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Identification and Detection of the Common 65-kb Deletion Breakpoint in the Nephropathic Cystinosis Gene (*CTNS*)

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The most common mutation in the cystinosis gene, *CTNS*, is a 65-kb deletion thought to have originated in Germany. Although homozygotes for this deletion are detectable by the absence of the *D17S829* polymorphic marker, no method exists to identify heterozygotes. We identified the 65-kb deletion breakpoints and used flanking PCR primers to amplify a 423-bp fragment present only in the deletion alleles. Using this method, we determined that 121 of 216 (56%) cystinosis alleles examined bore the 65-kb deletion. We found no non-Europeans with the deletion, and the deletion size and breakpoints appeared identical in all patients studied, supporting the concept of a founder effect. The addition of *D17S829* primers (266 bp apart) to the PCR created a multiplex PCR system useful for diagnosing cystinosis patients homozygous and heterozygous for the 65-kb deletion.

Key Words: **cystinosis; deletion; multiplex PCR; sample sequencing; founder effect.**

Nephropathic cystinosis (MIM 219800) is a storage disorder due to the defective transport of the disulfide amino acid, cystine, out of lysosomes (1–3). Its incidence has been estimated to be 1 in 100,000–200,000 live births. Affected patients are normal at birth, but typically exhibit failure to thrive, acidosis, dehydration, and hypophosphatemic rickets at 6 to 12 months of age (4, 5). These symptoms reflect renal tubular Fanconi syndrome, but renal glomerular damage soon supervenes, leading to kidney failure at approximately 10 years of age unless long-term cystine-depleting therapy is instituted. Hypothyroidism and photophobia due to corneal crystals often manifest late in childhood. After renal transplantation, a variety of nonrenal complications, including a distal vacuolar myopathy (6), impaired swallowing (7), retinal blindness (8), pancreatic insufficiency (9, 10), neurological deterioration (11, 12), and gonadal insufficiency in males (13), occur with differing frequencies.

The treatment of cystinosis includes replacement of renal losses due to the Fanconi syndrome and supplementation with L-thyroxine for those patients with hypothyroidism (4, 5). However, the mainstay of therapy involves early initiation of treatment with cysteamine (14), a free thiol that reacts with intralysosomal cystine to form cysteine and the mixed disulfide cysteine–cysteamine (15). These compounds exit the cystinotic lysosome via a process not requiring the defective cystine carrier (16). The consequent cystine depletion prevents renal deterioration and growth retardation (17, 18), depletes muscle of cystine (19), and obviates the need for thyroid hormone replacement (20). Cysteamine eyedrops effectively dissolve existing corneal crystals (21, 22).

In 1995, the cystinosis gene, presumed to encode the defective cystine carrier, was mapped by linkage analysis to chromosome 17p13 (23). After progressive narrowing of the critical region (24–26), a polymorphic marker in the area, *D17S829,* was found to be absent in several cystinosis patients (27). This apparent deletion facilitated the isolation of the cystinosis gene, called *CTNS*. The mRNA of *CTNS* is

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~2.6 kb in size. *CTNS* contains 12 exons that are distributed across ~23 kb of genomic DNA and is predicted to encode a 367 amino acid protein called cystinosin. Cystinosin has seven predicted transmembrane domains and eight potential glycosylation sites (27).

Although 31 different mutations in *CTNS* have already been identified, between 33 and 44% (27, 28) of patients are reported to be homozygous for a 65-kb deletion that encompasses the *D17S829* marker located within intron 3. The homozygous deletion state is associated with the failure to amplify a product with PCR primers specific for *D17S829,* but a method for establishing the heterozygous state for this deletion has yet to be reported. Therefore, we determined the breakpoints of the common 65-kb deletion and used primers immediately distal to these breakpoints for designing a PCR assay that detects heterozygotes for the deletion. Our method has allowed the frequency of this common deletion among cystinosis alleles to be established. It also provides a diagnostic test for the most common *CTNS* mutation in individuals of Northern European descent.

MATERIALS AND METHODS

All patients were enrolled in an Institutional Review Board-approved protocol, and they or their parents gave informed consent. DNA extraction and agarose gel electrophoresis were performed as described (28, 29). BAC RG147P12 (Research Genetics, Huntsville, AL) was sequenced using the "shotgun" method (30). Direct, automated DNA sequencing was performed using the ABI Prism dRhodamine terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. The conditions for multiplex PCR amplification are provided in the Results. The PCR primers used in this study are listed in Table 1. Sequence comparisons were performed using PowerBLAST (31).

RESULTS

Determination of the deletion breakpoints. The 3′ breakpoint of the common 65-kb deletion was reported by Town *et al.* (27) to reside within intron 10 of *CTNS.* We chose two reverse sequences in IVS 10 and IVS 11 that flanked exon 11 (see Table 1). These sequences served as the 3′ primers for PCR amplification across the deletion. Since these primers were 174 bp apart, the resulting PCR products generated using these primers along with suitable 5′ primers should differ in size by ~170 bp.

TABLE 1
Primers for Multiplex PCR Amplification

Name	Sequence
Flanking exon 11	
Reverse-1 (IVS10)	5′-AGACAGACGGACAGACAGAC-3′
Reverse-2 (IVS11)[a]	5′-GCCCGATGCCCCAGC-3′
LDM_1[b]	
Forward	5′-CTAACAGTATCACCGGAGTC-3′
Reverse	5′-GGCCATGTAGCTCTCACCTC-3′
LDM_2	
Forward	5′-ACCTCTCTGATGTGTCCAAG-3′
Reverse	5′-AGCCAAAGGCATCAGGAAAG-3′
D17S829	
Forward	5′-CTAGGGGAGCGTGTTAGCAT-3′
Reverse	5′-TGTAAGACTGAGGCTGGAGC-3′
Exon 4	
Forward	5′-GTCATTGATTTGGGTCC-3′
Reverse	5′-TAGGGCTTGTCTTACAGGTA-3′

[a] Reverse 2 (IVS11) is identical to the reverse primer for exon 11 of *CTNS,* previously described (27).

[b] LDM, large deletion marker. The reverse primer of LDM_1 is identical to the reverse primer for exon 10 of *CTNS* (27).

Potential PCR primers for the 5′ end of the breakpoint were selected based upon data acquired in the search for the cystinosis gene. Sequence data for BAC RG147P12, which contains the 5′ portion of the deleted sequence, was available (manuscript in preparation). In addition, the 5′ deletion breakpoint had been placed within 4 kb of EST T85505 (NID g713857) by Town *et al.* (27). Hence, candidate 5′ primer sequences, chosen at 1-kb increments on either side of T85505, were screened using the two 3′ primers flanking exon 11; DNA from a homozygous deletion patient served as template.

One 5′ primer, LDM_1 Forward (Table 1), yielded PCR products that were 2.22 and 2.05 kb in size (data not shown), indicating amplification across the deletion. The 2.22-kb product was sequenced from both ends. Its 5′ portion matched the sequence of BAC RG147P12 for 360 bases (or 361 bases, depending upon whether the C nucleotide at the junction was from the 5′ or the 3′ border of the deletion) (Fig. 1). The remaining sequence contained the last 5 (or 4) bases of exon 10 and the beginning of IVS 10. (Note that the entire 1683-bp sequence of IVS 10 was previously determined by sequencing the PCR product formed using primers from exon 10 and exon 11.) The 3′ portion of the 2.22-kb fragment was

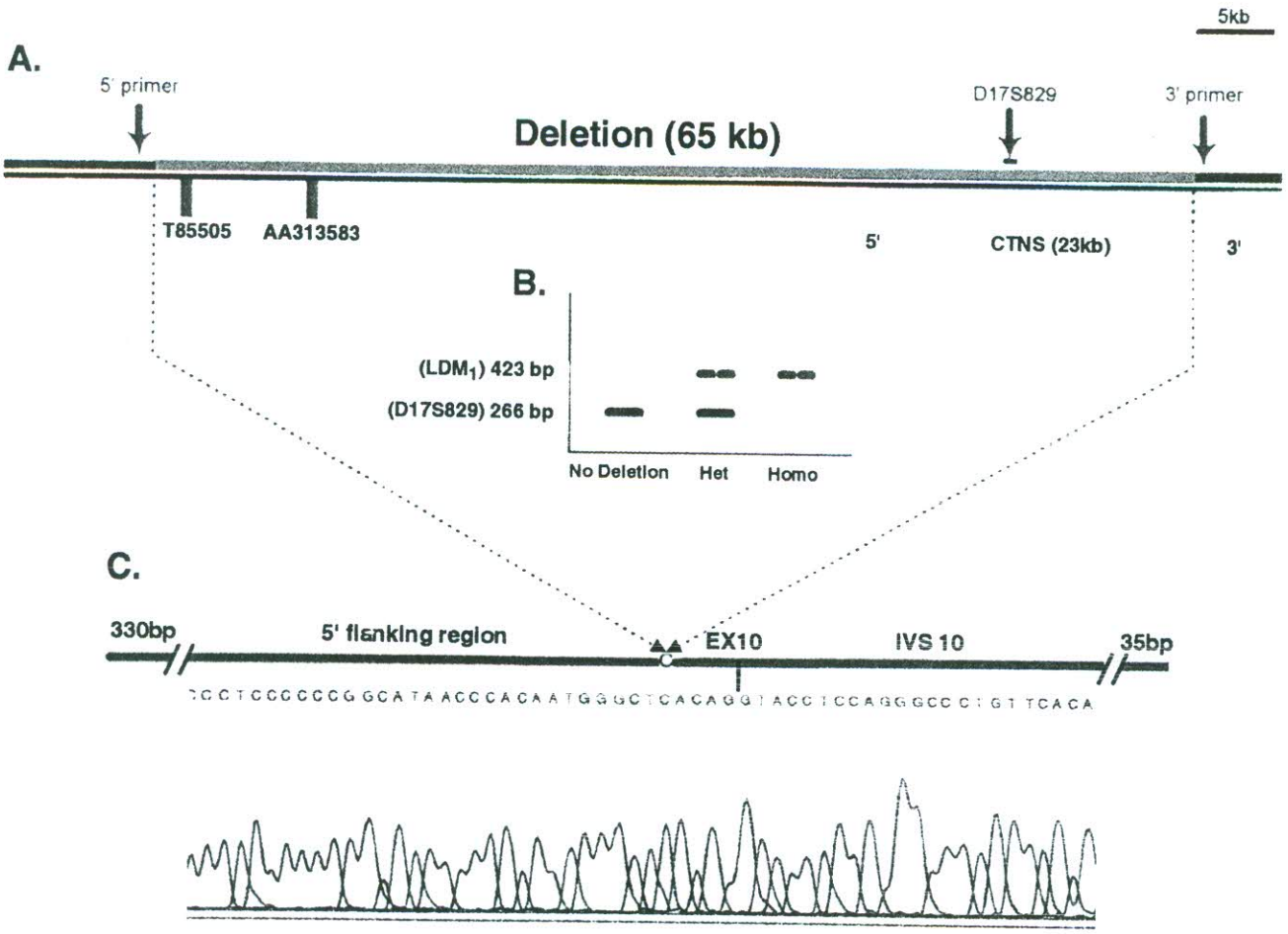

FIG. 1. Map of the region encompassing the 65-kb deletion and portions of *CTNS*. (A) Schematic representation of the 65-kb deletion, whose 5′ breakpoint occurs approximately 1.1 kb upstream of EST T85505 and whose 3′ breakpoint occurs in exon 10 of *CTNS*. Blue and red arrows indicate the annealing positions of the 5′ and 3′ LDM_1 primers, and a green arrow indicates the annealing position of the pair of *D17S829* primers. (B) Cartoon of gel-separated PCR products using large deletion (LDM_1) and *D17S829* primers. Using DNA from a nondeletion patient as template (lane 1), only a 266-bp band is apparent. Using DNA from a homozygous deletion patient (lane 3), only a 423-bp band is visible. DNA from a patient heterozygous for the deletion (lane 2) yields both bands. (C) Expanded view of the PCR fragment formed using LDM_1 primers. The breakpoint occurred at the cytidine indicated by the converging arrows. The 5′ region contains 360 bases upstream of the deletion. The 3′ portion contains 62 bases downstream of the deletion, including 4 bp of exon 10 and 58 bp of IVS 10. See text for details.

identical to the sequence of exon 11 and the 3′ portion of IVS 10. The GenBank accession number for the sequence of the 5′-breakpoint region in BAC RG147P12 is AF112441. The GenBank accession number for the 3′-breakpoint sequence, including IVS 10, is AF112442.

In summary, the 3′ breakpoint of the deletion was 5 (or 4) bases from the end of *CTNS* exon 10. The 5′ breakpoint was approximately 1.1 kb 5′ of T85505 (Fig. 1). The region of the deletion breakpoint was sequenced on 24 different chromosomes, and the identical sequence at the breakpoint was seen in all cases.

Multiplex PCR amplification. In light of the above findings, PCR amplification across the 65-kb deletion was performed using LDM_1 Forward (as the 5′ primer) and LDM_1 Reverse, the previously reported reverse primer for exon 10 (27) (as the 3′ primer) (Table 1). To ascertain the presence of a nondeleted allele, *D17S829* primers (Table 1) were also included. The deletion primers yield a 423-bp PCR product, while the *D17S829* primers yield ~266-bp product (Fig. 1B).

For the PCR, all reagents were obtained from Qiagen (Valencia, CA). The 25-μl reaction mixture

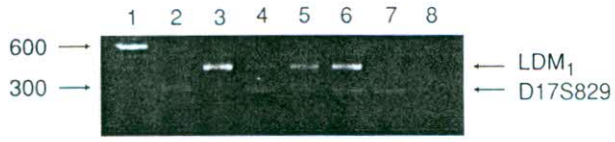

FIG. 2. Agarose gel electrophoresis of multiplex PCR products generated with LDM_1 and *D17S829* primers. Arrows point to the 423-bp band of the large deletion marker (LDM_1) and the 266-bp band of the *D17S829* marker. Lane 1, DNA size markers (GIBCO-BRL-Life Technologies, Gaithersburg, MD); lane 2, normal control; lane 3, homozygous deletion patient; lane 4, heterozygous deletion patient (No. 7 in Ref. 28)—the second mutation is 537del21bp; lane 5, heterozygous deletion patient—the second mutation is W138X (28); lane 6, heterozygous deletion patient—the second mutation has not been determined; lane 7, homozygous for 985insA (T216N, patient 14 in Ref. 28); lane 8, negative control (no DNA).

contained 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM each primer, 1.25 U of HotStarTaq DNA polymerase, and 500 ng of template DNA. After an initial activation step at 94°C for 15 min, PCR was conducted for 30 cycles of 94°C for 30 s, 55°C for 40 s, and 72°C for 1 min. An elongation step at 72°C for 10 min finalized the procedure. The PCR products were electrophoresed in 2% agarose and stained with ethidium bromide. For reactions not using the HotStarTaq DNA polymerase, an initial denaturation step at 94°C for 4 min replaced the 15-min activation step.

An example of the multiplex PCR amplification using the LDM_1 primers is provided in Fig. 2. A normal individual (lane 2) yields only the 266-bp product, reflecting the presence of *D17S829* and the inability to amplify between the LDM_1 primers when no deletion is present. The same product is generated from a patient homozygous for a nondeletion *CTNS* mutation, 985insA (lane 7). In contrast, only a 423-bp product is made from a homozygous deletion patient (lane 3). Finally, DNA from cystinosis patients carrying the 65-kb deletion allele as well as another *CTNS* mutation yields both bands (lanes 4–6). In the examples shown in Fig. 2, one patient was previously considered to be homozygous for the 537del21bp mutation (lane 4), another was considered homozygous for W138X (lane 5), and another had no known mutation (lane 6).

Figure 3 illustrates segregation of the deletion allele with the disease in a cystinosis family. Both parents and one child are heterozygous for the deletion, while the child with cystinosis inherited the deletion from each parent. Note that the polymorphic marker *D178S829* is smaller in the mother than in the father; the heterozygous child inherited the father's nondeletion allele and, by inference, the mother's deletion allele.

As an alternative to the use of LDM_1 primers, a second set of deletion-specific primers (called LDM_2) was designed (Table 1). LDM_2 Forward, located 140 (or 139) bp 5′ of the breakpoint, and LDM_2 Reverse, located 302 (or 303) bp 3′ of the breakpoint, yield a 442-bp product. In addition to the LDM_2 primers, exon 4 primers (Table 1) could be used to amplify a fragment of approximately 250 bp from nondeleted alleles (data not shown). The PCR conditions used were identical to those used with the LDM_1 primers.

Frequency of the 65-kb deletion. In a previous paper (28), we reported *CTNS* mutations in 108 patients with the caveat that "some of the mutations noted. . .as homozygous actually may be hemizygous, with a second allele carrying the large deletion." The ability to detect heterozygotes for the 65-kb deletion using the deletion primers described above (Table 1) allowed us to refine our estimates of the frequency of the deleted allele. For example, of 11 patients apparently homozygous for W138X, 7 proved to be hemizygous, each containing the 65-kb deletion as their second allele. Of 13 individuals deemed homozygous for various other mutations, 5 carried the 65-kb deletion. Specifically, patients 3, 6, 7, 8, and 18 (in Table 3 of Ref. 28) were hemizygous for their respective mutations. Thus, 1 of 6 patients in whom only one mutation had been identified was found to also carry the deletion, while 11 of 20 patients in whom no mutation had been recognized were heterozygous for the deletion.

In total, 50% (12/24) of "homozygous" nondeletion patients were actually hemizygous. Of 50 patients homozygous for deletion of *D17S829,* 1 showed no PCR amplification with LDM_1 primers and another

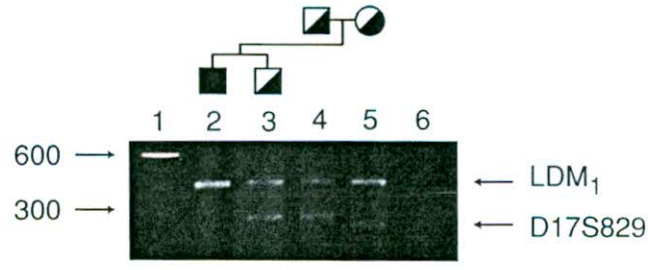

FIG. 3. Multiplex PCR amplification of the common deletion allele in a cystinosis family. Lanes 1 and 6 contain DNA size markers; lanes 2–5 represent the respective family members' PCR products. The deletion product (LDM_1) and the *D17S829* band are indicated by arrows.

was heterozygous for the deletion. Both of these two patients carried a smaller deletion that included the *D17S829* marker. In total, 97 of 100 alleles, presumed by *D17S829* testing to bear the complete 65-kb deletion, actually did. Of 216 cystinosis alleles, the 65-kb deletion was identified in 121 (56%), with 48 homozygous and 25 heterozygous for the deletion.

Finally, of 100 random alleles from normal individuals of unknown ethnicity, none was heterozygous for the 65-kb deletion.

DISCUSSION

The identification of suitable PCR primers flanking the common 65-kb deletion in *CTNS* allowed the design of a vastly improved method for detecting the deletion. First, the method can be used to detect heterozygosity for the deletion or hemizygosity for a different *CTNS* mutation. This new diagnostic capacity, for example, allowed us to revise our estimate of the frequency of the W138X mutation in cystinosis patients from 25 of 216 alleles (12%) (28) to 18 of 216 alleles (8%). Previously, it was impossible to distinguish between homozygosity and hemizygosity for a nondeletion allele. Second, the new method generates positive evidence for the deletion (i.e., a 423-bp band) rather than relying upon the absence of a PCR product using *D17S829* primers (27, 28). Conversely, in nondeletion patients, the presence of a control band confirmed the adequacy of the DNA template. Finally, the percentage of 65-kb deletion alleles in American-based cystinosis patients can be accurately estimated at 56%. These patients, of course, are largely of Northern European ancestry, and the 65-kb deletion has not been associated with non-European-derived populations. Hence, the use of a frequency of at least 56% for the deletion corresponds only to patients of Northern European heritage. In addition, as with other multiplex PCR methods, the intensity is not always equal among amplified bands, and separate PCR amplifications may occasionally be required.

With these reservations, the multiplex PCR technique identifies patients with zero, one, or two alleles bearing the 65-kb deletion and can be used in several situations. For families with a child who is homozygous for the deletion, subsequent prenatal diagnosis can be performed, since this technique distinguishes homozygotes from heterozygotes. For couples whose family history places them each at risk for carrying the deletion, PCR amplification can be used to ascertain whether or not they carry the deletion alleles. This constitutes a significant advance over current heterozygote detection by leukocyte cystine measurement (4), a tedious procedure seldom performed. For all cystinosis patients, including those with variants of the disease (4, 5), multiplex PCR amplification provides a simple and reliable diagnostic test that can eliminate the need for more extensive, sequence-based analyses. We generally employed the LDM_1 primers, but for verification of critical results, the LDM_2 primers can be used in addition.

In their paper describing the *CTNS* gene identification, Town *et al.* (27) state that the 65-kb "deletion was detected only in patients of European origin suggesting a founder effect." We agree (28), having verified the absence of the deletion in 11 of 11 non-European cystinosis alleles. Moreover, PCR amplification using the described assays has yielded products of apparently identical size in 73 of 73 patients and of absolutely identical sequence in 24 of 24 alleles. These findings support the hypothesis that a founder effect accounts for the high frequency of the deletion and may obviate the need to invoke one of the mechanisms of repeated gene rearrangement recently delineated (32).

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REFERENCES

1. Gahl WA, Tietze F, Bashan N, Steinherz R, Schulman JD. Defective cystine exodus from isolated lysosome-rich fractions of cystinotic leucocytes. *J Biol Chem* **257:**9570–9575, 1982.
2. Gahl WA, Bashan N, Tietze F, Bernardini I, Schulman JD. Cystine transport is defective in isolated leukocyte lysosomes from patients with cystinosis. *Science* **217:**1263–1265, 1982.
3. Jonas AJ, Smith ML, Schneider JA. ATP-dependent lysosomal cystine efflux is defective in cystinosis. *J Biol Chem* **257:**13185–13188, 1982.
4. Gahl WA, Schneider JA, Aula P. Lysosomal transport disorders: cystinosis and sialic acid storage disorders. In The Metabolic and Molecular Bases of Inherited Disease, 7th ed. (Scriver CR, Beaudet AL, Sly WS, Valle D, eds). New York, McGraw–Hill, pp 3763–3797, 1995.
5. Gahl WA. Cystinosis coming of age. *Adv Pediatr* **33:**95–126, 1986.
6. Charnas LR, Luciano CA, Dalakas M, Gilliat RW, Bernardini I, Ishak K, Swik VA, Fraker D, Brushart TA, Gahl WA.

Distal vacuolar myopathy in nephropathic cystinosis. *Ann Neurol* **35:**181–188, 1994.

7. Sonies BC, Ekman EF, Andersson HC, Adamson MD, Kaler SG, Markello TC, Gahl WA. Swallowing dysfunction in nephropathic cystinosis. *N Engl J Med* **323:**565–570, 1990.
8. Kaiser-Kupfer MI, Caruso RC, Minckler DS, Gahl WA. Long-term ocular manifestations in nephropathic cystinosis post-renal transplantation. *Arch Ophthalmol* **104:**706–711, 1986.
9. Fivush B, Green OC, Porter CC, Balfe JW, O'Regan S, Gahl WA. Pancreatic endocrine insufficiency in post-transplant cystinosis. *Am J Dis Child* **141:**1087–1089, 1987.
10. Fivush B, Flick JA, Gahl WA. Pancreatic exocrine insufficiency in a patient with nephropathic cystinosis. *J Pediatr* **112:**49–51, 1988.
11. Ehrich JHHH, Stoeppler L, Offner G, Brodehl J. Evidence for cerebral involvement in nephropathic cystinosis. *Neuropaediatrie* **10:**128–137, 1979.
12. Fink JK, Brouwers P, Barton N, Malekzadeh MH, Sato S, Hill S, Cohen WE, Fivush B, Gahl WA. Neurologic complications in longstanding nephropathic cystinosis. *Arch Neurol* **46:**543–548, 1989.
13. Chik CL, Friedman A, Merriam GR, Gahl WA. Pituitary–testicular function in nephropathic cystinosis. *Ann Intern Med* **119:**568–575, 1993.
14. Thoene J, Oshima R, Crawhall J, Olson D, Schneider J. Cystinosis: Intracellular cystine depletion by aminothiols *in vitro* and *in vivo*. *J Clin Invest* **58:**180–189, 1976.
15. Gahl WA, Tietze F, Butler JDeB, Schulman JD. Cysteamine depletes lysosomal cystine by the mechanism of disulfide interchange. *Biochem J* **228:**545–550, 1985.
16. Pisoni RL, Thoene JG, Christensen HN. Detection and characterization of carrier-mediated cationic amino acid transport in lysosomes of normal and cystinotic human fibroblasts. *J Biol Chem* **260:**4791–4798, 1985.
17. Gahl WA, Reed GF, Thoene JG, Schulman JD, Rizzo WB, Jonas AJ, Denman DW, Schlesselman JJ, Corden BJ, Schneider JA. Cysteamine therapy for children with nephropathic cystinosis. *N Engl J Med* **316:**971–977, 1987.
18. Markello TC, Bernardini IM, Gahl WA. Improved renal function in children with cystinosis treated with cysteamine. *N Engl J Med* **328:**1157–1162, 1993.
19. Gahl WA, Charnas L, Markello TC, Bernardini I, Ishak KG, Dalakas MC. Parenchymal organ cystine depletion with long term cysteamine therapy. *Biochem Med Metab Biol* **48:**275–285, 1992.
20. Kimonis VE, Troendle J, Rose SR, Yang ML, Markello TC, Gahl WA. Effects of early cysteamine therapy on thyroid function and growth in nephropathic cystinosis. *J Clin Endocrinol Metab* **80:**3257–3261, 1995.
21. Kaiser-Kupfer MI, Fujikawa L, Kuwabara T, Jain S, Gahl WA. Removal of corneal crystals by topical cysteamine in nephropathic cystinosis. *N Engl J Med* **316:**775–779, 1987.
22. Kaiser-Kupfer MI, Gazzo MA, Datiles MB, Caruso RC, Kuehl EM, Gahl WA. A randomized placebo-controlled trial of cysteamine eye drops in nephropathic cystinosis. *Arch Ophthalmol* **108:**689–693, 1990.
23. The Cystinosis Collaborative Research Group. Linkage of the gene for cystinosis to markers on the short arm of chromosome 17. *Nature Genet* **10:**246–248, 1995.
24. Jean G, Fuchshuber A, Town MM, Gribouval O, Schneider JA, Broyer M, van't Hoff W, Niaudet P, Antignac C. High-resolution mapping of the gene for cystinosis, using combined biochemical and linkage analysis. *Am J Hum Genet* **58:**535–543, 1996.
25. McDowell G, Isogai T, Tanigami A, Hazelwood S, Ledbetter D, Polymeropoulos MH, Lichter-Konecki U, Konecki D, Town MM, Van't Hoff W, Weissenbach J, Gahl WA. Fine mapping of the cystinosis gene using an integrated genetic and physical map of a region within human chromosome band 17p13. *Biochem Mol Med* **58:**135–141, 1996.
26. Peters U, Senger G, Rählmann M, Du Chesne I, Stec I, Köhler MR, Weissenbach J, Leal SM, Koch HG, Deufel T, Harms E. Nephropathic cystinosis (CTNS-LSB): construction of a YAC contig comprising the refined critical region on chromosome 17p13. *Eur J Hum Genet* **5:**9–14, 1997.
27. Town M, Jean G, Cherqui S, Attard M, Forestier L, Whitmore SA, Callen DF, Gribouval O, Broyer M, Bates GP, van't Hoff W, Antignac C. A novel gene encoding an integral membrane protein is mutated in nephropathic cystinosis. *Nature Genet* **18:**319–324, 1998.
28. Shotelersuk V, Larson D, Anikster Y, McDowell G, Lemons R, Bernardini I, Guo J, Thoene J, Gahl WA. *CTNS* mutations in an American-based population of cystinosis patients. *Am J Hum Genet* **63:**1352–1362, 1998.
29. Sambrook J, Fritsch EF, Maniatis T (Eds.). Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
30. Wilson RK, Mardis ER. Shotgun sequencing. In Analyzing DNA (Birren B, Green ED, Klapholz S, Myers RM, Roskams J, Eds.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp 397–454, 1997.
31. Zhang J, Madden TL. PowerBLAST: a new network BLAST application for interactive or automated sequence analysis and annotation. *Genome Res* **7:**649–656, 1997.
32. Lupski JR. Genomic disorders: structural features of the genome can lead to DNA rearrangements and human disease traits. *TIG* **14:**417–422, 1998.