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High-Resolution Mapping of the Gene for Cystinosis, Using Combined Biochemical and Linkage Analysis

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Summary

Infantile nephropathic cystinosis is an autosomal recessive disorder characterized biochemically by an abnormally high intracellular content of free cystine in different organs and tissues due to a transport defect of cystine through the lysosomal membrane. Affected children present with the Fanconi syndrome and usually develop progressive renal failure within the 1st decade of life. Measurement of free cystine in purified polymorphonuclear leukocytes provides an accurate method for diagnosis and detection of heterozygous carriers. In order to localize the gene locus for cystinosis we performed linkage analysis in 18 cystinosis families. However, since 17 of these were simplex families, we decided to include the phenotypes of the heterozygous carriers previously determined by their leukocyte cystine content in the linkage analysis. This approach allowed us to obtain highly significant results, confirming the localization of the cystinosis gene locus recently mapped to the short arm of chromosome 17 by the Cystinosis Collaborative Research Group. Crucial recombination events allowed us to refine the interval of the cystinosis gene to a genetic distance of 1 cM. No evidence of genetic heterogeneity was found. Our results demonstrate that the use of the previously determined phenotypes of heterozygous carriers in linkage analysis provides a reliable method for the investigation of simplex families in autosomal recessive traits.

Introduction

Infantile nephropathic cystinosis, the most frequent form of cystinosis, is an autosomal recessive disorder caused by a defect in the transport of cystine through the lysosomal membrane to the cytosol, where it is normally reutilized (Gahl et al. 1982; Jonas et al. 1982). Cystine is poorly soluble and forms crystals in the lysosomes as its concentration increases. Cystinosis affects 1/100,000–1/200,000 children (Bois et al. 1976). Patients develop a de Toni-Debré-Fanconi syndrome and suffer a progressive decline of glomerular filtration rate, with end-stage renal failure occurring before the age of 10 years (Lemire and Kaplan 1984). Extrarenal symptoms are due to intracellular accumulation of cystine in different tissues and organs, resulting in photophobia, retinopathy, hypothyroidism, insulin-dependent diabetes, muscular weakness, and CNS involvement (Gahl et al. 1995).

Therapy of cystinosis consists of supportive treatment to replace substances lost from renal tubules, administration of cysteamine or phosphocysteamine, which reduce the intracellular cystine content and have been shown to limit renal deterioration, and renal transplantation in children with end-stage renal disease (Markello et al. 1993).

The disease can be diagnosed by the detection of large amounts of intracellular free (acid-soluble) cystine (~100 times normal) in peripheral blood leukocytes or fibroblasts of affected individuals (Schneider et al. 1967*a*, 1967*b*). Cells from heterozygotes contain about five times the usual amount of free cystine (Schneider et al. 1967*a*), although they are clinically unaffected. The measurement of free cystine in purified polymorphonuclear leukocytes (PMNL) by use of a cystine binding protein provides an accurate method, which is sensitive enough to allow the detection of heterozygous carriers (Oshima et al. 1974; Smith et al. 1987).

Although there is strong evidence that the biochemical defect in cystinosis is localized within the lysosomal membrane, in the absence of potential candidate genes, we employed linkage analysis to map a gene responsible for cystinosis. Since most of the examined families were simplex families, including the results of intracellular cystine content in the linkage analysis considerably changed the lod scores to highly significant values. We

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had already excluded 50% of the entire human genome as a possible gene locus, when the assignment of the cystinosis locus was initially demonstrated by the Cystinosis Collaborative Research Group to be on the short arm of chromosome 17 (Cystinosis Collaborative Research Group 1995). We confirmed linkage to this chromosomal region by examination of nine microsatellite markers with 18 cystinosis families.

Subjects and Methods

Subjects

Eighteen two- or three-generation families (1 multiplex and 17 simplex families) were included in this study. Affected individuals presented with infantile cystinosis before 1 year of age, with proximal tubular damage, elevated leukocyte cystine levels, and progressive renal failure. Two families originated from Portugal (families 15 and 17), 2 from Morocco (families 2 and 16), 1 from Belgium (family 18), and 13 from France (families 1 and 3–14), including 2 from Brittany (families 8 and 13). A total of 160 meioses were assessed, 19 of which were affected individuals. Three of 18 families were consanguineous (families 13, 16, and 17).

Detection of Heterozygotes

Subjects were divided into unaffected individuals (*normals*), obligate heterozygotes (*OblHT*), at-risk individuals (*at risk*), and affected homozygotes (*homozygotes*). Since nonpaternity was excluded in all cases, individuals were defined as *OblHT* for nephropathic cystinosis if they were parents of an affected individual. Grandparents were identified as carriers (assigned to the group of *OblHT*) or noncarriers according to their cystine levels. If one grandparent was identified as a carrier, his or her spouse was assigned to the control group of *normals*. Clinically unaffected siblings of affected individuals or of their parents were defined as *at risk*. Heterozygotes for cystinosis were detected by measuring the cystine content in purified PMNL from heparinized blood (Smolin et al. 1987), by use of the cystine binding protein assay (Oshima et al. 1974; Smith et al. 1987). Cystine assays were performed in two different laboratories. Fifteen families (families 1, 2, 4, and 7–18, comprising 123 individuals and 5 controls) were tested in the INSERM laboratory (group 1) and three families (families 3, 5, and 6, comprising 37 individuals and 2 controls) were analyzed in J.A.S.'s laboratory (group 2).

In brief, PMNL from fresh blood were isolated (within 2 hours) using a discontinuous gradient consisting of Histopaque®-1119 and -1077 (Ficoll-Hypaque, Sigma). A pellet of PMNL was sonicated in 150 μl of 5 mM *N*-ethylmaleimide in phosphate buffer (pH 7.2) and mixed with 12% sulfosalicylic acid. Cystine isotope dilution assay was performed on the supernatant. In the presence of the cystine-binding protein (Analytical Applications), nonradioactive cystine contained in the samples competes with exogenous ^{14}C cystine (307 mCi/mmol, New England Nuclear) resulting in a reduction in the expected amount of bound radioactivity, allowing the direct calculation of nonradioactive cystine concentrations in unknown samples. Protein content was determined colorimetrically as described elsewhere (Lowry et al. 1951). In order to validate the cystine measurements, all individuals of each family were tested in conjunction with the same control individuals. The final values represent the average of two assays. The results were expressed as nmol 1/2 cystine/mg protein. Geometric mean and SD values were calculated for *normals* and *OblHT*, as were the 75th and 95th percentiles for *normals* and the 5th and 25th percentiles for *OblHT*.

Microsatellite Genotyping

Peripheral blood samples were obtained following informed consent from all individuals or from their parents. Genomic DNA was extracted either directly from whole blood samples or after Epstein-Barr virus transformation of blood lymphocytes, according to standard procedures. Polymorphic microsatellite markers were analyzed using PCR and PAGE. PCR was performed either in our laboratory in a volume of 25 μl containing 50 ng of genomic DNA, 25–100 ng of the specific primers, 0.2 mM of each dNTP, and 0.2 U of *Taq* polymerase (H.T. Biotechnology) in 1 × *Taq* buffer or in the Généthon laboratory as described elsewhere (Gyapay et al. 1994).

Genotyping was performed either by radiolabeling using markers from the Généthon collection (Gyapay et al. 1994; C. Dib, personal communication) or by laser-based techniques with fluorescently labeled primers (Reed et al. 1994), using an automated 373A DNA sequencer with Genescan™ 672 and Genotyper softwares (Perkin Elmer). The program Lodview (Hildebrandt et al. 1993) was used for the evaluation of exclusion mapping from lod score results.

Linkage and Haplotype Analysis

The phenotypic status was established according to cystine-assay results. Linkage analysis was carried out using the previously determined phenotypes. In a first approach, individuals whose cystine levels were found to range over the 75th percentile of the *normals* and below the 25th percentile of the *OblHT* were considered as having an indeterminate status (*not determined*). A second analysis was performed, in which the individuals were assigned to four liability classes. The two-point lod scores were calculated between the disease locus and seven microsatellite markers (D17S926, D17S1529, D17S1583, D17S1828, D17S1584, D17S513, and D17S796) by use of the FASTLINK package, version

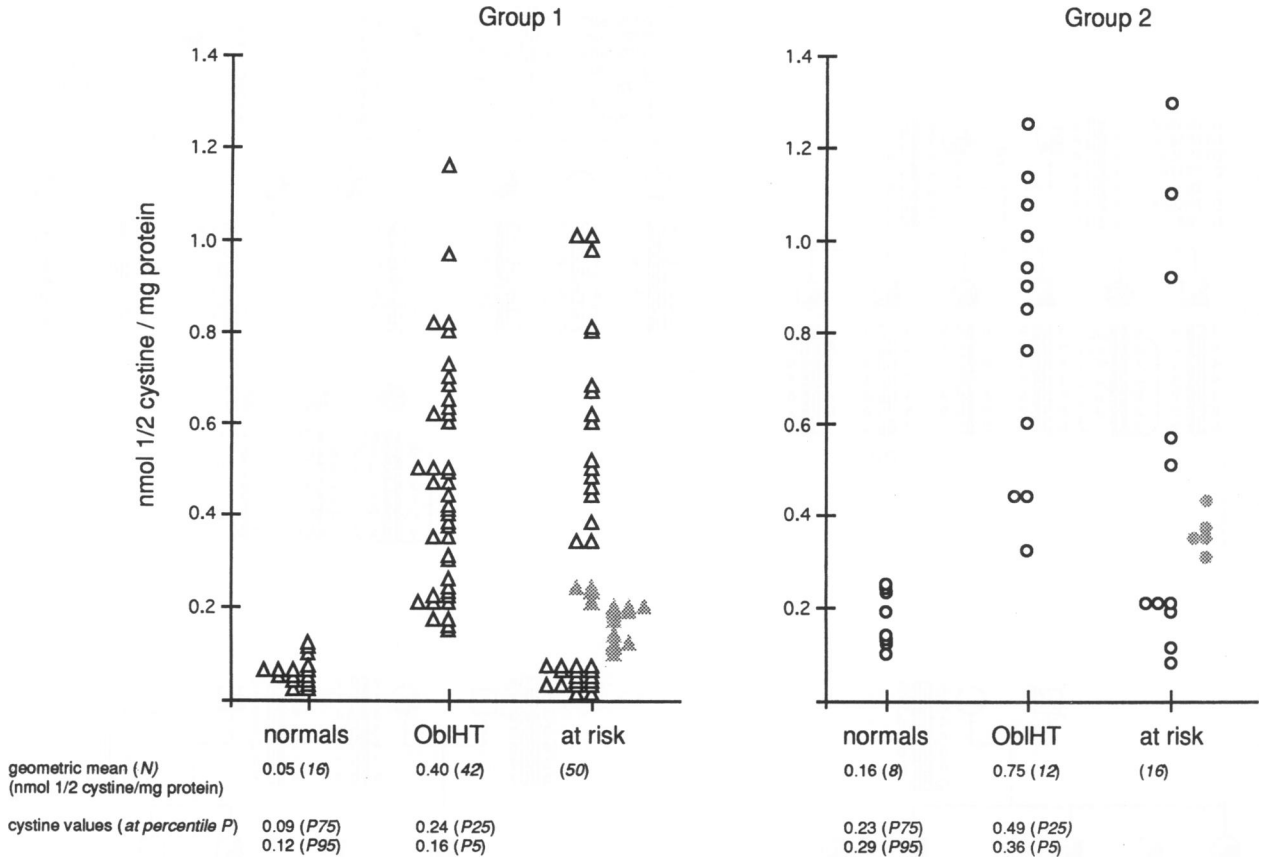

Figure 1 Cystine values for normal individuals (normals), obligate heterozygotes (OblHT), and at-risk individuals (at risk), measured in PMNL. Cystine assays, performed in the INSERM laboratory (group 1) and J.A.S.'s laboratory (group 2), are represented separately. Dotted symbols represent values for at-risk individuals excluded for linkage analysis.

2.2 (Lathrop et al. 1985; Cottingham et al. 1993). The cystinosis gene frequency was estimated to be .001. The true population-allele frequencies were estimated for all markers used except D17S926 and D17S796, for which equal allele frequencies were assumed, since allele sizes have not been determined. Multipoint analysis was carried out using the program LINKMAP from the FASTLINK package. Computation was facilitated by reducing the number of alleles to four without taking into account consanguinity in families 13, 16, and 17. Haplotype

Table 1

Pairwise Lod Scores for the Cystinosis Locus with Microsatellites on Chromosome 17

		Recombination Fraction at θ								
Locus	Name	.00	.01	.05	.10	.15	.20	.25	Z_{max}	θ_{max}
D17S926	AFM 207xa11	−∞	−1.90	2.09	3.17	3.36	3.18	2.81	3.36	.14
D17S1529	AFM 022xb6	−∞	−2.90	2.48	3.95	4.14	3.80	3.21	4.16	.14
D17S1583	AFM b307zg5	−∞	7.28	8.22	7.74	6.88	5.88	4.82	8.22	.05
D17S1828	AFM b013zb1	−∞	12.50	12.46	11.26	9.82	8.29	6.74	12.76	.02
D17S1584	AFM a061za9	−∞	6.87	9.42	9.22	8.26	7.03	5.69	9.50	.06
D17S513	12G6CA/GT	−∞	3.18	6.69	7.05	6.54	5.69	4.68	7.08	.08
D17S796	AFM 177xh6	−∞	4.56	8.46	8.74	8.03	6.96	5.73	8.81	.08

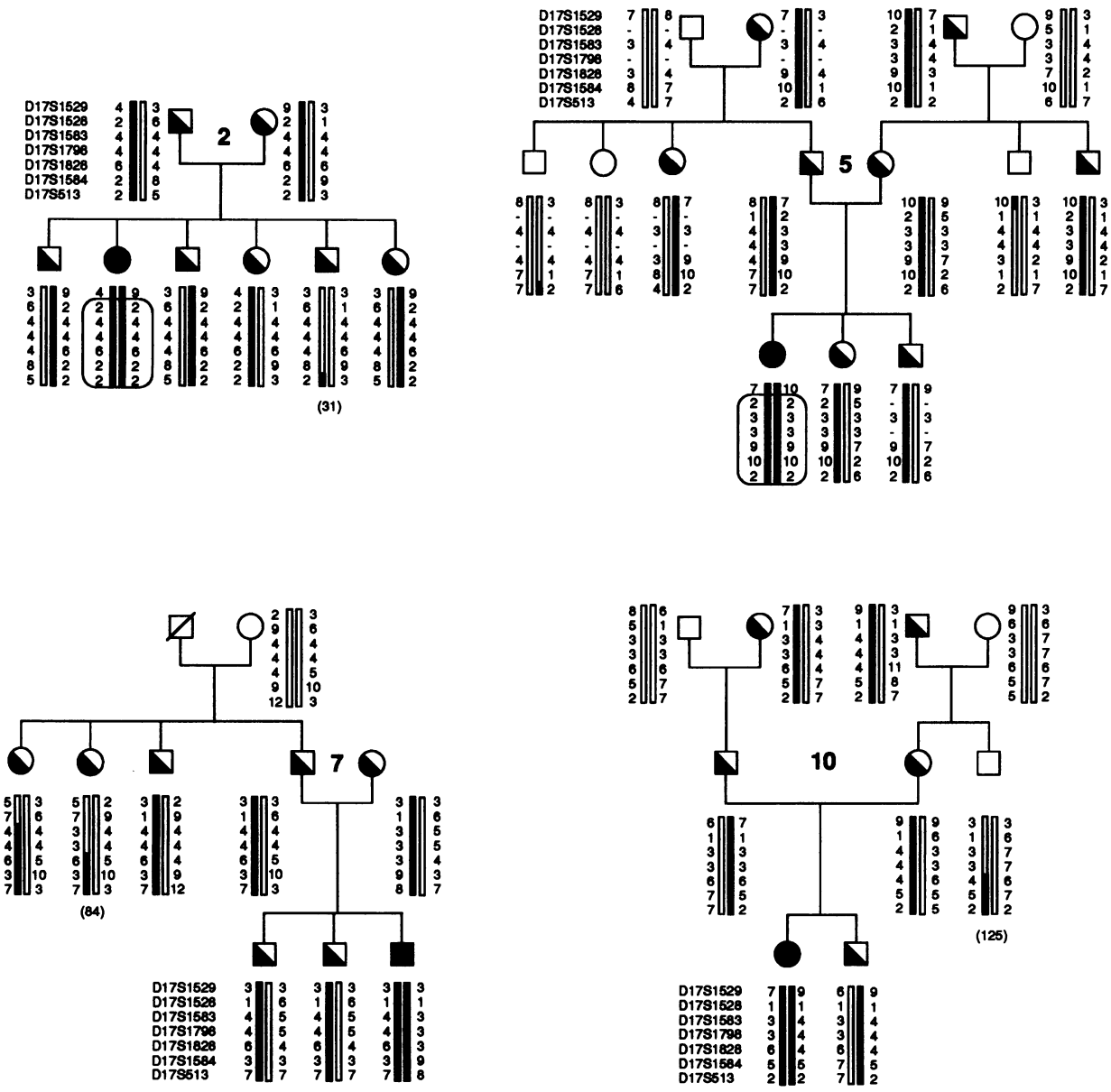

Figure 2 Haplotypes of families 2, 5, 7, 10, 11, 13, and 17. Genotypes are indicated for seven polymorphic microsatellite loci. Haplotypes that are associated with cystinosis are blackened, and the regions of homozygosity are framed. Numbers in brackets are reference numbers of individuals referred to in the text.

analysis was performed with the same markers, although two additional markers (D17S1528 and D17S1798) were examined in those families exhibiting crucial recombination events.

Linkage Disequilibrium

In order to look for a potential founder effect in our population, we examined the possibility of linkage disequilibrium. The allelic associations in affected and nonaffected chromosomes were studied for four informative markers: D17S1583, D17S1798, D17S1828, and D17S1584. Allele frequencies of each marker on normal and carrier chromosomes were calculated by simple counting. Since in consanguineous marriages, the disease gene and the associated chromosomal region are usually derived from a single ancestral chromosome, in these cases, the disease-associated allele was counted only once (Aksentijevich et al. 1993). Control-allele distributions were drawn from the noncarrier chromosomes in the same families. Significance of allelic association was evaluated using a χ^2 test.

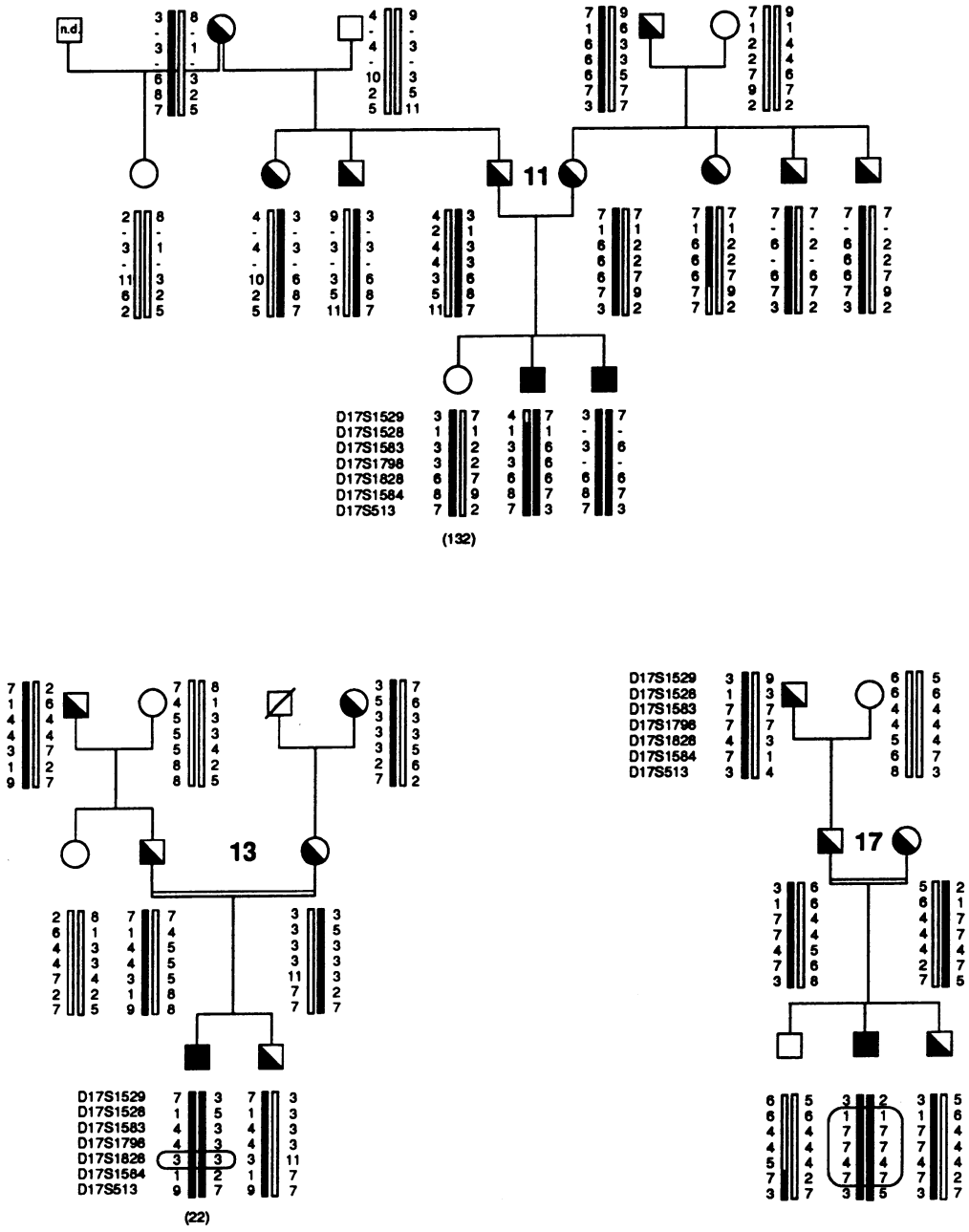

Figure 2 (continued)

Results

Detection of Heterozygotes

Cystine assays were performed in 128 individuals in group 1 and 39 in group 2, which included 19 *homozygous,* 54 *OblHT,* 70 *at risk,* and 24 *normals.* Cystine levels of the *normals* were found to range between 0.02 and 0.12 nmol 1/2 cystine/mg protein in group 1 (geometric mean = 0.05; n = 16) and between 0.10 and 0.25 nmol 1/2 cystine/mg protein in group 2 (geometric mean = 0.16; n = 8) (fig. 1). Values for *homozygotes* of both groups ranged between 1.0 and 12.8 nmol 1/2 cystine/mg protein, the lowest cystine levels being found in patients treated with cysteamine. Cystine levels of the *OblHT* ranged between 0.15 and 1.16 nmol 1/2 cystine/mg protein in group 1 (geometric mean = 0.40; n = 42) and between 0.32 and 1.25 in group 2 (geometric mean = 0.75; n = 12). Values of *at-risk* subjects were found to range between 0.01 and 1.01 nmol 1/2 cystine/mg protein in group 1 and 0.08 and 1.32 in group 2. Four *at-risk* individuals from group 1 with nonreproducible cystine values were considered as *not determined.* Non-

paternity was excluded in all cases. No overlap was observed between values for *normals* and *OblHT* in the two groups.

Linkage and Haplotype Analysis

Linkage analysis was performed taking into account the previously determined phenotypes of the individuals. Seventeen of 50 *at-risk* subjects in group 1 and 6 of 16 in group 2 were determined as *normals* (the respective values were found to range <75th percentile of the *normals*), whereas 17 *at-risk* subjects of group 1 and 5 of group 2 were considered to be heterozygous (values >25th percentile of the *OblHT*). However, 16 individuals in group 1 and 5 in group 2, whose cystine values were found to be >75th percentile of the *normals* and <25th percentile of the *OblHT*, could not be phenotyped. Considering the 4 individuals with nonreproducible results, phenotypes of 25/70 *at-risk* individuals were finally considered as *not determined* for linkage analysis.

Initially, 110 microsatellite markers located on different autosomes were tested for linkage to the cystinosis locus. A total, nonoverlapping exclusion zone of ≥1,800 cM—~50% of the human genome—was established from the cumulative exclusion intervals for each marker, on the basis of their respective published locations (Gyapay et al. 1994) (data not shown).

In order to confirm linkage to the recently mapped cystinosis locus on the short arm of chromosome 17 (Cystinosis Collaborative Research Group 1995) in our families, seven microsatellites markers located in this region were selected: D17S926, D17S1529, D17S1583, D17S1828, D17S1584, D17S513, and D17S796 (D17S926 was analyzed in only 15 families) (table 1). The highest lod scores were obtained with marker D17S1828, with a maximum lod score of 12.76 at θ_{max} = .02. In order to show that a significant lod score would not have been possible without taking into account the cystine values of the individuals, linkage analysis was also carried out without indicating the phenotypes of the heterozygotes. The maximal lod score obtained with the same marker was reduced to 2.52 at θ_{max} = .05 (data not shown).

Haplotype analysis, carried out with nine markers, revealed inconsistencies between phenotypes and haplotypes in two individuals of group 1: one *at-risk* individual of pedigree 2 (individual 31) with a cystine level within the heterozygous range (0.34 nmol 1/2 cystine/mg protein) was found to have inherited two nonaffected alleles, whereas an apparently noncarrier of pedigree 11 (individual 132) (0.07 nmol 1/2 cystine/mg protein) was heterozygous for the cystinosis region (fig. 2). No additional discrepancy was found when the cystine values <95th percentile for *normals* and >5th percentile for *OblHT* were taken into account.

Of the 137 clinically unaffected individuals tested (excluding 7 controls and 4 nonreproducible values), these two false results represent a risk error of 1.5%. We therefore reanalyzed the data by using the affection status modus. Instead of attributing a previously determined phenotype to each person, individuals were assigned to four liability classes according to their leukocyte cystine values. A risk error of 1.5% was estimated for the liability classes 1 (cystine values <75th percentile of the *normals*) and 3 (values >25th percentile of the *OblHT*). Individuals of liability class 2 (*at risks* whose cystine values were found to be >75th percentile of the *normals* and <25th percentile of the *OblHT*) were attributed a 50% probability of being either nonaffected or heterozygous. *Homozygotes* were included in class 4. The lod scores obtained by this method increased by 0.7–1.3 compared to the former analysis. The maximum lod score with marker D17S1828 increased from 12.76 at θ_{max} = .02 to 14.05 at θ_{max} = .01. Furthermore, the values with markers D17S1583, D17S1828 and D17S1584 became positive at θ = .0 (data not shown).

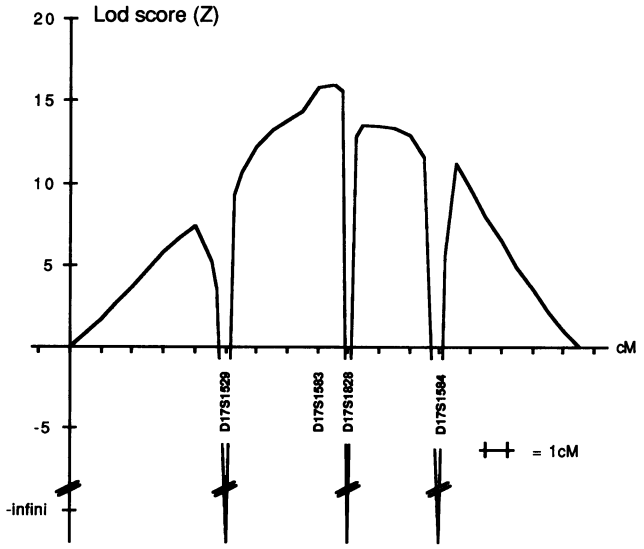

Figure 3 Multipoint linkage analysis with four markers in 18 cystinosis families. For practical reasons, in LINKMAP analysis computation was facilitated by reducing the number of alleles to four without taking into account consanguinity in families 13, 16, and 17.

Multipoint analysis was carried out using four markers selected according to the haplotype analysis results with following intermarker distances: D17S1529–3 cM–D17S1583–1 cM–D17S1828–3 cM–D17S1584 (Cystinosis Collaborative Research Group, personal communication) (fig. 3). The highest location score peak value of 72.83 (lod score = 15.83) at θ_{max} = .02 telomeric to D17S1828 confirmed the location of the cystinosis gene between markers D17S1828 and D17S1583.

The smallest region of homozygosity found in the con-

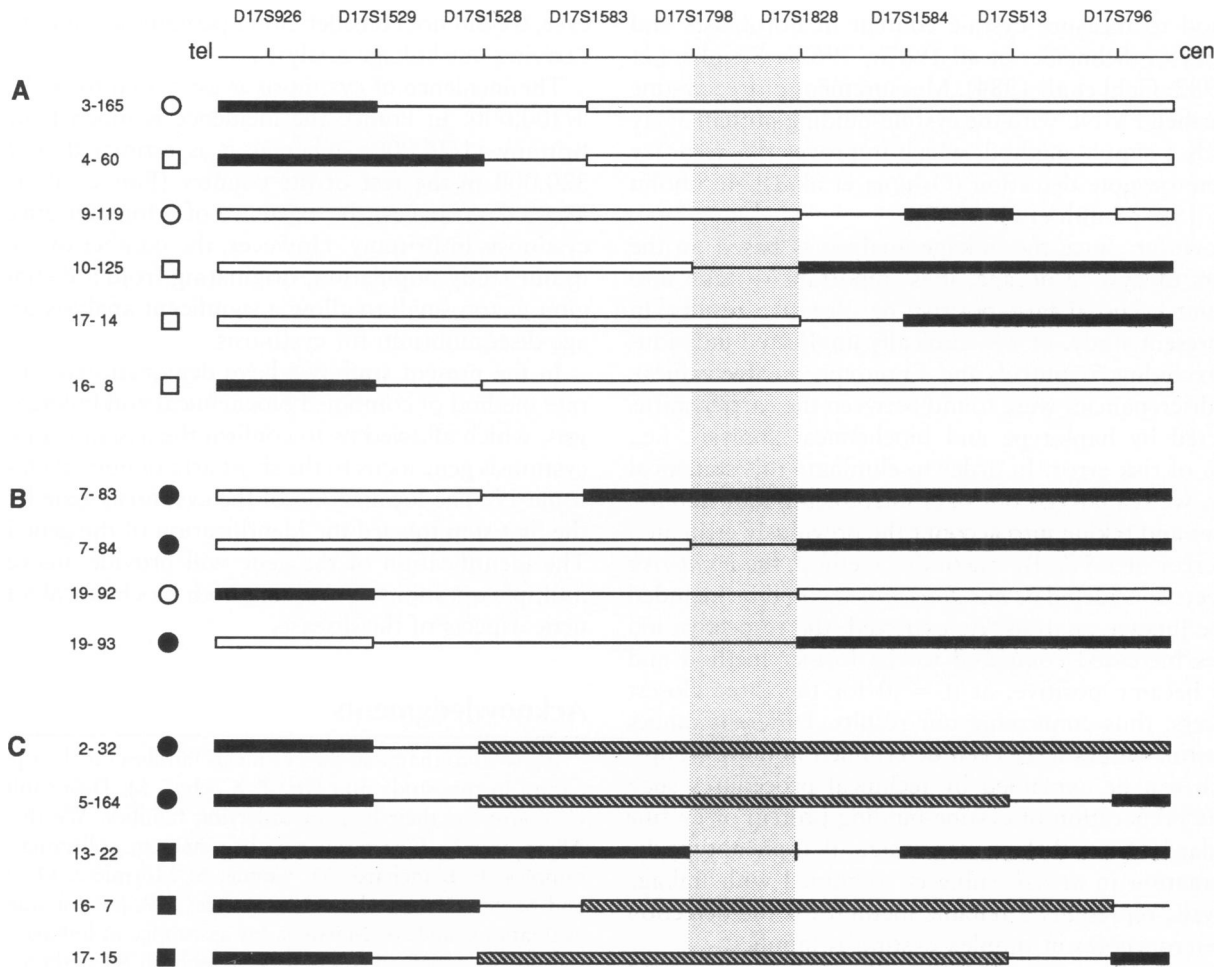

Figure 4 Recombination events observed in 15 individuals between D17S926 and D17S796. The order of the nine markers tested are represented on the top scale. The numbers on the left indicate family and individual numbers. *A*, Recombinant alleles found in normal individuals. *B*, Recombinant alleles found in heterozygote individuals. *C*, Regions of homozygosity found in affected inbred patients. Blackened symbols and boxes represent affected alleles; open symbols and boxes represent unaffected alleles; hatched boxes represent regions of homozygosity in inbred patients; thin lines represent the regions of crossing-over. The genetic boundaries of the cystinosis region are indicated by the dotted area.

sanguineous pedigree 13 (individual 22) with D17S1828 and crucial recombination events with D17S1828 in pedigree 10 (individual 125) and D17S1798 in pedigree 7 (individual 84) suggest that the gene is located between markers D17S1828 and D17S1798 (estimated order of tested markers: telomere–D17S926–D17S1529–D17S1528–D17S1583–D17S1798–D17S1828–D17S1584–D17S513–D17S796–centromere) (fig. 4).

A region of homozygosity spanning parts of the region was detected in two pedigrees, which suggests that they may be consanguineous (families 2 and 5, fig. 4). None of the markers tested showed a significant allelic association to cystinosis chromosomes ($P \geq .14$; data not shown). The pedigrees and haplotypes of the most informative families are represented in figure 2.

Discussion

The best approach to identification of the cystinosis gene was positional cloning by linkage analysis. However, since cystinosis is an autosomal recessive disease, linkage analysis with simplex families did not produce significant results. In such cases, accurate heterozygote identification according to the cystine dosage is crucial. Other authors have used biochemical and linkage analyses for heterozygote detection, for instance in hemophilia A (Lillicrap et al. 1988; Cappello et al. 1992) and in Hunter syndrome (Ben Simon-Schiff et al. 1993), demonstrating the advantages of a combination of the results of both techniques. We decided to take into account the phenotypes of *at-risk* subjects, determined by cystine dosage, when carrying out linkage analysis. Different attempts have been made to establish an adequate

method to measure cystine content in fibroblasts and leukocytes (Schneider et al. 1967*a*, 1967*b*; Steinherz et al. 1982; Gahl et al. 1984). Measurement of free cystine in purified PMNL with the cystine-binding protein assay affords a simple method, which improves the accuracy of heterozygote detection (Oshima et al. 1974; Smolin et al. 1987; Smith et al. 1987).

However, since the linkage analysis is based on the results of cystine dosage, it is important to take into account eventual false-positive or -negative results. In the present study, of 137 clinically unaffected individuals (excluding 7 controls and 4 nonreproducible values), two discrepancies were found between the carrier status detected by haplotype and biochemical analysis, i.e., 1.5% of risk error. In order to eliminate this statistical error, we reanalyzed our data introducing four liability classes and taking into account the previously estimated risk error of 1.5%. By use of this method, the 25 *at-risk* subjects considered as *not determined* could be included in the linkage analysis. As expected, the respective lod scores increased compared to the former method and even became positive, at $\theta = .0$ for the three closest markers, thus confirming our results. Different ranges of cystine values have been determined in both groups, which can be explained by technical procedures such as the preparation of cystine-binding protein or cystine standards. Nevertheless, we consider that phenotype determination in *at-risk* subjects combined with linkage analysis, represents a reliable method for the detection of heterozygotes in simplex cystinosis families.

The cystinosis gene was recently located on a 6-cM interval on the short arm of chromosome 17 between markers D17S1583 and D17S796 by the Cystinosis Collaborative Research Group (1995). Our results confirm linkage to the cystinosis locus. Multipoint analysis provided evidence that the gene is located between markers D17S1583 and D17S1828. Examination of additional markers enabled us to refine the cystinosis interval between markers D17S1798 and D17S1828, spanning ~1 cM. However, since the centromeric border of the region is defined by a crucial recombination event of a single allele of an *at-risk* subject (family 10, patient 125), the investigation of additional families may be necessary to confirm this result.

Homozygosity mapping is known to be a powerful tool for mapping recessive traits in inbred pedigrees. In inbred affected children, both mutated alleles are expected to be inherited from one common ancestor. Thus, these cases must be homozygous for markers closely linked to the disease locus. This may also be true for markers that are located in the vicinity of the linked markers. In affected children of two families (family 2, originally from Morocco, and French family 5) we found a region of homozygosity, thus suggesting consanguinity, which is frequently found in Northern Africa. However, we did not consider either pedigree as inbred when carrying out linkage analysis.

The incidence of cystinosis is estimated to be 1/100–1/200,000. In France the incidence is much higher in Brittany (1/26,000), whereas it is estimated to be 1/320,000 in the rest of the country (Bois et al. 1976). These data suggest the existence of a founder effect for cystinosis in Brittany. However, the number of families in our study population, originating from a distinct region, is too small to allow a significant analysis of linkage disequilibrium for cystinosis.

In the present study we have demonstrated an accurate method of combined biochemical and linkage analysis, which allowed us to confirm the assignment of the cystinosis gene locus to the short arm of human chromosome 17. The localization of the cystinosis gene locus is the first step toward the identification of the gene itself. The identification of the gene will provide answers to multiple questions concerning both biochemical and genetic aspects of the disease.

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