. cDNA corresponding to exons 16–19 was amplified with 5'-gcttctgctgatatcacgcgagc-3' and 5'-gtggcaatgtgtcattcaggc-3'. cDNA corresponding to exons 16–21 was amplified with 5'-gcttcctgctgatatcacgcgagc-3' and 5'-gcatcacatcacaagacatctc-3'. PCR parameters were as follows: 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 45 s. The PCR products from GM01759, GM02425, and GM1006 (LT) were separated from primers by use of QIAquick PCR Purification Kit and were sequenced with the same primers used for PCR. The PCR products from F2954, GM02558, and GM03685 were gel purified and sequenced with primers 1288 and 1289 (table 2).

Expression Plasmids for Mutants

Expression plasmids encoding mutant α/β -subunits precursors—K4Q, D190V, L1168fsX1172 (FS1172X), or W1201fsX1202 (FS1202X)—were constructed by replacing the restriction-enzyme-flanked region in the wild-type sequence in pcDNA6/V5/His-A. Mutant sequences were generated by PCR with the primers described below and with the wild-type expression plasmid as the template. The K4Q expression plasmid was constructed by replacing the 5'-terminal *NheI-XhoI* fragment with an *NheI/XhoI*-cleaved PCR fragment amplified with 5'-cgtggcgggctagccaccatgctgttccagctcctgcagag-acaacc-3' and 5'-ctaaaggtaggcaagtggctc-3' (Q^4 is introduced by underlined nucleotides). The D190V plasmid was constructed by replacing the 5'-terminal *NheI-XhoI* fragment with an *NheI/XhoI*-cleaved PCR product amplified by sequential PCR. The first two reactions were performed with primers T7 (5'-taatcagactcactataggg-3') and 5'-aaccttggtactgtcaaaaacaact-3' and with primers 5'-ccaaggtgttgaagatgccactctg-3' and 5'-ctaaaggtaggca-

Table 2**Oligonucleotide Sequences of Primers Used for PCR Amplification and Sequencing of *GNPTAB***

Primer ID	Oligonucleotide Sequence (5'→3')
1076	cctggtgaactcattctcaagacc
1077	gctaaagtgaacacatcagatgggc
1078	cataatctctgggttaaacctgtg
1082	aacctccccagtgcaagc
1083	aagctctatcttggagttgg
1084	gctgtttgtcttctttgtgc
1085	tgctgctttaatagctctcagaatg
1086	atggaccacaagaaaagaatcacac
1087	cgctcgtcgccggagctgcaatg
1088	ggcaaacccccgtctctaataatg
1089	gtgatgatgcagctctgtagtc
1092	aggactccatctttaaagtctgc
1103	gtggtaggcagtaagtgaatac
1105	ctctaagcaaacctcttgg
1106	cacaaatcctgtgtacc
1107	ctcactctctgtaagtccc
1109	gatttgctaaagtactccacgc
1118	ctgctctcttctaactgtcc
1119	caatcccttttgaggttaaggcc
1120	cgctcagtaagaacggtaacg
1121	gtttggttaagtctatagcacatgttc
1122	caccagtcagaaactgtctttc
1123	gctaaagtaaatctgcttggtcc
1125	caagtggaaaaccatccacc
1126	tccaagtacgcttctgtgag
1128	cttctgggctctccttggg
1129	tgctcctaataagagttcgtgac
1132	gtttgctgtgttcagaactaagg
1134	tgtagcactgcgctgaccag
1135	ccacagtcattactacaatgcgc
1136	cctgctgtagttctgaagtgc
1137	attgccatgttcaactctgatg
1138	gtccaacactgcctgcaactg
1139	cccatagctaaaaggccatctacc
1140	gtatacactcaccacacatgc
1141	gaagtctctctcctgctgg
1142	ctgaagacactgtgctcaactca
1162	gggattacaggtgtgagccacc
1163	tgctctctcagcagcacatatac
1164	cctgagcatgagaagaatgagg
1215	cttacctccagagacatagaatc
1216	tcctatctcactcaaccac
1219	ggctcttagaaagtgtgac
1233	ggcgtgaaaggggtgatgctgttc
1257	gcatcacatcacaagacatctc
1259	ccacaaaacagcatgtactgg
1349	acatactgtatcggggcatc

gtggctc-3' (V¹⁹⁰ is introduced by underlined nucleotides). The second PCR was performed with primers T7 and 5'-ctaaagtgtaggcaagtggctc-3' with the use of a mixture of the products from the first reactions as template. Expression plasmids for FS1172X and FS1202X were constructed by replacing the 3'-terminal *EcoRV-XbaI* fragment with *EcoRV/XbaI*-cleaved PCR fragments amplified with primers 5'-gcttctgctgatatcacgcagc-3' and 5'-catggatttctagaggagccctgaacagcctcactgctgagcatc-3' (for FS1172X) or primers 5'-tttgtctctctagactcaacattctcgcagctcatgatga-3' (for FS1202X) (stop codons are underlined).

Expression of the Mutant α/β -Subunits Precursor cDNA in 293T Cells

The human embryonic kidney cell line 293T was grown in DMEM with 10% (v/v) FBS at 37°C and 5% CO₂/95% air. Cells were transfected with empty vector or with vector containing cDNAs encoding wild-type or mutant α/β -subunits precursor, by use of FuGene 6 (Roche). Cells were harvested at 72 h after transfection, and GlcNAc-phosphotransferase activity was measured as described for mucopolipidosis cell lines.

Results*Source and Characterization of Cell Lines*

Fibroblast or lymphoblast cell lines from 15 patients with a diagnosis of MLII or MLIIIA (and, when possible, their relatives) were obtained from repositories or clinicians as described in the "Material and Methods" section. Cells from NIGMS have a family number assigned when several cell lines are available from a family. There are no data available about any possible genetic relationships between the pedigrees. In three pedigrees with MLII, the cells from the proband were not available; however, cells from both parents were available in two of the three pedigrees. GlcNAc-phosphotransferase activity in cell lysates was measured by an assay for the transfer of [³²P]GlcNAc-phosphate to α -methylmannoside as described in the "Material and Methods" section. Activity was expressed as a percentage of the specific activity in the normal fibroblast line (table 1). In this assay, patients with MLII lacked detectable activity, whereas patients with MLIIIA had reduced but detectable activity (Varki et al. 1982; Mueller et al. 1985). In table 1, patients and available relatives are listed along with available clinical information, measured GlcNAc-phosphotransferase activity, and mutations identified by this study. Specific activity of the normal fibroblast lysate was 80–110 pmol/mg/h, comparable to that reported elsewhere (Varki et al. 1982).

*Strategy for the Identification of Mutations in *GNPTAB**

To identify as many mutations as possible, including those that altered splicing, we chose to amplify and sequence all 21 exons and their flanking intronic sequences, as described in the "Material and Methods" section. The PCR products were sequenced and compared with the corresponding *GNPTAB* sequence in BAC 14951. Mutations were identified in the uncloned PCR product as a second sequence along with the wild-type sequence. For some mutations, additional experiments, such as RT-PCR and expression of mutant cDNAs, were performed to confirm the effect of mutations. The genomic sequence for *GNPTAB* is num-

Table 3**PCR and Sequencing Primers and PCR Conditions for Amplification of *GNPTAB***

EXON(S)	PCR PRIMER ^a		ANNEALING TEMPERATURE (°C)	ELONGATION TIME (s)	PCR PRODUCT SIZE (bp)	SEQUENCING PRIMERS ^a
	Forward	Reverse				
1	1087	1088	60	45	386	1088, 1233, 1349
2	1076	1077	60	45	536	1103, 1107
3 and 4	1078	1082	60	90	1,832	1078, 1105, 1162, 1106
5	1118	1119	60	45	737	1083, 1084
6 and 7	1085	1086	58.5	45	646	1085, 1086
8, 9, and 10	1089	1109	58	60	1,433	1092, 1107
11	1120	1121	60	45	424	1120, 1121
12	1122	1123	60	45	522	1123, 1122
13	1163	1164	60	60	1,345	1163, 1125, 1126, 1128, 1164
14 and 15	1129	1134	60.5	45	829	1129, 1132
16	1135	1136	60.5	45	267	1136, 1135
17 and 18	1215	1216	60	45	708	1215, 1216
19	1139	1140	60	45	436	1139, 1140
20	1141	1142	60	45	717	1141, 1142
21	1219	1259	58.5	45	297	1219, 1257

^a Primer sequences are listed in table 2.

bered starting from the first base in the initiation codon in BAC 14951. The cDNA is numbered according to the cDNA sequence reported elsewhere (GenBank accession number AY687932), and amino acids are numbered starting from the initiator methionine. Mutations are described in accordance with the nomenclature suggested by den Dunnen and Antonarakis (2000). Polymorphisms identified in *GNPTAB* during mutation analysis are listed in table 4.

Mutations Identified in Patients with MLII and Their Parents

Eight unique mutations were found in the nine pedigrees with clinically diagnosed MLII (table 1). These mutations are listed in table 5, and their locations in the

cDNA are shown diagrammatically in fig. 1. Two mutant alleles were found in each patient or family, except for family 38, for which only the maternal cell line was available. Only one patient with MLII was homozygous for a mutation. When parental samples were available, each parent carried a mutant allele in common with the patient. One mutation (FS1172X) was predominant and was found in all the pedigrees with MLII except family 34.

All the mutations, except for the one in F2954, were insertions or deletions of nucleotide(s) causing frameshift and truncation of the protein product. These frameshift mutations were named after the location of the introduced stop codons (table 5). One allele in F2954 carried a substitution at the exon 17–intron 17 junction (IVS17+1G→A) in the invariant (GT...AG) splice-site sequence (Breathnach and Chambon 1981), whereas the other allele carried FS1172X. To determine the outcome of this substitution, cDNA corresponding to exons 16–21 was amplified by RT-PCR by use of RNA from F2954 fibroblasts as template and was sequenced. Two distinct products were found at equal abundance, and each product contained a single mutation. The FS1172X allele contained a 2-nt deletion in the cDNA, as expected. The IVS17+1G→A allele led to aberrant splicing with deletion of exon 17 (fig. 2), resulting in truncation at amino acid 1084—namely, *FS1085X (type 1) (an asterisk [*] indicates that the frameshift is caused by aberrant splicing).

Five of the mutations, FS211X (type 1), FS288X, FS546X, FS588X, and FS737X, truncate the protein in the α -subunit, yielding truncated α -subunit and no β -subunit. The rest of the mutations, FS1081X, FS1172X,

Table 4**Polymorphisms in *GNPTAB***

Genomic DNA ^a	cDNA ^b	Location
del-42_-40CGG	del108_110CGG	Exon 1, 5' UTR
27A→G	191A→G	Exon 1 (silent)
33801A→G	282-117A→G	Intron 1
del42226_42228GTG	IVS4+94_96GTG	Intron 4
63255A→G	IVS10-166G→A	Intron 10
65373G→C	IVS12-3G→C	Intron 12
65696A→G	2096A→G	Exon 13 (silent)
69559T→C	IVS+5T→C	Intron 15
73124G→A	IVS17+14G→A	Intron 17
73347T→C	IVS17-25T→C	Intron 17

^a In the genomic sequence, the first nucleotide of the initiation codon in BAC clone 14951 (GenBank accession number AC005409) is numbered 1.

^b In the cDNA sequence, the first nucleotide of the cDNA (GenBank accession number AY687932) is numbered 1.

Table 5

Mutations Identified in GNPTAB

Mucopolidosis Classification and Mutation ^a	cDNA Sequence ^b	Genomic Sequence ^c	Location	Consequence	Patients and Families with Mutation
MLII:					
FS211X (type 1)	773_776delCAGA	g-50096_50100delCAGA	Exon 6	Frameshift T206fsX211	C.M., R.S.
FS288X	1012delA	g-59600delA	Exon 8	Frameshift T284fsX288	Family 34
FS546X	1744delC	g-64558delC	Exon 12	Frameshift C528fsX546	Family 34
FS588X	1902_1905tripTATA ^d	g-65502_65505tripTATA	Exon 13	Frameshift S581fsX588	Family 469
FS737X	2352T→AAA	g-65952delTinsAAA	Exon 13	Frameshift L730fsX737	Family 1909
FS1081X	3395_3398dupCTAC	g-70633_70636dupCTAC	Exon 16	Frameshift Y1079fsX1081	Family 36
FS1172X	3665_3666delTC	g-77209_77210delTC	Exon 19	Frameshift L1168fsX1172	Families 36, 38, 469, 1908, 1909; C.M., R.S., F2954
*FS1085X (type 1)	IVS17+1G→A	g-73111G→A	Intron 17	Skipped exon 17; P1084fsX1085	F2954
MLIIIA:					
K4Q	174A→C	g-10A→C	Exon 1	Missense K4Q	Family 1202, GM00113
*FS211X (type 2)	733A→T	g-44667A→T	Exon 5	4-nt del from 3' end of exon 5; D190fsX211/missense D190V	GM01759, GM02425
Q278X	996C→T	g-59584C→T	Exon 8	Stop codon Q278X	Family 501
FS745X	2215_2219delACTCA	g-65815_65819delACTCA	Exon 13	Frameshift S685fsX745	GM02065
FS1172X	3665_3666delTC	g-77209_77210delTC	Exon 19	Frameshift L1168fsX1172	Family 1012, GM01759, GM02425, GM03685, GM1006 (LT)
R1205X	3777C→T	g-81501C→T	Exon 20	Stop codon R1205X	F1515
*FS1085X (type 2)	IVS17+6T→G	g-73116T→G	Intron 17	Skipped exon 17; P1084fsX1085	Family 501, GM02065, GM03685
FS1202X	IVS19-1G→A	g-81490G→A	Intron 19	Skipped exon 20; W1201fsX1202	GM1006 (LT)

^a An asterisk (*) before the mutation indicates that the frameshift is caused by aberrant splicing.

^b The first nucleotide of the cDNA (GenBank accession number AY687932) is numbered 1.

^c The first nucleotide of the initiation codon in BAC clone 14951 (GenBank accession number AC005409) is numbered 1.

^d TATA sequence is repeated three times as TATATATATA.

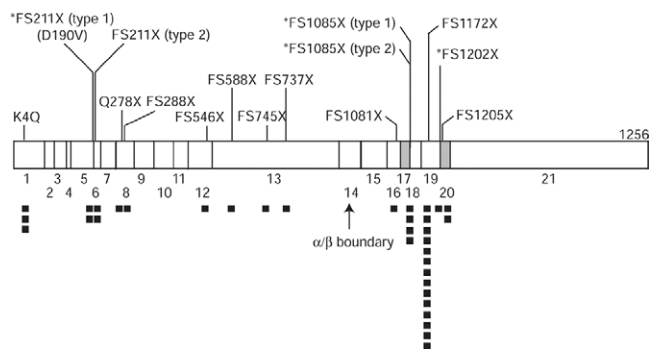


Figure 1 Location of mutations on the GlcNAc-phosphotransferase α/β -subunits precursor cDNA. The vertical lines indicate positions of mutations. Numbers below the boxes are exon numbers. Exons that are deleted as a result of splice-site mutations are shaded. An asterisk (*) before the name of a mutation indicates that the frameshift is caused by aberrant splicing. Blackened squares under the cDNA show the frequency of the mutant alleles in this study.

and *FS1085X (type 2), truncate the protein in the β -subunit, yielding intact α -subunit and truncated β -subunit (fig. 1). To examine the effect of truncation on the GlcNAc-phosphotransferase activity, the expression plasmid for the longest product (FS1172X; 1171-aa product) was constructed. Transient transfection of 293T cells with the FS1172X plasmid did not increase GlcNAc-phosphotransferase activity, compared with that of mock-transfected cells. The absence of additional activity in the cells transfected with FS1172X plasmid was consistent with the absence of activity in the fibroblast GM01586, which was homozygous for the FS1172X mutation. Since the other mutations generate proteins shorter than FS1172X, the protein from these mutations is likely to be inactive as well. The lack of GlcNAc-phosphotransferase activity from mutations found in patients with MLII is consistent with the very low activity ($\leq 1\%$ of normal fibroblasts) detected in fibroblasts of patients with MLII.

Mutations Identified in Patients with MLIIIA and Their Parents

Six unique mutations were found in the seven pedigrees with clinically diagnosed MLIIIA (table 1). The mutations are listed in table 5, and their locations in the cDNA are shown diagrammatically in fig. 1. Two mutant alleles were found in each patient or family. Only one patient with MLIIIA was homozygous for the mutation. When parental samples were available, each parent carried a mutant allele in common with the patient.

Four of the mutations were single-nucleotide substitutions, and the other two were nucleotide deletions. Each single-nucleotide substitution affects the protein product in different ways, whereas the nucleotide dele-

tions cause a frameshift and yield inactive enzyme in the same way as mutations found in MLII.

The first substitution, c.174A→C, causes the amino acid substitution K4Q. This mutation was homozygous in GM00113 and heterozygous in GM01494. The effect of the K4Q substitution was examined by transient transfection of 293T cells with K4Q plasmid. Transfection resulted in ~20%–40% activity when compared with cells transfected with the wild-type sequence. We conclude that the K4Q substitution reduced but did not eliminate GlcNAc-phosphotransferase activity. GlcNAc-phosphotransferase activity in GM00113 fibroblast lysate, homozygous for K4Q, was 12% (table 1), which is consistent with K4Q being a mild mutation. It is interesting to note that the diagnosis had been made by the time the patient was 2 years old, although the GM00113 fibroblasts have very high activity (12%). GlcNAc-phosphotransferase activity in GM01494 fibroblast lysate, heterozygous for K4Q, was 14% of normal fibroblasts (see data for MLIIIA in table 1). Since the other mutant allele was the null FS1172X, the product of the K4Q mutation could be directly observed and was reasonably consistent with K4Q being a mild mutation. The reported age at which fibroblasts were obtained, 9 years, is also consistent with the relatively high enzyme activity.

The second substitution, c.733A→T, is located 3 nt upstream from the 3'-end of exon 5 (table 5). This mutation was heterozygous in GM01759 and GM02425. The effect of this substitution is complex. This nucleotide substitution should change amino acid Asp¹⁹⁰ (GAT) to Val (GTT). However, when a cDNA encoding the D190V substitution was expressed in 293T cells, it produced 86% of GlcNAc-phosphotransferase activity. Therefore, the substitution of valine for aspartic acid at position 190 does not significantly affect the enzyme activity. To investigate potential other effects of the c.733A→T substitution, the RNA was analyzed. RT-PCR amplification of exons 3–7 from GM01759 and GM02425 RNA revealed two RNA sequences in both cell lines. The mRNA from the c.733A→T allele contained a 4-nt deletion resulting in FS211X (type 2) at the 3'-end of the exon 5 sequence (figs. 2 and 3). Sequencing the cDNA product revealed that a new splice site was generated between G⁴⁴⁶⁶⁵ and G⁴⁴⁶⁶⁶ instead of the original site between G⁴⁴⁶⁶⁹ and G⁴⁴⁶⁷⁰ (fig. 2). Even though the original splice site is unaltered, the new site appears to be preferred. Since the other allele in GM01759 and GM02425 is FS1172X, GlcNAc-phosphotransferase activity in these patients is from the c.733A→T allele. GlcNAc-phosphotransferase activity of GM01759 and GM02425 fibroblasts was 0.8% and 1%, respectively, which is consistent with the lack of full-length enzyme. Fibroblasts from probands GM01759 and GM02425 were collected when the patients were aged 13 and 15 years, respec-

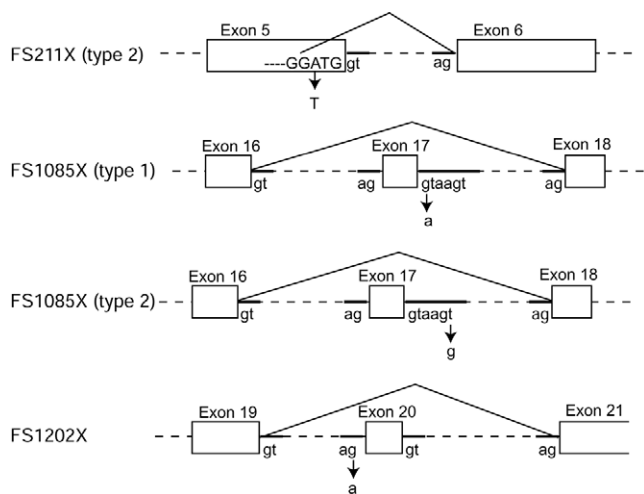


Figure 2 Structure of mutations causing aberrant splicing. Mutations and the resulting mRNA splicing that cause F211X (type 2), FS1085X (type 1), FS1085X (type 2), and FS1202X are shown. Nucleotide substitutions are indicated (arrows). RNA was analyzed by RT-PCR with oligo(dT) or random hexamer primers, and the amplified products were sequenced.

tively, which strongly suggests that the diagnosis of MLIIIA is correct. The long clinical course and very low fibroblast enzyme activity may suggest that splicing is especially disrupted in these fibroblasts.

The third substitution, c.996C→T, is a nonsense mutation truncating the enzyme at amino acid 277—namely, Q278X. This mutation probably results in enzyme with no activity. This mutation was heterozygous in the proband GM02558 and affected sister GM02559.

The fourth substitution, IVS17+6T→G, is located at the junction of exon 17 and intron 17. This mutation was found in GM02558, GM02559, GM02065, and GM03685. Although the substitution is outside the invariant sequence, it alters splicing. RT-PCR amplification of cDNA containing exons 16–21 from GM02558 mRNA revealed two sequences; one was wild type derived from the Q278X allele, and the other contained a deletion of exon 17 (fig. 2). Skipping of exon 17 results in a frameshift at amino acid 1084 and termination at amino acid 1085 (FS1085X, type 2). The same result was previously observed in a patient with MLII (F2954), caused by IVS17+1G→A (FS1085X, type 1). Although mRNA sequences derived from these two mutations are the same (fig. 2), the GlcNAc-phosphotransferase activity and clinical outcomes are different. Fibroblasts with FS1085 (type 2) exhibited 1%–3% GlcNAc-phosphotransferase activity, whereas F2954 fibroblasts with FS1085X (type 1) exhibited <0.1% activity (table 1). Since, in these fibroblasts, the other alleles are null alleles, the observed difference is the difference between FS1085X types 1 and 2. This difference may be because

of the location of the substitutions. It seems probable that the substitution outside the invariant splice site may occasionally allow the correct splicing, although we could not demonstrate the presence of a product with the wild-type sequence in fibroblasts.

The final two mutations are deletions of nucleotides. One is FS1172X, which was also found in patients with MLII. The other is FS745X, which truncates the protein in the α -subunit. These two frameshift mutations and the nonsense mutation Q278X are thought to not yield active enzyme. These three null mutations were always found heterozygous with other mutations that yield active enzyme in patients with MLIIIA. At least one of the alleles in each patient with MLIIIA is K4Q, *FS211X (type 2), or *FS1085X (type 2), which allow generation of some active enzyme.

Mutations Identified in Patients with Reclassified MLIIIA

Patients GM1006 and F1515 were given the clinical diagnosis of MLII. On the basis of the GlcNAc-phosphotransferase activity and mutations identified in these patients (table 1), we propose that they should be classified as having MLIIIA. Mutations found in these patients are listed in table 5 with those of the other patients with MLIIIA, and the locations in the cDNA are shown diagrammatically in fig. 1.

In patient GM1006, one allele carried the common FS1172X mutation, and the other carried a substitution at the intron 19–exon 20 junction, IVS19–1G→A. The substitution was in the invariant (GT...AG) splice-site sequence. A region containing exons 16–21 of mRNA from GM1006 was amplified by RT-PCR, which demonstrated that the intron 19–exon 20 splice-site mutation led to aberrant splicing, resulting in a deletion of exon 20 (fig. 2). This causes a frameshift and truncates the protein at amino acid 1201 (FS1202X). Because of the proximity of the two mutations found in this patient (exon 19 and intron 19), it was possible to demonstrate that these two mutations were always found on different transcripts. Nearly 100% of the transcripts from patient GM1006 contained the exon 20 deletion, suggesting that the mRNA containing the 2-nt deletion in exon 19 is poorly expressed or unstable. This is in contrast to the result for patient F2954, who is heterozygous for FS1172X mutation, in which both transcripts were expressed at a similar level as the transcript in normal human fibroblasts. Fibroblasts from proband GM1006 expressed 5.1% GlcNAc-phosphotransferase activity, which is well above the maximum typically observed in patients with MLII (<1%), suggesting that the classification should be MLIIIA. Since one allele carries the FS1172X null mutation, this unexpectedly high activity should be from FS1202X deleting the final 55 aa. Tran-

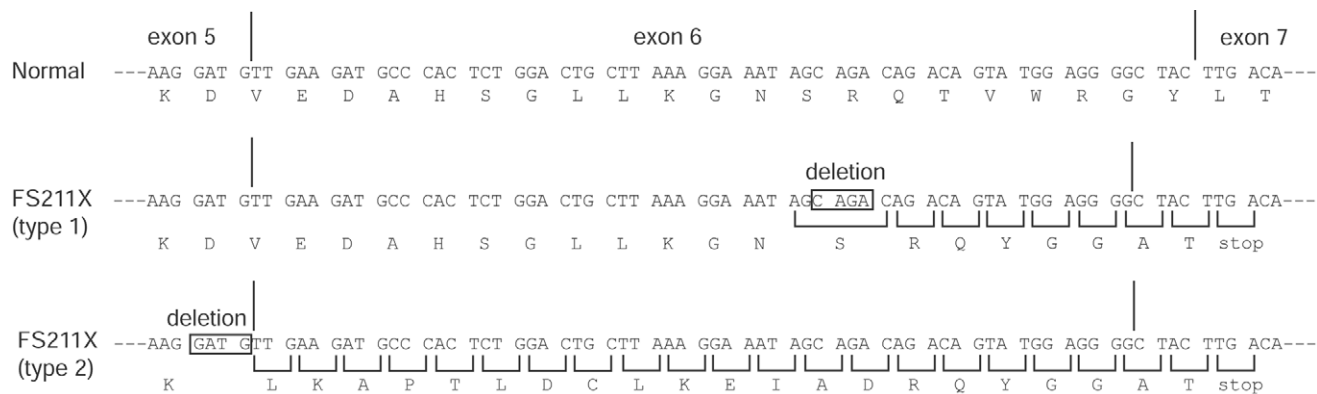


Figure 3 Comparison of frameshift mutations that cause termination at amino acid 211. Deletions in the cDNA sequence change the reading frame in FS211X (type 1) and FS211X (type 2). Nucleotides deleted from the cDNA are boxed. The wild-type reading frame is indicated by spacing; the abnormal reading frame, by brackets.

sient transfection of 293T cells with FS1172X or FS1202X plasmids resulted in 0% and 2.8% increase in activity, respectively, compared with cells transfected with the wild-type sequence. Therefore, the observed activity in patient GM1006 resulted from the FS1202X allele. This enzyme activity in fibroblasts could be the result of normal transcript from leaky splicing at the original or alternative splice sites, although we could not demonstrate the presence of the wild-type sequence by RT-PCR.

Patient F1515 appeared to be homozygous for R1205X caused by a substitution in exon 20; however, since a family study was not possible, a genomic deletion could not be excluded. F1515 fibroblasts expressed 4.4% GlcNAc-phosphotransferase activity (table 1), suggesting that the correct diagnosis is MLIIIA. Since we demonstrated that truncation at amino acid 1201 retains 2.8% of wild-type GlcNAc-phosphotransferase activity when expressed in 293T cells, we believe R1205X is likely to retain a small amount of enzyme activity. This mutation was also reported by Tiede et al. (2005), who found the mutation in a patient with MLII.

Patient with MLIIC

To confirm that MLIIC is not caused by *GNPTAB* mutations, DNA from GM03392 (MLIIC) fibroblasts was sequenced. No mutations in *GNPTAB* were found. This result supports the hypothesis that the MLIIC phenotype is exclusively caused by mutations in the γ -subunit gene. Fibroblast GlcNAc-phosphotransferase activity was 122% of normal, consistent with the MLIIC diagnosis (table 1).

Discussion

In a previous study, we demonstrated that mutations in the γ -subunit of GlcNAc-phosphotransferase were the cause of MLIIC (Raas-Rothschild et al. 2000). This was subsequently expanded by Raas-Rothschild et al. (2004). To understand the relationship between MLII and MLIIIA, this study involved sequencing of *GNPTAB* from pedigrees with MLII or MLIIIA, as well as measuring GlcNAc-phosphotransferase activity in their fibroblasts by use of a specific and sensitive assay. These studies allowed us to elucidate the complex molecular genetics of these disorders.

Nine pedigrees with MLII and nine with MLIIIA were analyzed in this study. Two mutant alleles were identified in 17 of the 18 pedigrees. In the remaining family (family 38), only a maternal cell line was available, and, consequently, only the maternal mutant allele could be identified. The identification of two mutant *GNPTAB* alleles in each of the 17 adequate pedigrees strongly suggests that the MLII or MLIIIA phenotypes are only caused by *GNPTAB* mutations. Since both pedigrees with MLII and pedigrees with MLIIIA always had *GNPTAB* mutations, this confirmed that these two diseases are allelic, which is consistent with the fact that GlcNAc-phosphotransferase activity is affected in both diseases and that MLII and MLIIIA are not complemented by each other, as discussed below. This study provides the criteria to distinguish between MLII and MLIIIA on the basis of the location and type of the mutations.

In the 18 pedigrees with MLII or MLIIIA, 15 different mutations were identified, including 2 nonsense mutations, 8 frameshift mutations (insertions and/or deletions), 1 missense mutation, and 4 mutations that affect splicing. Mutations causing aberrant splicing were con-

firmed by RT-PCR. The missense mutation and some of the frameshift mutations near the 3'-terminus of the cDNA were tested for GlcNAc-phosphotransferase activity after transient expression in 293T cells, to confirm the effect on enzyme activity. Five of the 15 different mutations were found in multiple patients or pedigrees. The remaining 10 mutations were found in only a single patient or family. The most common mutation was the FS1172X null mutation found in 13 of 18 families, including 9 pedigrees with MLII and 4 pedigrees with MLIIIA. This is the only mutation found in both MLII and MLIIIA. FS1172X was always caused by the same 2-nt deletion in exon 19. Since the mutation was a deletion of CT from a repeating CTCT sequence, this may be the result of a mismatch-repair error. This frameshift causes truncation of the enzyme at amino acid 1171 in the β -subunit and appears to be a null allele. When a cDNA with this mutation was expressed, no activity could be demonstrated. Additionally, fibroblasts from patient GM01586, who is homozygous for this mutation, exhibited the very low value of <0.1% GlcNAc-phosphotransferase activity. Since FS1172X is the most common mutant allele identified in both MLII and MLIIA in our study (13 of 32 cell lines), it is surprising that only a single homozygote (GM01586) was identified. This might be the result of adverse selection or our small sample size. In the remaining 11 pedigrees with this allele, there was never evidence of enzyme activity from this allele. The second frequent mutation was FS1085X (type 2), shared by three pedigrees with MLIIIA. The remaining three shared mutations, FS211X (type 1), FS211X (type 2), and K4Q, were found in two pedigrees each.

Given the multiple mutations and the presence of the same mutation in pedigrees with MLII and MLIIA, how can these two diseases be distinguished molecularly? In the 11 pedigrees listed by the repositories or clinicians as having MLII, the following mutations were found. Nine pedigrees with MLII shared the common FS1172X allele, which was homozygous in one case (family 1908) and present in a single maternal sample in another case (family 38). In six of the remaining seven pedigrees, the FS1172X was paired with a second mutation: FS211X (type 1) (two cases), FS588X, FS737X, FS1081X, or FS1085X (type 1). These are all due to insertion or deletion of nucleotide(s), except for the FS1085X (type 1) that is a substitution in the invariant splice-site (GT...AG) sequence. None of these mutations were predicted to generate active GlcNAc-phosphotransferase. In the only MLII pedigree that lacks the FS1172X mutation (family 34), the proband was heterozygous for FS288X and FS546X. Unexpectedly, no amino acid substitutions were found as a cause of MLII. All fibroblasts or lymphoblasts from patients with MLII lacked GlcNAc-phosphotransferase activity ($\leq 1\%$) and had mutations that

precluded the production of mRNA coding for full-length or active GlcNAc-phosphotransferase. We propose that these criteria—very low GlcNAc-phosphotransferase activity and mutations yielding truncated proteins—should define the MLII diagnosis. We believe that the two patients (GM1006 and F1515) clinically diagnosed with MLII were misclassified, and the proper classification is MLIIIA. This is based on enzyme activity ($\sim 5\%$ of normal), which we believe to be incompatible with a diagnosis of MLII.

In the nine pedigrees with MLIIIA, including the two reclassified cases, the mutations determined were qualitatively different. Although the common FS1172X null mutation was found in five pedigrees, and additionally two patients were heterozygous for Q278X or FS745X, these null mutations were always paired with a mild mutation, such as an amino acid substitution, truncation of the enzyme near the carboxyl-terminus, or a leaky splice-site mutation. Two patients were homozygous for a mutation: K4Q or R1205X. Fibroblast GlcNAc-phosphotransferase activity in patients with MLIII varied from 0.8% to 14% of normal and overlapped with the values found for patients with MLII ($\leq 1\%$). When the patients' ages were available, all except one were in the range from 7 to 15 years. Given the age range, it is unlikely that any of these cases represent misclassified MLII. There does not appear to be any obvious correlation between age and fibroblast GlcNAc-phosphotransferase activity. We propose that MLIIIA is defined by the presence of at least one mild mutation that can produce active GlcNAc-phosphotransferase.

Genetic heterogeneity within the mucopolidoses has been investigated by cell fusion studies (heterokaryon analysis) (Honey et al. 1982; Shows et al. 1982; Mueller et al. 1983; Little et al. 1986). These studies demonstrated several important features about the mucopolidoses, including the presence of multiple complementation groups. On the basis of this technique, MLIII was divided into MLIIIA, MLIIIB, and MLIIIC, confirming the previous identification of a variant form of MLIII by Varki et al. (1981) that was based on enzyme activity with different substrates. Group B is represented by a single patient whose cell line was subsequently lost. Group A represents classical MLIII, and group C represents the variant form. In this study, we demonstrated that MLIIIA is caused by mutations in *GNPTAB* on human chromosome 12. We previously showed that MLIIIC was caused by mutations in the γ -subunit gene on chromosome 16 (Raas-Rothschild et al. 2000, 2004); therefore, the molecular basis for the complementation is obvious. Mueller et al. (1983) also demonstrated complementation between MLII and MLIIIC but not between MLII and MLIIIA nor between MLII and MLIIIB. Again, these results are expected because we now know that MLII and MLIIIA are allelic. Less un-

derstandable is the complementation within MLII reported by Shows et al. (1982). This complementation was only observed when the fusion partner was fibroblasts from GM1006 (LT). To investigate this observation, we obtained the GM1006 (LT) cell line from Dr. Shows. Our analyses strongly suggested that this patient should be classified as having MLIIIA, not MLII, on the basis of the GlcNAc-phosphotransferase activity (5.2% of normal) and the FS1202X mutation that was active on transfection. Since we reclassified patient GM1006 (LT), we conclude that complementation within MLII was not demonstrated by Shows et al. (1982).

Since we now know the α - and β -subunits are derived from one gene, intragenic complementation in MLII or MLIIIA is possible in principal. For example, if one allele carries an α -subunit mutation and the other carries a β -subunit mutation, active GlcNAc-phosphotransferase might be formed from the active subunits. In our study, the only patient that might demonstrate intragenic complementation is patient GM01494. In GM01494 cells, an active α -subunit (amino acids 1–928) might be provided by the FS1172X allele, and an active β -subunit (amino acids 929–1256), by the K4Q allele. However, GlcNAc-phosphotransferase activity of fibroblast GM01494 was only 14% of normal. This is only slightly higher than the average of the activity in GM00113 (K4Q/K4Q) (12%) and GM01586 (FS1272X/FS1272X) (<0.1%), which suggests that other factors, such as unstable mRNA, may prevent complementation in this case. Additionally, persons with fully complementing mutations would not be expected to display symptoms that would identify them as candidates for *GNPTAB* sequencing.

Our study proved that MLII and MLIIIA are allelic on the basis of gene sequence, and we proposed criteria to distinguish them. MLII is defined by two severe mutations, whereas MLIIIA is defined by at least one mild mutation that can produce active GlcNAc-phosphotransferase. This approach allowed us to reclassify as MLIIIA two cases that were previously misdiagnosed as MLII, and it provided a molecular basis to understand a large body of complementation data. The identification of 15 different mutations, including 5 common mutations, provides a basis for screening, carrier testing, prenatal diagnosis, genetic counseling, and prediction of prognosis.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for BAC 14951 containing *GNPTAB* [accession number AC005409], cDNA encoding GlcNAc-phosphotransferase α/β -subunit precursor [accession number AY687932], and the following exons and the flanking intronic sequences: exon 1 [accession number BV677459], exon 2 [accession number BV677460], exons 3 and 4 [accession number BV677461], exon 5 [accession number BV677462], exons 6 and 7 [accession number BV677463], exons 8, 9, and 10 [accession number BV677464], exon 11 [accession number BV677465], exon 12 [accession number BV677466], exon 13 [accession number BV677467], exons 14 and 15 [accession number BV677468], exon 16 [accession number BV677469], exons 17 and 18 [accession number BV677470], exon 19 [accession number BV677471], exon 20 [accession number BV677472], and exon 21 [accession number BV677463])

IUBMB (International Union of Biochemistry and Molecular Biology) Enzyme Nomenclature, <http://www.chem.qmul.ac.uk/iubmb/enzyme/> (for accession numbers EC 2.7.8.17 and EC 3.1.4.45)

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for MLII, MLIIIA, and MLIIIC)

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