JCI The Journal of Clinical Investigation

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J Clin Invest. 1983;72(3):1016-1023. https://doi.org/10.1172/JCI111025.

Research Article

The genetic relationships between the multiple variants of mucolipidosis II (I-cell disease) and mucolipidosis III (pseudo-Hurler polydystrophy) were investigated with a sensitive genetic complementation analysis procedure. These clinically distinct disorders have defects in the synthesis of a recognition marker necessary for the intracellular transport of acid hydrolases into lysosomes. Both disorders are associated with an inherited deficiency of a uridine diphosphate-N-acetyl-glucosamine: lysosomal enzyme precursor N-acetyl-glucosamine-phosphate transferase activity. We had previously shown that both disorders are genetically heterogeneous. Complementation analysis between mucolipidosis II and III fibroblasts indicated an identity of mucolipidosis II with one of the three mucolipidosis III complementation groups (ML IIIA), suggesting a close genetic relationship between these groups. The presence of several instances of complementation within this group suggested an intragenic complementation mechanism. Genetic complementation in heterokaryons resulted in increases in N-acetyl-glucosamine-phosphate transferase activity, as well as in the correction of lysosomal enzyme transport. This resulted in increases in the intracellular levels of several lysosomal enzymes and in the correction of the abnormal electrophoretic mobility pattern of intracellular beta-hexosaminidase. The findings demonstrate that a high degree of genetic heterogeneity exists within these disorders. N-acetyl-glucosamine-phosphate transferase is apparently a multicomponent enzyme with a key role in the biosynthesis and targeting of lysosomal enzymes.

FIGURE 2

Mucolipidosis II and III

THE GENETIC RELATIONSHIPS BETWEEN TWO DISORDERS OF LYSOSOMAL ENZYME BIOSYNTHESIS

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ABSTRACT The genetic relationships between the multiple variants of mucolipidosis II (I-cell disease) and mucolipidosis III (pseudo-Hurler polydystrophy) were investigated with a sensitive genetic complementation analysis procedure. These clinically distinct disorders have defects in the synthesis of a recognition marker necessary for the intracellular transport of acid hydrolases into lysosomes. Both disorders are associated with an inherited deficiency of a uridine diphosphate-*N*-acetyl-glucosamine: lysosomal enzyme precursor *N*-acetyl-glucosamine-phosphate transferase activity. We had previously shown that both disorders are genetically heterogeneous. Complementation analysis between mucolipidosis II and III fibroblasts indicated an identity of mucolipidosis II with one of the three mucolipidosis III complementation groups (ML III_A), suggesting a close genetic relationship between these groups. The presence of several instances of complementation within this group suggested an intragenic complementation mechanism. Genetic complementation in heterokaryons resulted in increases in *N*-acetyl-glucosamine-phosphate transferase activity, as well as in the correction of lysosomal enzyme transport. This resulted in increases in the intracellular levels of several lysosomal enzymes and in the correction of the abnormal electrophoretic mobility pattern of intracellular β-hexosaminidase. The findings demonstrate that a high degree of genetic heterogeneity exists within these disorders. *N*-acetyl-glucosamine-phosphate transferase is apparently a multicomponent enzyme with a key role in the biosynthesis and targeting of lysosomal enzymes.

INTRODUCTION

Mucolipidosis (ML)[1] II (I-cell disease) and ML III (pseudo-Hurler polydystrophy) are inherited childhood neurometabolic disorders associated with defects in the biosynthesis of acid hydrolases and their targeting into lysosomes. Both disorders have the clinical manifestations of a connective tissue disorder. Children with ML II are affected at birth with severe psychomotor retardation, multiple skeletal dysplasias, hypotonia, and organomegaly. They rarely survive the first decade of life (1). The symptoms of ML III are subclinical until 3–5 yr of age and include milder skeletal dysmorphisms, growth retardation, joint stiffness, and absent-to-moderate mental retardation with survival of affected subjects generally extending into adulthood (2).

Both disorders have intracellular deficiencies of multiple hydrolases and characteristic inclusion bodies in certain cell types (3). Abnormally high levels of these enzymes are detected in the sera of affected subjects and in the medium in which ML II and ML III fibroblasts are cultured (4). Lysosomal enzymes secreted from these fibroblasts lack a recognition marker involved in receptor-mediated uptake from the extra-

This work was supported by National Institutes of Health grants HD 05196 to Dr. Shows; NS 12138 and Easter Seal Research Foundation grant N-7910 to Dr. Miller.

Received for publication 13 October 1982 and in revised form 12 April 1983.

[1] *Abbreviations used in this paper:* αFUC, α-fucosidase; αGAL, α-galactosidase; αMAN, α-mannosidase; βGAL, β-galactosidase; βHEX, β-hexosaminidase; GlcNAc, *N*-acetyl-glucosamine; GlcNAc-P transferase, uridine diphosphate *N*-acetyl-glucosamine: lysosomal enzyme precursor *N*-acetyl-glucosamine-1-phosphate transferase; ML, mucolipidosis; UDP, uridine diphosphate.

cellular space (5). This marker was identified to be mannose-6-phosphate groups present on the oligosaccharide chains of these enzymes (6–8). It also allows the binding of newly synthesized enzymes to intracellular receptors that transport them into lysosomes (9, 10). The lack of the mannose-6-phosphate marker (11, 12) results in the enhanced secretion of these enzymes and, consequentially, severe intracellular deficiencies. The primary defect in both ML II and ML III was shown to be a deficiency of uridine diphosphate *N*-acetyl glucosamine: lysosomal enzyme precursor *N*-acetyl-glucosamine-1-phosphate transferase (GlcNAc-P transferase) activity, which synthesizes the marker (13–17).

Although both the ML II and ML III disorders are associated with the GlcNAc-P transferase deficiency and share many of the biochemical characteristics of the affected lysosomal enzymes (18), there is biochemical and genetic evidence for heterogeneity within these disorders. In addition to the distinct differences in the severity of the ML II and ML III disorders, there are suggestions of clinical heterogeneity within the ML III disorder itself, particularly in the extent of mental retardation (2, 19). We have reported variations within both disorders in the residual activity of lysosomal enzymes, in their electrophoretic mobility, and lectin binding affinity that suggest heterogeneity (20, 21). Varki et al. (22), using an assay with an artificial acceptor molecule, α-methyl mannoside, have described an unusual variant of ML III that has a catalytically active GlcNAc-P transferase.

Previously, we and others have used genetic complementation analysis to demonstrate that both ML II and ML III are genetically heterogeneous (23–25). We now present complementation studies that explore the genetic relationship between these two disorders. Complementation was found to result in increases in GlcNAc-P transferase activity, the primary defect in these disorders, as well as in corrected lysosomal enzyme processing.

METHODS

Heterokaryon formation and enrichment. Human skin fibroblasts were derived from normal or clinically diagnosed ML II (24) and ML III (25) subjects. Cells with a GM prefix were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). All ML II and ML III fibroblasts have characteristic inclusion bodies visible under phase microscopy and intracellular deficiencies of multiple lysosomal hydrolases (Table I). Fibroblasts were cultured in Ham's F-12 growth medium with 10% fetal calf serum and antibiotics. Heterokaryons were formed by seeding flasks with $\sim 5 \times 10^6$ cells of each of two different parental cultures at a confluent density and incubating overnight. Cultures were fused with 42% (wt/vol) polyethylene glycol 1000 containing 9% (vol/vol) dimethyl sulfoxide in Dulbecco's minimal essential medium without serum (26). The fused culture was harvested after an additional 1–3 d culture, and the multinucleated cells were enriched by sedimentation velocity as previously described (27). The cells were allowed to sediment through a linear gradient of 1% bovine serum albumin to 5% Ficoll 400 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) in a Sta-Put apparatus (Johns Scientific, Toronto, Canada). The most rapidly sedimenting cell fractions containing polykaryocytes were isolated, confirmed to be multinucleated by phase-contrast microscopy, and cultured an additional 10–14 d before harvesting for analysis. This procedure allowed the nondestructive isolation of $\sim 10^6$ multinucleated cells that were enriched five- to eightfold. Each fusion was accompanied by an identical mixture of parental cells that was co-cultivated and harvested at the same time as fused cultures.

Enzyme assays and electrophoresis. The GlcNAc-P transferase activity toward exogenous acceptor was determined according to methods modified from Varki et al. (22) and Waheed et al. (17). Fibroblast cell pellets were homogenized in 62.5 mM Tris-HCl (pH 7.45), including 0.94% Triton X-100, 312.5 μM dithiothreitol, 2.5 mM iodoacetamide, and 125 μM leupeptin with a dounce-type homogenizer with a Teflon pestle. 40 μl of homogenates containing 150–300 μg protein and 10 μl of 1 M α-methyl mannoside were added to 1.5-ml centrifuge tubes containing 25 pmol uridine diphosphate (UDP)-[^{3}H]-*N*-acetyl-glucosamine (GlcNAc) (0.6 μCi, New England Nuclear, Boston, MA), 250 nmol ATP, 200 nmol ADP, 500 nmol cytosine diphosphate choline, 1 μmol $MgCl_2$, and 500 nmol $MnCl_2$ (these components were previously added and the solvent removed in vacuo) and incubated for 30 min at 37°C. Identical tubes containing, in addition, 50 μl of 50 mM EDTA were included as blanks. Reactions were terminated by adding 50 μl of 40 mM EDTA and heating tubes for 5 min in a boiling water bath. Samples were diluted with 1 ml of 2 mM Tris base, centrifuged at 12,000 *g* for 10 min, and applied to 0.5 × 2.5-cm columns of QAE-Sephadex equilibrated with 2 mM Tris base. Columns were washed with 3 ml 2 mM Tris base and the reaction product, [^{3}H]GlcNAc-P-α-methyl mannoside, selectively eluted with 6 ml 30 mM NaCl in 2 mM Tris base and quantitated by liquid scintillation. The rate of product formation was constant over the 30-min incubation and was proportional to the amount of fibroblast homogenate added up to 300 μg protein. GlcNAc-P transferase activity toward endogenous lysosomal enzyme precursors was determined as described by Reitman and Kornfeld (13), except that [^{32}P]UDP-GlcNAc was synthesized according to Owada and Neufeld (28). Both activities are expressed in picomoles of product formed per hour per milligram of homogenate protein.

The intracellular activities of lysosomal β-hexosaminidase (βHEX), β-galactosidase (βGAL), α-fucosidase (αFUC), α-galactosidase (αGAL), and α-mannosidase (αMAN) were measured on aqueous fibroblast homogenates with appropriate fluorigenic 4-methylumbelliferyl derivatives as described (24) and are expressed in nanomoles of substrate enzymatically hydrolyzed per hour per milligram of homogenate protein. Protein concentration was determined using a method modified from Lowry et al. (29).

Cellulose acetate electrophoresis was performed and the βHEX isoenzymes visualized with a fluorigenic enzyme-specific stain as previously described (20).

Homokaryon mixtures. To assess the effect of the fusion and purification procedures on lysosomal enzyme activities, a series of 14 homokaryon mixtures were prepared. Each consisted of two different ML II or ML III cultures that were fused separately (forming homokaryons), then combined, co-

purified, and co-cultivated exactly as described for the heterokaryon-forming fusions. Unfused, co-cultivated mixtures of the parental cells corresponding to each homokaryon mixture were also prepared and were used as the basis for comparing enzyme activities. The calculated variation in the enzyme activities of each homokaryon mixture with respect to its co-cultivated mixture of parental cells was, at most, 6% of the activity of normal cells. This is apparently the range of variability in enzyme levels due to the fusion and enrichment procedures, the variations in culture conditions, and the amount of error in the assay procedure. Increases in activity in heterokaryon-forming fusions greater than this range, therefore, indicated complementation. In all fusions scored complementation positive, the increases in at least four of the five enzymes measured were greater than the range of homokaryon mixtures.

RESULTS

Correction of GlcNAc-P transferase activity in complementing fusions. All the ML II and ML III fibroblast cultures are severely deficient in GlcNAc-P transferase activity toward endogenous (lysosomal enzyme precursor) acceptors (Table I). Most of these are also deficient in an assay with α-methyl mannoside as an exogenously added acceptor, although several ML III cultures have normal GlcNAc-P transferase activity with this assay, as previously noted (22, 25). The exogenous assay routinely used to score complementation utilized UDP-GlcNAc and α-methyl mannoside as the donor and acceptor molecules, respectively. This assay uses a commercially available tritiated donor rather than the [^{32}P]UDP-GlcNAc used in the Varki et al. (22) procedure, which requires synthesis and purification before use. The isotope used does not affect the measured GlcNAc-P transferase activity of either normal, ML II, or ML III fibroblasts using the exogenous acceptor. Fibroblasts derived from obligate ML II heterozygotes have activities intermediate between the ranges of normal and affected fibroblasts in this assay (0.80–1.92 pmol/h per mg), as expected for these autosomal recessive disorders.

The correction of GlcNAc-P transferase activity in ML II × ML III and ML II × ML II fusion experiments was scored by determining the enzyme activity in enriched heterokaryon-containing polykaryocytes and in corresponding co-cultivated mixtures of parental cells

TABLE I
ML II and III Fibroblast Enzyme Activities

	GlcNAc-P transferase		Lysosomal hydrolases				
Fibroblasts	Exogenous	Endogenous	βHEX	βGAL	αFUC	αGAL	αMAN
	pmol/h/mg		*nmol/h/mg*				
Normal (n = 10)	3.86±0.87	1.44±0.67	11,949±1,257	725±178	287±168	120±18	228±60
ML II							
LT	0.09	<0.02	734	100	5	23	20
CM	0.01	<0.02	427	7	4	5	16
MD	<0.01	<0.02	506	22	4	7	12
OA	<0.01	0.14	481	4	2	5	14
VT	<0.01	0.47	438	7	5	4	10
ML III							
Group A							
CW	0.37	<0.02	1,138	144	19	20	30
MB	1.96	<0.02	1,981	105	136	16	54
GM2425	0.07	<0.02	558	39	15	8	15
Group B							
RW	0.35	<0.02	805	136	11	9	20
Group C							
SR	3.94	—	470	53	2	13	8
AA	7.56	<0.02	1,398	171	1	44	32
TA	4.55	<0.02	573	66	1	10	7
GM3391	2.12	0.06	962	79	2	22	10

All enzyme activities were measured on fibroblast homogenates harvested at confluency. GlcNAc-P transferase activity was determined with either exogenous α-methyl mannoside or endogenous lysosomal enzyme precursors as acceptors and is expressed in picomoles per hour per milligram of homogenate protein (±SD). Lysosomal enzyme activities are the mean of at least five determinations and are expressed in nanomoles per hour per milligram of homogenate protein (±SD).

(Table II). An increase in the activity of the polykaryocyte fraction was detected in certain fusions, indicating that the defects complemented. The activity of a fused and enriched culture of normal fibroblasts was not significantly changed from normal activity, indicating that the GlcNAc-P transferase was not affected by the fusion or polykaryocyte enrichment procedures. Complementations were scored as positive when the activity of polykaryocytes increased by at least 7% of the activity of normal cells. The activity in complementing fusions was increased to approximately the level found in heteroygotes for these disorders. These fusions also showed evidence of corrected lysosomal enzyme processing, as discussed below.

Correction of intracellular lysosomal enzyme retention. The criteria for scoring fusions on the basis of the correction of lysosomal enzyme processing have been discussed in detail (25). All of the ML II and ML III fibroblasts studied are deficient in the intracellular activities of βHEX, βGAL, αFUC, αGAL, and αMAN (Table I). These activities are, in general, <15% of the activity of normal fibroblasts. The correction of intracellular enzyme activities was determined by measuring the activities of enriched polykaryocytes and comparing them with those of a co-cultivated mixture of parental cells for each fusion experiment. Increased intracellular enzyme retention was detected in complementing fusion experiments within 2 d and reached a maximum 10–14 d after fusion (Fig. 1), which appears to be close to the average turnover time of lysosomal enzymes (30). The activities of four different enzymes increased in concert, suggesting that assembly of the corrected gene product is the rate-limiting step in the restoration of intracellular lysosomal enzymes.

The changes in the intracellular enzyme levels due to complementation in 32 different ML II × ML III fusion experiments are listed in Table III. Increases in activity resulting from complementation were distinguished from variations in enzyme activity due to culture conditions and the fusion and enrichment procedures by comparing with the activity changes measured in a series of 14 different homokaryon mixtures (see Methods). Those experimental fusions that resulted in an average intracellular activity increase greater than that of the homokaryon mixtures were

TABLE II
GlcNAc-P Transferase Activity in ML II × ML II and ML II × ML III Fusions

	Co-cultivated	Fused	Percent change	Complementation
	pmol/h/mg			
Normal × normal	3.84	3.47		
II × III_A				
CM × CW	0.23	0.27	+1	−
CM × 2425	0.10	0.00	−2	−
OA × CW	0.30	0.05	−7	−
VT × 2425	0.00	0.00	0	−
LT × 2425	0.27	0.00	−8	−
LT × CW	0.24	1.65	+41	+
II × III_B				
MD × RW	0.00	0.69	+20	+
OA × RW	0.28	0.52	+7	+
VT × RW	0.09	0.40	+9	+
II × III_C				
CM × TA	3.40	4.75	+39	+
MD × SR	1.80	3.75	+56	+
VT × TA	1.23	2.02	+23	+
LT × SR	2.45	2.84	+11	+
II × II				
LT × CM	0.11	1.36	+36	+
LT × KZ	0.32	0.67	+10	+
LT × MD	0.00	0.00	0	−
LT × VT	0.09	0.07	−1	−
CM × VT	0.00	0.00	0	−
MT × VT	0.16	0.18	+1	−

The GlcNAc-P transferase activity of co-cultivated mixtures of parental cells and the multinucleated heterokaryon-containing cell fractions enriched from fused cultures were determined with an exogenous acceptor and are expressed in picomoles per hour per milligram of homogenate protein. The activity change is calculated as the difference between these activities, divided by that of normal cells.

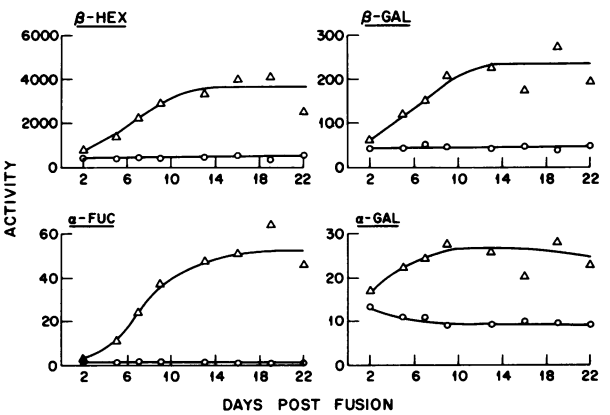

FIGURE 1 The time course of intracellular lysosomal enzyme correction. A culture consisting of an equal mixture of ML II (VT) and ML III (SR) fibroblasts was fused (day 0) and the multinucleated cell fraction was enriched by sedimentation velocity (day 1) as described in Methods. The intracellular activities of four lysosomal enzymes were determined on the heterokaryon-containing cell fraction (Δ) as well as a co-cultivated mixture of parental VT and SR cells (O) at various times following fusion and are expressed in nanomoles of substrate enzymatically hydrolyzed per hour per milligram of homogenate protein.

TABLE III

Correction of Intracellular Lysosomal Enzyme Activities

Fusions	Change in hydrolase activity						Correction of βHEX mobility	Complementation
	βHEX	βGAL	αFUC	αGAL	αMAN	Average		
	%							
Homokaryon mixtures								
Range (n = 14)	−3.0 to 5.0	−3.5 to 5.8	−5.1 to 4.0	−3.5 to 5.6	−1.6 to 4.0	−2.3 to 4.0	−	−
Heterokaryon fusions								
ML II × ML III_A								
CM × MB	−1.7	−2.6	10.9	−4.9	3.4	1.0	−	−
CM × CW	2.2	−0.6	0.4	1.0	0.9	0.8	−	−
CM × 2425	0.6	−2.0	−2.8	−0.8	−1.2	−1.2	−	−
MD × MB	0.9	−0.1	3.3	0.4	0.0	0.9	−	−
MD × CW	0.9	−1.4	3.8	1.4	0.6	1.1	−	−
MD × 2425	−1.5	−3.7	−7.5	−1.0	−4.2	−3.6	−	−
OA × MB	0.9	0.5	3.6	0.8	2.2	1.6	?	−
OA × CW	2.2	2.7	0.9	2.4	3.2	2.3	−	−
OA × 2425	−1.2	−1.9	−2.8	−0.6	−0.7	−1.6	−	−
VT × MB	3.0	1.5	5.3	2.0	1.0	2.6	−	−
VT × CW	−0.5	0.5	—	0.3	−10.0	−2.4	−	−
VT × 2425	−2.0	0.3	−0.7	0.8	−0.4	−0.4	−	−
LT × MB	12.1	20.8	28.6	16.2	16.4	18.8	?	+
LT × CW	6.6	13.8	8.3	9.5	8.8	9.4	+	+
LT × 2425	1.5	1.4	−4.0	2.2	−5.5	−0.9	−	−
ML II × ML III_B								
CM × RW	6.1	19.0	4.9	4.6	4.7	7.9	?	+
MD × RW	10.3	25.1	8.7	9.2	18.4	14.3	+	+
OA × RW	10.2	17.8	8.4	11.0	18.8	13.2	+	+
VT × RW	5.4	17.5	21.5	6.8	18.7	14.0	+	+
LT × RW	11.5	18.3	11.6	7.1	27.0	15.1	+	+
ML II × ML III_C								
CM × SR	5.2	12.2	6.7	9.6	3.2	7.4	+	+
CM × TA	27.6	34.5	41.6	32.8	23.9	32.1	+	+
CM × 3391	6.4	9.6	13.7	10.7	14.0	10.9	+	+
MD × SR	11.2	13.7	10.2	21.9	14.5	14.3	+	+
MD × AA	13.6	7.6	16.4	13.1	20.5	14.2	+	+
OA × SR	6.1	7.2	10.1	17.0	14.6	11.0	+	+
OA × AA	21.0	12.8	10.1	16.0	30.3	18.0	+	+
VT × SR	21.4	22.9	17.0	17.8	12.8	18.4	+	+
VT × TA	16.9	17.0	26.0	20.1	19.1	19.8	+	+
VT × 3391	10.3	15.6	10.3	9.3	10.8	11.3	+	+
LT × SR	8.1	7.6	4.4	7.3	11.6	7.8	+	+

The change in hydrolase activities is expressed as the difference in the activities of the co-cultivated mixture of parentals and the enriched multinucleated fraction, divided by the mean activity of normal cells for each enzyme. The activity changes for five different enzymes are averaged for each fusion experiment. The range of activity changes in 14 different pairs of co-cultivated homokaryons is listed as a control range. Activity increases greater than this range are considered significant increases due to complementation. The correction of the β-HEX mobility is scored qualitatively for each fusion as illustrated in Fig. 2.

scored complementation positive. 18 of the ML II × ML III fusions resulted in significant intracellular enzyme activity increases (Table III), including all of those fusions demonstrating corrections of GlcNAc-P transferase activity.

Correction of enzyme processing was also scored on the basis of the intracellular βHEX electrophoretic mobility pattern. The residual activity of ML II and III fibroblasts has an abnormal βHEX band intermediate to the normal A and B isozyme positions on cel-

lulose acetate gels (20). Complementing fusions resulted in a disappearance of this band and increases in the strength of the normal A and B isoenzymes (Fig. 2). Each of the ML II × ML III fusions were scored qualitatively (+ or −) for correction of this pattern (Table III). Several fusions could not be clearly scored and are marked "?". Except for these, there was complete correlation with the results of both GlcNAc-P transferase and lysosomal enzyme activity measurements.

Relationship between I-cell disease and pseudo-Hurler polydystrophy. The results of complementation analysis within ML II (24), between ML II and ML III, and within ML III (25) are summarized in Fig. 3. Our complementation analysis of ML III indicated that three distinct complementation groups exist within this disorder, designated as ML III_A, ML III_B, and ML III_C. Complementation analysis between ML II and each of these ML III groups indicates a close genetic relationship between the mutations of most of the ML II lines and those of the ML III_A group. Of the 16 fusions between ML II and ML III_A lines, only two were scored complementation positive (Table III), and these both involved the ML II line LT. This indicates an identity of the ML II and ML III_A complementation groups, suggesting that these clinically distinct disorders are the results of mutations that are alleles at the same locus. The fusions between ML II cells and those of ML III_B and ML III_C groups always resulted in detectable complementation (Fig. 3), indicating that the mutations in these complementation groups most likely occurred within different genes.

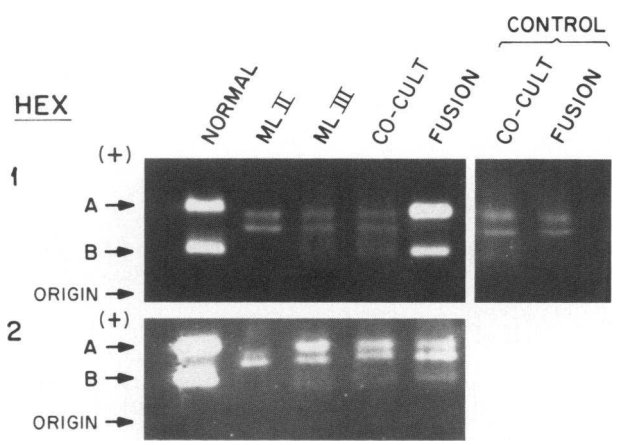

FIGURE 2 Correction of βHEX electrophoretic mobility pattern. Cellulose acetate electrophoresis was performed on fibroblast homogenate extracts and the βHEX isoenzymes visualized within a fluorigenic enzyme-specific stain. Gel 1 shows, from left to right: normal, CM, TA, CM plus TA co-cultivated, enriched CM × TA heterokaryons, CM plus TA co-cultivated, and an enriched mixture of CM and TA homokaryons. Gel 2 shows normal, OA, GM2425, OA + 2425 co-cultivated, and enriched OA × 2425 heterokaryons.

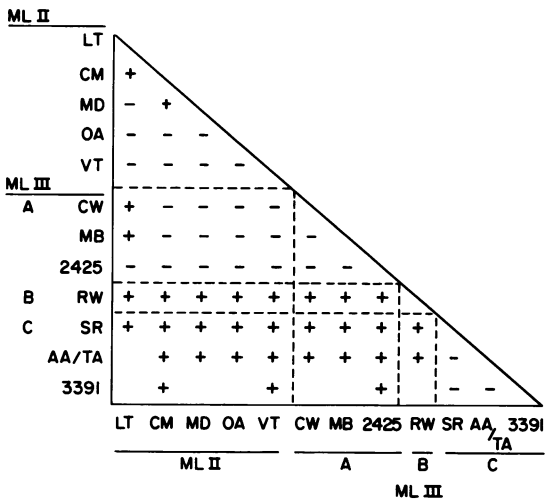

FIGURE 3 Summary of complementation tests within ML II and III. Complementation scores were based on (*a*) correction of GlcNAc-P transferase activity of 7% or more (as in Table II), (*b*) significant increases in the intracellular activities of several lysosomal enzymes (Table III) or (*c*) the qualitative correction of the βHEX electrophoretic mobility pattern (Fig. 2 and Table III). Where possible, all three tests were applied and were found to correlate.

The ML III_B group has RW as its sole member. The ML III_C group consists of three sibships (AA/TA, SR/TR, and GM3391/GM3392) out of the 12 ML III sibships examined thus far (25). The latter group of variants, therefore, may make up a significant subgroup of ML III subjects in the general population. They are readily distinguished from other ML III complementation groups by having normal GlcNAc-P transferase activity with α-methyl mannoside as the acceptor molecule (Table I).

Genetic heterogeneity within the ML II/ML III_A complementation group. We have described several instances of complementation in fusions within the ML II/ML III_A group. These included LT × CM, LT × KZ (Table II and reference 24), and CM × MD (unpublished data; 15.2% average increase of five lysosomal enzymes) among ML II × ML II fusions; also LT × MB and LT × CW among ML II × ML III_A fusions (Tables II and III). However, no distinct complementation groups have emerged after exhaustive analysis (Fig. 3). For example, the LT × CW (ML II × ML III_A) clearly showed evidence of complementation with a 41% increase in GlcNAc-P transferase activity and significant increases in intracellular lysosomal enzyme levels, yet fusions involving LT or CW with most other ML II or ML III_A fibroblasts did not complement (Fig. 3 and others [24]). These findings suggest that the mutations in all the ML II and ML III_A fibroblasts apparently occur in the same gene product or GlcNAc-P transferase component, but those mutations in the

fibroblasts involved in complementing fusions are somewhat structurally or functionally separated, allowing intragenic complementation and at least partial correction of GlcNAc-P transferase activity.

DISCUSSION

Although all of the ML II and ML III sibships studied have a deficiency of GlcNAc-P transferase activity, there is considerable genetic and biochemical heterogeneity within these disorders (19–25). We have demonstrated the existence of three distinct complementation groups within ML III (25) and somewhat less defined heterogeneity within ML II (24) that indicate the involvement of several genes. In this report we investigated the genetic relationships between these two clinically distinct disorders. Most of the ML II fibroblasts do not complement with those in one of the ML III groups (A), suggesting that they are due to mutations within the same gene. There is therefore no obvious genetic explanation for the clinical differences between these variants of ML II and ML III. These findings may be analogous to those in several other inherited enzyme deficiency disorders, i.e., the infantile and juvenile onset variants of G_{M1} gangliosidosis (31), Niemann-Pick disease (32), the sialidoses (33, 34), and the Hurler and Scheie syndromes (unpublished observation), where there are no apparent biochemical or genetic explanations for variations in the severity of a disorder. Analysis of these mutations on a molecular level may explain these phenomena.

The detection of several instances of complementation within the ML II/ML III_A group extends our previous findings of heterogeneity within ML II (24). None of the fibroblasts involved in the five complementing fusions formed a distinct group (Fig. 3), suggesting that the complementations within this group may be due to an intragenic mechanism. One of the ML II fibroblasts involved in several of these fusions (LT) has distinctly different residual activities (Table I) and electrophoretic mobilities of several lysosomal enzymes (20, 24). It is possible that this variant belongs in a different complementation group and that complementation was prevented by some unknown mechanism in the several fusions between LT and other ML II or ML III_A fibroblasts that were scored negative. Even so, the CM × MD fusion clearly resulted in complementation and it remains as a likely instance of intragenic complementation.

The genetic heterogeneity in these disorders results in variations in the residual levels of lysosomal enzymes (Table I) and in their biochemical properties (21, 35). In many instances, complementation resulted in large activity increases in certain enzymes (i.e., βGAL in several fusions) and somewhat smaller increases in other enzymes (Table III). These discrepancies from a coordinated increase may be due to variations in the rates of processing and maturation among different enzymes. The rate of maturation of α-iduronidase, for instance, was shown to be much slower than other enzymes (36).

The GlcNAc-P transferase is apparently a key enzyme in the biosynthesis and intracellular transport of lysosomal enzymes. The genetic heterogeneity in the human disorders involving this enzyme suggests that it is a complex multisubunit enzyme. The biochemical analysis of the mutation in each of the genetic variants may indicate the function of the defective enzyme components. Varki et al. (22), using α-methyl mannoside as the acceptor, have described an ML III variant, GM3392 (sibling of GM3391, ML III_C), which has normal GlcNAc-P transferase activity, yet it shows no activity toward endogenous lysosomal enzymes. They show that GM3392 has a normal Michaelis-Menten constant (K_m) toward purified lysosomal enzyme oligosaccharides, indicating that the binding of these substrates is unaffected. They suggest that the GlcNAc-P transferase may also be involved in the recognition mechanism by which lysosomal enzyme precursors are distinguished from other glycoproteins (which are not phosphorylated), and that it is this enzyme component that is defective in GM3392. Our complementation analysis has identified two other sibships (SR/TR and AA/TA) with similar characteristics (25, Table I), suggesting that these variants may make up a significant subgroup of the ML III disorder. Our preliminary kinetic analysis of the GlcNAc-P transferase deficiency in the ML II/ML III_A and ML III_B complementation groups also suggests that each group has a characteristic mutation (unpublished observation). The precise identification of the mutations in each of the ML II and ML III variants will further our understanding of lysosomal enzyme biosynthesis and will suggest avenues toward treatment of these disorders.

ACKNOWLEDGMENT

The authors would like to thank Ms. C. Young for expert secretarial assistance in the preparation of this manuscript.

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