Am. J. Hum. Genet. 43:756–763, 1988

Identification of a Mutation in the Structural α-L-Fucosidase Gene in Fucosidosis

Patrick J. Willems,*,† John K. Darby,‡,§ Richard A. DiCioccio,‖ Phil Nakashima,‡ Christine Eng,# Keith A. Kretz,* Luca L. Cavalli-Sforza,‡ Eric M. Shooter,§ and John S. O'Brien*

*Department of Neurosciences, University of California, San Diego; † Department of Medical Genetics, University of Antwerp, Belgium; Departments of ‡Genetics and §Neurobiology; ‖Department of Gynecologic Oncology, Roswell Park Memorial Institute, Buffalo; and #Department of Medical Genetics, Mount Sinai Medical Center, New York

Summary

Fucosidosis is an autosomal recessive lysosomal storage disorder characterized by progressive neurological deterioration and mental retardation. The disease results from deficient activity of α-L-fucosidase (E.C.3.2.1.51), a lysosomal enzyme that hydrolyzes fucose from fucoglycoconjugates. In an attempt to identify the mutation(s) that result(s) in fucosidosis, we performed Southern blot analysis of the structural gene encoding α-L-fucosidase (FUCA 1) in 23 patients affected with fucosidosis. In five patients Southern blot analysis showed obliteration of an *Eco*RI restriction site in the open reading frame of FUCA 1 encoding mature α-L-fucosidase. This abnormality was not observed in 80 controls, and it may be the basic defect responsible for fucosidosis in these patients. Both patients with the severe type I form of fucosidosis and patients with the less severe type II were shown to be homozygous for this presumed mutation. In the remaining 18 patients the *Eco*RI site obliteration, major-gene deletions, or insertions were not detected. This suggests that at least two different mutations are involved in fucosidosis. The heterogeneity found at the DNA level was not present at the protein level, as all fucosidosis patients investigated had low fucosidase protein (<6% of normal) and negligible fucosidase activity in fibroblasts and lymphoblastoid cell lines.

Introduction

Fucosidosis is a rare inborn error of metabolism with autosomal recessive inheritance (Durand et al. 1966, 1969; Loeb et al. 1969; Kousseff et al. 1976). The clinical picture consists of progressive mental and motor deterioration, hepatosplenomegaly, dysostosis multiplex, angiokeratoma corporis diffusum, and growth retardation (for review, see Durand et al. 1982). At the biochemical level, fucosidosis results from nearly complete deficiency of α-L-fucosidase activity (Van Hoof and Hers 1968). This lysosomal hydrolase cleaves fucose from fucoglycoconjugates and appears to be the only α-L-fucosidase active in mammalian tissue. Deficient activity of this enzyme leads to accumulation of fucose-containing glycolipids and glycoproteins in various tissues (Van Hoof 1973; Warner and O'Brien 1983).

In view of the observed clinical heterogeneity in fucosidosis, two major subtypes have been delineated (Gatti et al. 1973; Kousseff et al. 1973). The severe type I fucosidosis is characterized by rapidly progressive neurological deterioration leading to decerebration and death before the age of 10 years (Durand et al. 1969; Loeb et al. 1969). In the less severe type II, neurological deterioration is slower, survival into adulthood is common, and most patients develop angiokeratoma corporis diffusum (Patel et al. 1972; Kousseff et al. 1976). The clinical heterogeneity is not due to different nonallelic mutations, as complementation studies between type I and type II did not restore α-L-fucosidase activ-

Received November 5, 1987; revision received May 16, 1988.
Address for correspondence and reprints: John S. O'Brien, M.D., Department of Neurosciences, Center for Molecular Genetics (M-034), University of California–San Diego, La Jolla, CA 92093.
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0002-9297/88/4305-0024$02.00

ity (Beratis et al. 1977). The possible existence of different coallelic mutations in the fucosidase gene, resulting in different clinical phenotypes, has not yet been investigated.

We have previously cloned and sequenced several cDNAs for the structural α-L-fucosidase gene (FUCA 1) (de Wet et al. 1984; Fukushima et al. 1985; O'Brien et al. 1987). Using RFLPs identified at the FUCA 1 locus (Darby et al. 1986), we found evidence that the fucosidosis mutation resides in FUCA 1 (Darby et al. 1988). Therefore, we performed Southern blot analysis of FUCA 1 to define and characterize possible fucosidosis mutation(s).

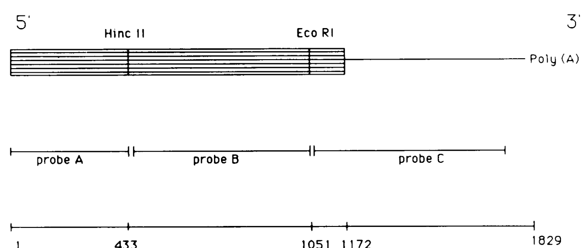

Figure 1 Schematic diagram showing the cDNA encoding human α-L-fucosidase (FUCA 1) and the three probes A–C used in the Southern blot analysis. The hatched box represents the open reading frame (1–1172 bp), which lacks the 5′ end encoding the NH_2 terminus of α-L-fucosidase. The poly $(A)^+$ tail starts at bp 1807. A *Hinc*II site separates probe A from probe B. The only internal *Eco*RI site present in the cDNA at bp 1047–1053 separates probe B from probe C.

Material and Methods

Cell Lines

Cultured fibroblast cell lines of fucosidosis patients were obtained from P. Durand (SU, MI, and ST), D. Wenger (FV, BL, FC, JC, and LA), J. Libert (SS, MS, and MB), R. Martin-Jimenez (DG and RP), P. Ferreira (EN), C. Garcia (RL), L. Poenaru (DS and ZE), J. Kunze (CE), S. Puck (JT), J. A. Lowden (JB), G. Donnell (MZ and GZ), B. Echenne (DM and SM), K. Hirschhorn (GM and RM), and J. Troost (CN). Lymphoblastoid cell lines were established from Epstein-Barr-transformed peripheral lymphocytes from fucosidosis patients of G. Donnell (MZ), K. Hirschhorn (RM and GM), and S. Puck (JT) after informed consent was obtained.

α-L-Fucosidase Assays

Prior to α-L-fucosidase assay, fibroblast and lymphoblastoid cell lines were harvested, lysed with 0.1% Triton X-100, and homogenized by gentle pipetting. The final fucosidase assay mixture contained 0.15 M acetate (pH 5.0) and 0.5 mM 4-methylumbelliferyl-α-L-fucopyranoside (Sigma Chemical Co., St. Louis). The specific enzymatic fucosidase activity was expressed in nanomoles 4-methylumbelliferone (4MU) liberated per minute per milligram total cellular protein. Total cellular protein was determined by the Bradford (1976) method using Bio-Rad reagent (Richmond, CA). Cross-reacting immunological material (CRIM) against α-L-fucosidase was determined using a quantitative ELISA assay employing antibody raised in rabbits against purified α-L-fucosidase (DiCioccio et al. 1986). CRIM was expressed as percent of total cellular protein. The α-L-fucosidase enzymatic activity was calculated from the ratio of specific enzymatic activity to CRIM and expressed in nanomoles 4MU liberated per minute per milligram of fucosidase protein.

Southern Blot Analysis

DNA from buffy coats prepared from whole blood of fucosidosis patients and controls and from cultured fibroblasts and lymphoblasts from fucosidosis patients was extracted as described in the accompanying paper (Darby et al. 1988). DNA (10–15 μg) was digested with 18 different restriction enzymes (*Eco*RI, *Pvu*II, *Bgl*I, *Hin*dIII, *Taq*I, *Tth*III, *Msp*I, *Mbo*I, *Bgl*II, *Rsa*I, *Sac*I, *Hin*fI, *Pst*I, *Xba*I, *Hin*cII, *Sty*I, *Sau*3A, and *Bsm*I). Electrophoresis, Southern blot transfer, prehybridization, hybridization, washing, and autoradiographing were performed as described in the accompanying paper (Darby et al. 1988). Three adjacent DNA fragments—A–C—of a cDNA encoding the human structural fucosidase gene FUCA 1 (O'Brien et al. 1987) were used as probes (fig. 1).

Results

α-L-Fucosidase Assay

The α-L-fucosidase-specific enzymatic activity in 21 fibroblast cell lines and three lymphoblastoid cell lines of a total of 22 patients affected with fucosidosis was negligible (table 1). CRIM against mutant α-L-fucosidase was 2.2 ± 1.3% of normal in fibroblasts and 1.3 ± 0.9% of normal in lymphoblastoid cell lines. No biochemical heterogeneity in this group of 22 fucosidosis patients was discovered (table 1). Six obligate carriers of fucosidosis had specific enzymatic fucosidase activity of 29.22 ± 15.97% of normal and 53.05 ± 50.58% of normal in fibroblasts and lymphoblastoid

Table 1

¼-L-Fucosidase in Fibroblast and Lymphoblastoid Cell Lines

Source	Specific Enzymatic Activity		CRIM		Enzymatic Activity	
	Total Protein (nmol 4MU/min/mg)	Control (%)	Total Protein (%)	Control (%)	Fucosidase Protein (nmol 4MU/min/mg)	Control (%)
Fibroblasts:						
Controls (N = 9)[a]	1.59 ± .70	100.00 ± 43.84	.01098 ± .00475	100.0 ± 43.3	14,309 ± 1,424	100.0 ± 10.0
Patients						
FC[c]	Negligible[b]	Negligible[b]	.00018	1.6		
JC[c]			.00017	1.5		
RL			.00056	5.1		
SU			.00020	1.8		
MI			.00029	2.6		
ZE			.00031	2.8		
DS			.00027	2.4		
DG			.00006	.5		
EN			.00029	2.6		
CE			.00017	1.5		
ST			.00018	1.6		
MS			.00012	1.1		
BL			.00007	.6		
LA			.00019	1.7		
GM			.00023	2.1		
RM			.00033	3.0		
SM			.00063	5.7		
JT			.00022	2.0		
FV			.00026	2.4		
MB			.00015	1.4		
MZ			.00033	3.0		
Mean ± SD			.00025 ± .00014	2.2 ± 1.3		
Heterozygotes:						
Father FC/JC	.6730	42.42	.00692	63.0	9,722	68.0
Mother FV	.2521	15.89	.00157	14.3	15,978	111.7
	.8335	52.54	.00700	63.8	11,905	83.2
Father MZ/GZ	.3524	22.21	.00238	21.7	14,800	103.4
Mother MZ/GZ	.1770	11.16	.00103	9.4	17,166	120.0
Mother JG	.4938	31.13	.00333	30.3	14,814	103.5
Mean ± SD	.4636 ± .2533	29.22 ± 15.97	.00370 ± .00264	33.7 ± 24.0	14,064 ± 2,753	98.3 ± 19.2

Lymphoblasts:						
Controls (N = 19)[a] . . .	2.5 ± 1.5	100.00 ± 59.85	.01116 ± .0060	100.0 ± 53.8	22,353 ± 2,515	100.0 ± 11.3
Patients:						
MZ	Negligible[b]	Negligible[b]	.00005	.5		
GM	—	—	.00012	1.1		
RM			.00024	2.2		
Mean ± SD	.0007 ± .0004	.03 ± .02	.00014 ± .000010	1.3 ± .9	543 ± 94	2.4 ± .4
Heterozygotes:						
Mother GM/RM . . .	3.1833	127.32	.01280	114.7	24,834	111.1
Father GM/RM	1.0500	42.00	.00450	40.3	23,333	104.4
Father MZ/GZ	.65	25.92	.00333	29.8	19,444	87.0
Mother MZ/GZ . . .	.4242	16.97	.00212	19.0	20,000	89.5
Mean ± SD	1.33 ± 1.27	53.05 ± 50.58	.00569 ± .00484	50.9 ± 43.4	21,903 ± 2,602	98.0 ± 11.6

[a] Data are mean ± SD.
[b] Raw data were less than double background in assays employing 10–50 times more protein than assays with extracts from control cell lines.
[c] Affected sibs.

cell lines, respectively. CRIM levels were 33.7 ± 24.0% of normal in fibroblasts and 50.9 ± 43.4% of normal in lymphoblasts. The enzyme activity per milligram fucosidase protein was normal in both heterozygote cell lines.

Southern Blot Analysis

When genomic DNA extracted from lymphoblastoid cell lines of four fucosidosis patients (GM, RM, JT, and MZ) from three different families, (M, T, and Z) was cut with a panel of 11 different restriction endonucleases and hybridized successively to three adjacent cDNA probes A–C (fig. 1), no abnormalities on the Southern blots were detected. When *Eco*RI digests of DNA from two affected sibs RM and GM (family M) were blotted, an extra 6.0-kb band hybridized to probe B, and the intensity of the normal 4.4-kb band was reduced (fig. 2). As probe B has an *Eco*RI site at its 3′ end, we rehybridized the original filter with the adjacent probe C, which has this *Eco*RI site at its 5′ end (fig. 1). An extra band of exactly the same size (6.0 kb) was present (fig. 2). Therefore, the 6.0-kb fragment was generated by obliteration of the *Eco*RI site between the normal 4.4-kb fragment (visible on blots hybridized to probe B) and the normal 1.6-kb fragment (visi-

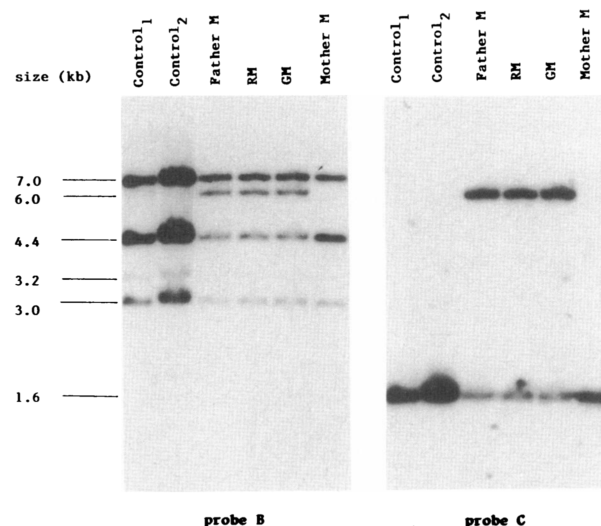

Figure 2 Southern blot analysis of *Eco*RI-digested DNA from two sibs (RM and GM), affected with fucosidosis, the father, the mother, and two controls. The same filter was hybridized successively with probe B and the adjacent probe C. In both panels an extra 6.0-kb band is present in the two patients and the father. The normal 7.0-, 4.4-, 3.2-, and 3.0-kb bands (probe B) and 1.6-kb band (probe C) are present in each individual.

ble on blots hybridized with probe C). Both RM and GM are heterozygous for the abnormality, since the 4.4-kb band (probe B) and the 1.6-kb band (probe C) are still present, although at approximately half intensity. The *Eco*RI abnormality segregated through the paternal line in family M and was detected in the two affected sibs, the father, the paternal aunt, and a brother of the paternal grandmother. The patients' mother and paternal grandfather had a Southern pattern identical to that of the controls. Complete cosegregation of the paternal fucosidosis mutation and the *Eco*RI abnormality was present in family M (fig. 3). The genotypes with respect to fucosidosis had been assigned previously (Turner et al. 1975) through pedigree analysis and isoelectric focusing.

In a panel of 17 additional unrelated patients (for a total of 20 unrelated patients) affected with fucosidosis, blots from three more patients—SU, DG, and RP—showed the extra 6.0-kb band (fig. 4; RP not shown). In contrast to GM and RM, however, the Southern blots did not show the normal 1.6-kb band after hybridization with probe C (fig. 4), nor the normal 4.4-kb band after hybridization with probe B (results not shown). Consequently, SU, DG, and RP are homozygous for the 6.0-kb band, while GM and RM are heterozygous for this abnormal band. In a survey of 80 normal subjects the abnormal 6.0-kb *Eco*RI band was not detected. All seven individuals (five patients and two heterozygotes) with the obliterated *Eco*RI site had the 6.0-kb allele for the *Pvu*II RFLP (results not shown).

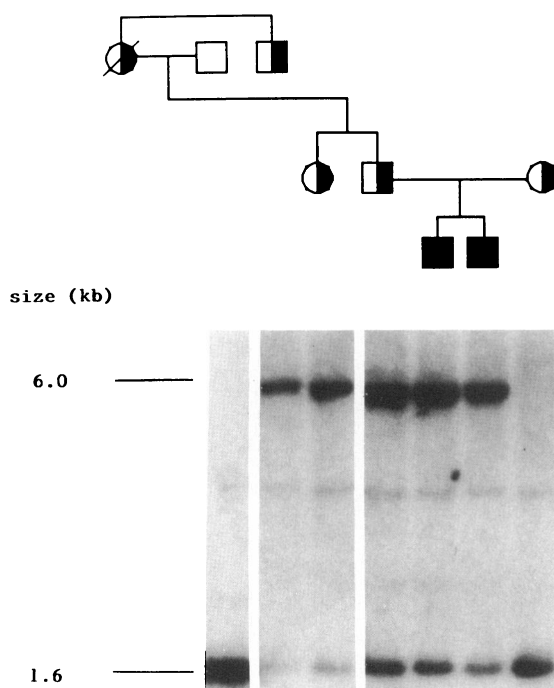

Figure 3 Pedigree and Southern blot analysis of family M. *Top:* Pedigree of family M with patients affected with fucosidosis (■) and carriers of the fucosidosis mutation (◑ and ◨). *Bottom:* Southern blot hybridization of probe C to *Eco*RI-digested DNA of corresponding family members. There is complete cosegregation of the fucosidosis mutation with the *Eco*RI abnormality.

Discussion

Fucosidosis is a heterogeneous disorder at the clinical level and has been classified into a severe type I and a less severe type II. Because of concordance in clinical type in sibs affected with fucosidosis, Kousseff et al. (1973) suggested that the phenotypic differences between the two types are genetically determined by different mutations in the fucosidase gene. This, however, is in contradiction with the presence of both clinical types within the same family, a situation described by Durand et al. (1982), Christomanou and Beyer (1983), and Willems et al. (1988).

At the protein level no heterogeneity was observed in 22 fucosidosis patients (table 1), since all were severely CRIM deficient. This is in agreement with the previously reported substantial reduction of fucosidase protein in fucosidosis patients studied by Thorpe and Robinson (1978), Alhadeff and Andrews-Smith (1980), Andrews-Smith and Alhadeff (1982), and Johnson and Dawson (1985). We did not determine CRIM against fucosidase in the cell culture medium after NH_4Cl treatment and cannot exclude the existence of a precursor protein, which is either processed into an unstable mature protein or not processed at all. Such a processing defect was reported by Johnson and Dawson (1985) in two fucosidosis patients (our patients SS and JC).

The absence of accurately measurable enzymatic activity of fucosidase despite the presence of detectable CRIM in all fucosidosis patients suggests the existence of a kinetically altered mutant enzyme. The reduced quantity of mutant enzyme may be due to instability. This is in accordance with earlier work showing increased thermolability (DiMatteo et al. 1976; Troost et al. 1976; Thorpe and Robinson 1978; Alhadeff and Andrews-Smith 1980), increased K_m values (DiMatteo et al. 1976; Troost et al. 1976; Alhadeff and Andrews-Smith 1980), abnormal pH optimum curves (Beratis et al. 1977), and a different pattern on isoelectric focusing (DiMatteo et al. 1976; Alhadeff and Andrews-Smith 1980) of mutant fucosidase enzyme. Since a polyvalent

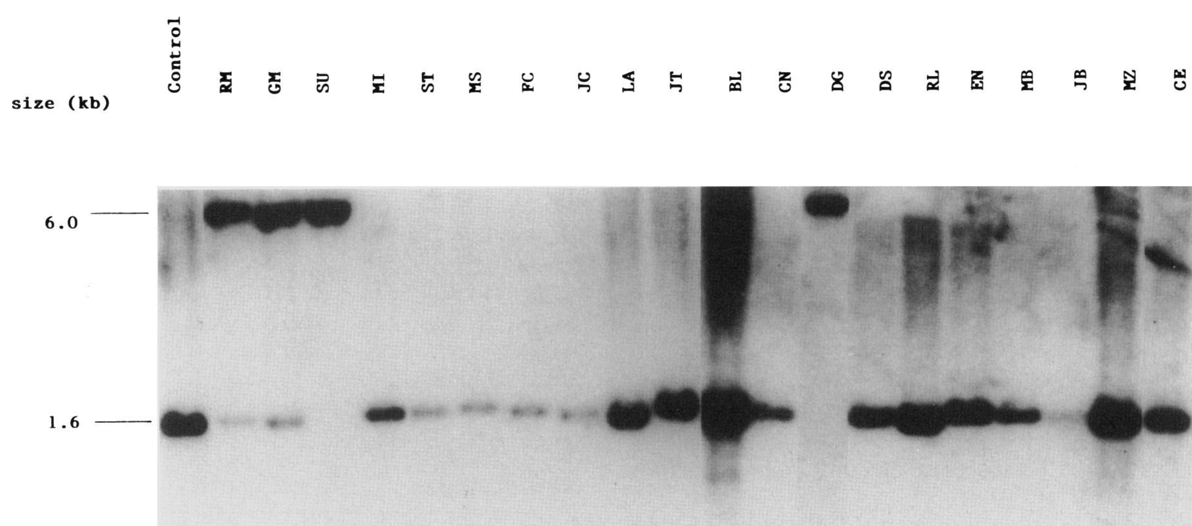

Figure 4 Southern hybridization of probe C to *Eco*RI-digested DNA from 20 different fucosidosis patients and a control. The normal 1.6-kb signal is absent in patients SU and DG, while the abnormal 6.0-kb fragment is present in patients RM, GM, SU, and DG.

fucosidase antibody in the ELISA assay was used (DiCioccio et al. 1986), the small amount of CRIM found in the fucosidosis patients could include some nonfucosidase CRIM, and some fucosidosis patients studied here may have a total absence of fucosidase protein in fibroblasts and/or lymphoblasts.

Extensive Southern blot analyis with the 1,829-bp cDNA FUCA 1 probe (O'Brien et al. 1987) did not detect large gene deletions or insertions in four fucosidosis patients for which lymphoblastoid cell lines were available. The only observed abnormality was the *Eco*RI site obliteration in four different families. This *Eco*RI site is located in the open reading frame encoding mature α-L-fucosidase at bp 1047–1053 of the fucosidase cDNA, separating probe B and probe C (O'Brien et al. 1987). Mapping of the normal structural α-L-fucosidase gene FUCA 1 showed that this *Eco*RI site is situated at the 3′ end of the gene (Darby et al. 1988). Evidence that the *Eco*RI-site alteration probably reflects the actual fucosidosis mutation in these patients, rather than an RFLP, was obtained in three ways. First, the mutation was detected in four of 20 unrelated fucosidosis patients and was not observed in 80 control individuals of Caucasian origin, making it statistically (Fisher's exact probability test: $P = .0001$) improbable that the *Eco*RI-site alteration is a harmless RFLP. In β-thalassemias and sickle cell anemia (Antonarakis et al. 1985), in carbamyl phosphate synthetase I deficiency (Fearon et al. 1985), and in phenylketonuria (DiLella et al. 1986), RFLPs are found in linkage disequilibrium with the mutation causing the disease. These situations are different from fucosidosis, as the alleles in linkage disequilibrium with these diseases are also found in the normal population. Only in α_1-antitrypsin deficiency has the disease mutation been found in linkage disequilibrium with a polymorphic allele that is not present in the control population (Cox et al. 1985). Second, the *Eco*RI abnormality segregated concordantly with the fucosidosis mutation in the families of GM and RM (fig. 3) and in DG and RP (total of 14 individuals). Third, the *Eco*RI site involved is located within the open reading frame of FUCA 1 encoding for mature α-L-fucosidase (O'Brien et al. 1987), whereas the majority of RFLPs are present in introns (Cooper and Schmidtke 1984). It is likely that the fucosidosis mutation is a small deletion or single base change obliterating the *Eco*RI site, since the addition of the normal 4.4-kb band (probe B) and the normal 1.6-kb band (probe C) equals the size of the 6.0-kb mutant band. Furthermore, the *Eco*RI site alteration was not detected with a panel of 17 additional restriction endonucleases. Two of those enzymes, *Sau*3A and *Bgl*II, have restriction sites close to the *Eco*RI site, respectively 10 bp and 12 bp to the 3′ end of the latter site. Of the 18 possible point mutations changing the *Eco*RI site, four were excluded because they would create new restriction sites that were shown not to be present (P. J. Willems, J. K. Darby, R. A. DiCioccio, P. Nakaskima, C. Eng, K. A. Kretz, L. L. Cavalli-Sforza, E. M. Shooter, and J. S. O'Brien, (unpublished data). Of the remaining possi-

bilities, four are transitions, of which one creates a stop codon in the open reading frame encoding mature fucosidase. This could encode for a truncated protein which might be inactive or be rapidly degraded.

The identification of a mutation in FUCA 1 in fucosidosis confirms that the disease resides in this gene. Further evidence was provided in two ways. First, the fucosidosis mutation in family M was assigned to chromosome 1 (Turner et al. 1978), in accordance with the mapping of α-L-fucosidase to chromosome 1p34.1–1p36.1 (Fowler et al. 1986). Second, linkage analysis using RFLPs identified at the FUCA 1 locus (Darby et al. 1986, 1988) revealed that the fucosidosis mutation is linked to the FUCA 1 locus in two different families, with significant lod scores at a recombination fraction of 0 (Darby et al. 1988).

The obliteration of the *Eco*RI site in FUCA 1 was not detected in the remaining 18 fucosidosis patients. Thus, at least two different mutations must be involved in fucosidosis. This is also evident in family M, where the two sibs appear to be compound heterozygotes for two different mutant alleles, and is compatible with the observed segregation of the two different fucosidosis mutations with different alleles of the *Pvu*II RFLP in this family (results not shown). As described in the accompanying paper (Darby et al. 1988), there is linkage disequilibrium between the *Eco*RI mutation(s) and the 6.0-kb allele of the *Pvu*II RFLP. All seven individuals with the obliterated *Eco*RI site were found to carry the 6.0-kb allele for the *Pvu*II RFLP, and it is unlikely that this is coincidental (Fisher's exact probability test: $P = .019$). One explanation is that the *Eco*RI-site obliteration occurred in an ancestor carrying the 6.0-kb allele of the *Pvu*II RFLP and that the five fucosidosis patients with the *Eco*RI-site obliteration are descendants of this individual.

The clinical phenotype of RM, GM, and RP is compatible with type II fucosidosis, as these three patients are still alive at the age of 18, 23, and 24 years, respectively, whereas SU and DG are affected with type I fucosidosis. SU died at the age of 4 years 9 mo. and DG died at age 10 years; DG's affected sibling also died at 4 years (Kessler et al. 1981). As RM and GM are compound heterozygotes of two different mutations, their clinical phenotype cannot be compared with that of RP, SU, and DG, who are homozygous for the mutation obliterating the *Eco*RI restriction site. However, RP, SU, and DG appear to have the same mutation, although their clinical phenotypes are clearly different. If it is true that the *Eco*RI abnormality is the primary mutation in these patients, the differences in their phenotypes may be due to environmental facotrs or other as yet unidentified allelic or nonallelic determinants.

Acknowledgments

We are indebted to G. Dawson, D. Wenger, C. Garcia, O. Poenaru, W. Lissens, J. Barranger, R. Martin-Jimenez, S. Puck, P. Ferreira, G. Donnell, R. Matalon, and M. Zabot for blood samples or fibroblast cell lines of fucosidosis patients. We also thank R. Martin-Jimenez, G. Romeo, R. Gatti, and P. Durand for clinical information. This work was funded by NIH grants NS 08682 and HD 18983 and Weingart Foundation support to J. S. O'Brien and by NIH grants GM 28428 to J. K. D., HD07105 to C. E., and DK 3216103 to R.D.

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