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Report

A Recessive Contiguous Gene Deletion of Chromosome 2p16 Associated with Cystinuria and a Mitochondrial Disease

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Deletions ranging from 100 Kb to 1 Mb—too small to be detected under the microscope—may still involve dozens of genes, thus causing microdeletion syndromes. The vast majority of these syndromes are caused by haploinsufficiency of one or several genes and are transmitted as dominant traits. We identified seven patients originating from an extended family and presenting with a unique syndrome, inherited in a recessive mode, consisting of cystinuria, neonatal seizures, hypotonia, severe somatic and developmental delay, facial dysmorphism, and lactic acidemia. Reduced activity of all the respiratory chain enzymatic complexes that are encoded in the mitochondria was found in muscle biopsy specimens of the patients examined. The molecular basis of this disorder is a homozygous deletion of 179,311 bp on chromosome 2p16, which includes the type I cystinuria gene (*SLC3A1*), the protein phosphatase 2Cβ gene (*PP2Cβ*), an unidentified gene (*KIAA0436*), and several expressed sequence tags. The extent of the deletion suggests that this unique syndrome is related to the complete absence of these genes' products, one of which may be essential for the synthesis of mitochondrial encoded proteins.

The vast majority of syndromes associated with deletions of 100 Kb to 1 Mb are caused by haploinsufficiency of one or several genes and are transmitted as dominant traits. Homozygous deletions are usually lethal and, in the heterozygote state, only a few dosage-sensitive genes cause the phenotype. A recessive contiguous gene deletion has been reported only once for a homozygous 122-kb deletion of 11p14-15, associated with profound, congenital sensorineural deafness, severe hyperinsulinemic hypoglycemia, enteropathy, and renal tubular dysfunction. This phenotype could be explained by the underlying absence of more than half of the *ABCC8* gene (MIM 600509) and partial deletion of the USHER syndrome type IC gene (*USH1C* [MIM 276904]; Bitner-Glindzicz et al. 2000).

Cystinuria (MIM 220100) is a relatively common autosomal recessive disorder of the transport of cystine and dibasic amino acids through the epithelial cells of the renal tubuli and the intestinal brush border (Segal and Their 1995). The clinical manifestations are predominantly renal and are attributed to cystine calculi. The frequency of neurological deficits is comparable to that in the general population (Gold et al. 1977). Mutations in either the light or the heavy chain of the dibasic amino acids and cystine transport system (bo,+AT) have been identified in cystinuric patients (Calonge et al. 1994; International Cystinuria Consortium 1999). The heavy chain, *rBAT,* is encoded by the *SLC3A1* gene (MIM 104614), which resides on chromosome 2 (Pras et al. 1994) and is linked by a disulfide bond to the catalytic light chain, (bo,+AT), which is encoded by the *SLC7A9* gene (MIM 604144) on chromosome 19 (Bisceglia et al. 1997).

Seven patients (4 male and 3 female) from three nuclear families of a small Bedouin clan (fig. 1*a*) participated in this study, after informed consent was obtained. They were followed until the oldest subject was aged 16 years. Their medical records were carefully reviewed, and details of their somatic growth, psychomotor development, clinical course, hospitalizations, and labo-

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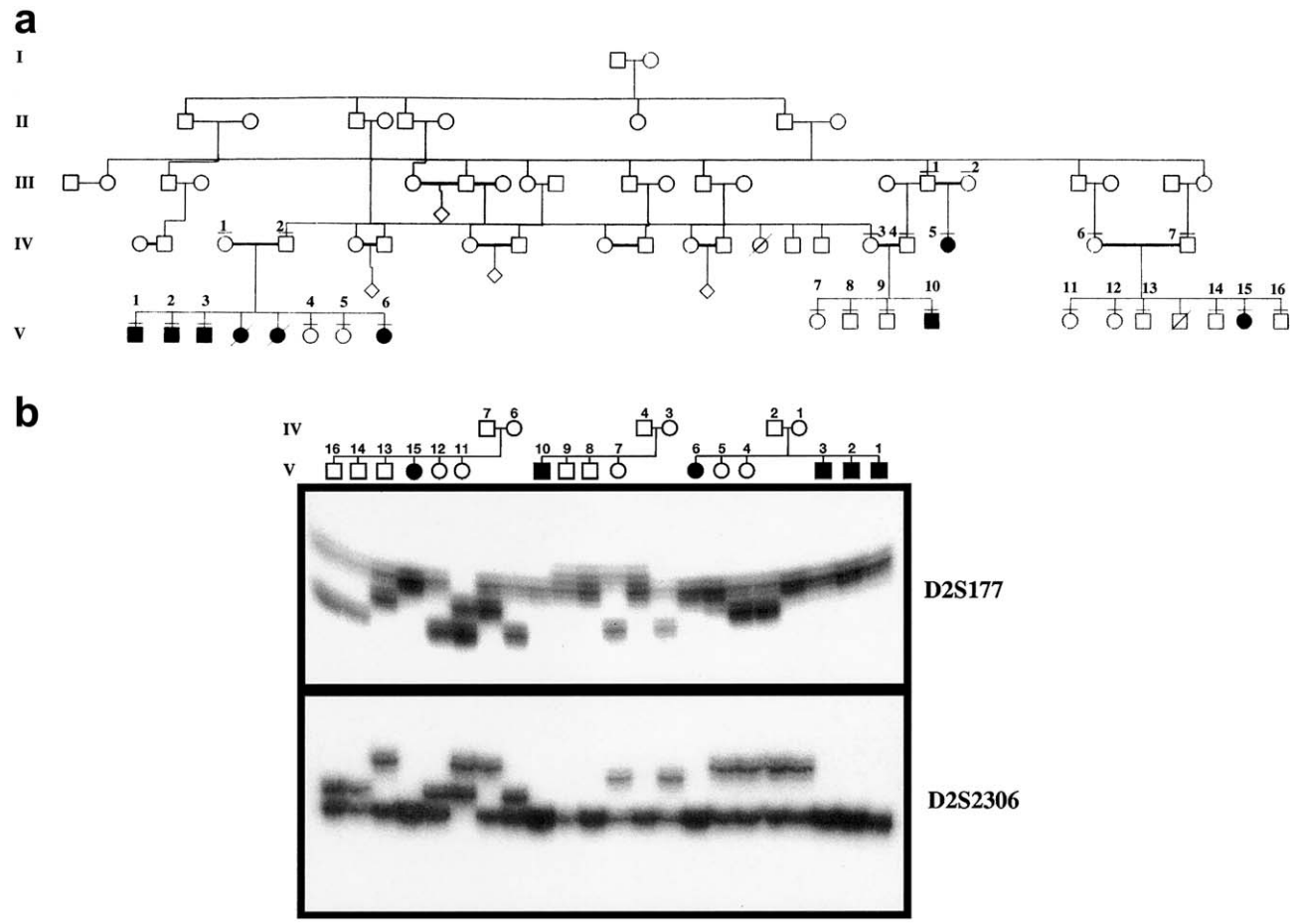

Figure 1 *a,* Pedigree of the extended Bedouin family with seven children affected with cystinuria, neonatal seizures, hypotonia, severe somatic and developmental delay, facial dysmorphism, mitochondrial disease, and lactic acidemia. Family members whose symbols are numbered and marked with a small horizontal line were available for the DNA studies. *b,* Linkage analysis to markers *D2S2306* and *D2S177,* showing one homozygous allele in all patients. The pedigree members' symbols, marked as in *a,* are arranged above each gel lane. Markers *D2S119* and *D2S2220* showed similar patterns.

ratory results were recorded. Their parents and siblings were interviewed and underwent complete physical examination and urine amino acid analysis.

Decreased fetal movements were reported in two patients. All the patients were born at term, with normal growth parameters. Nonetheless, all fed poorly and exhibited linear growth impairment and severe failure to thrive. At birth, all patients had generalized severe axial hypotonia and a weak cry, and five suffered from neonatal seizures. The postnatal global development was delayed; muscle tone improved after 2 years of age, but walking was achieved only at 5–7 years of age. Despite intact hearing, speech was incomprehensible, and moderate to severe mental retardation was evident in all the patients. Brain CT scan, which was performed in four patients, was normal in three patients and disclosed mild dilatation of the right ventricle, due to neonatal hemorrhage, in one patient. Dysmorphic facies, including frontal bossing, almond-shaped eyes, long eyelashes, depressed nasal bridge, and large, posteriorly rotated ears, were noted in all patients. Visceral organs were of normal size in all patients. Growth failure continued into childhood, and the oldest patients, a 16-year-old boy and a 13-year-old girl, do not yet show any pubertal signs.

Renal and/or bladder cystine calculi were detected in all patients as early as 9 months of age, and excretion of arginine, lysine, ornithine, and cystine in urine was markedly increased; response to urine alkalinization and penicillamine was inconsistent. Transient neonatal hypocalcemia and hypoglycemia were noted in four and two patients, respectively. Serum lactate level was elevated in four of the seven patients, and the urinary organic acids analysis suggested an impairment of mitochondrial fatty acids oxidation in one patient.

In the mitochondrial enriched fraction of the muscle

of one patient (fig. 1*a;* patient V10), the enzymatic activity of mitochondrial respiratory chain (MRC) complex I+III was 27%; complex II+III, 45%; complex IV, 39%; and complex V, 68% of the control mean, normalized for citrate synthase activity. Only complex II, which does not contain any mitochondrial encoded subunit, had normal activity (119%). In the mitochondrial enriched fraction of the muscle of a second patient (fig. 1*a;* patient IV5), the activity of MRC complex I+III activity was 55%; complex IV, 27%; and complex V, 36% of the control mean, normalized for citrate synthase activity. The nuclear encoded complex II activity was normal (90%). In a third patient (fig. 1*a;* patient V3) whose muscle was subjected to histochemical analysis only, ragged red fibers were demonstrated. These findings were compatible with an MRC defect. The ratio of the mitochondrial DNA (mtDNA) to nuclear DNA in muscle mitochondria of the first patient was 30% of the control mean.

Linkage to the *SLC7A9* gene (Bisceglia et al. 1997) was excluded by the finding of four recombination events with two adjacent genetic markers (*D19S433* and *D19S245*). Marker information and PCR amplification conditions were obtained from the Genome Database. Visualization of the PCR products was done by the addition of $\alpha[^{32}P]$-dCTP, as detailed in Parvari et al. (1998). Linkage analysis to the *SLC3A1* gene on chromosome 2p16 (Pras et al. 1994), using the polymorphic markers *D2S177, D2S2306, D2S2220,* and *D2S119,* revealed homozygosity for the same allele in all patients; this was not present in any of the healthy relatives tested (fig. 1*b,* which presents results for *D2S177* and *D2S2306;* results for *D2S2220* and *D2S119* are not shown.) Siblings appeared homozygous where one parent was not informative, but this homozygosity was not observed for the other marker; for example, at marker D2S2306, V8 and V9 are homozygous because their father, IV4, was homozygous. This result suggests that this locus is involved with the disease in this family. PCR amplification of the *SLC3A1* exons was performed according to Pras et al. (1996). Repeated failures to amplify the 10 exons of the *SLC3A1* gene with the patients' DNA, along with normal amplification products with the parents' DNA, indicated that the *SLC3A1* gene is deleted (fig. 2*a*). The presence in patients' DNA of multiple adjacent genes extracted from the database of GeneMap and the Unified Database for Human Genome Mapping was verified by PCR amplification. These included malate dehydrogenase (*MDH1* [MIM 154200]); butyrate response factor 2 (BRF2); 3-hydroxanthranilic acid dioxygenase (HAAO); Cdc42 effector protein 3 (CEP3); splicing factor arginine/serine-rich 7 (SFRS7 [MIM 600572]); guanine nucleotide exchanger factor (SOS1); glutamine cyclotransferase (QPCT); ATPase, H+ transporting, lysosomal (Vacuolar proton pump) 9kD (ATP6H [MIM 603931]); and cytochrome c oxidase subunit VII–related protein (COX7RP). Specifically, the mitochondrial translation initiation factor 2 (*MTIF2* [MIM 603766]) and the mitochondrial deoxyguanosine kinase (*DGUOK* [MIM 601465]) genes were also not included in the deletion; furthermore, both were normally transcribed, as determined by RT-PCR with patients' lymphoblastoid RNA (not shown), thus excluding their involvement in the MRC defect. However, the possibility remains that compromised transcription of other genes outside the region may be the cause of the observed phenotype.

To estimate the size of the deletion, we used the sequence available in GenBank to design primers for PCR amplification on both sides of the *SLC3A1* gene. Sequence-tagged sites (STSs) were designed on the basis of the genomic region available at GenBank after RepeatMasker and BLAST were used, against the nr and htgs databases, to confirm their unique sequences (available upon request). Absence of amplification after multiple attempts, in the presence of good amplification of control samples, was taken as evidence that the sequence of at least one of the primers was within the deletion. We then designed primers to amplify across the deletion (fig. 3*a;* primers "a," located on the left side of the deletion [5′-AGCTAGAAGAAACTGGGGAAG-3′] and "b," located on the right side of the deletion [5′-AATGCTAATACCACTTGGGTC-3′]). Sequencing across the deletion was done on the PCR product of primers a and b, which define the deletion borders. The PCR product was purified using the QIAquick kit (Qiagen), and sequencing was performed on an ABI 3700 machine, using the fluorescent dideoxy termination method. The result of the sequence indicated that the size of the deletion is 179,311 bp; either side of the deletion could contribute four nucleotides (CCTT) at the joining site, since they are present in both (fig. 3*b*). The deletion could have been caused by an excision transposition event through alignment of two Alu elements on clone RP11-559M23, whose ends reside 806 bp and 3,343 bp from the edge, to an Alu element on clone RP11-559M23, whose end resides 1,020 bp from the edge. A multiplex PCR reaction was designed using primers a and b, located on the margins of the deletion breakpoint, and primer "c" (5′-TAACTGGGTGGTGATGAGGAG-3′), located within the deleted region, and identifies the presence of the wild-type sequence (fig. 3*a*). This multiplex reaction confirmed that all eight parents were heterozygous and that all patients were homozygous for the deleted allele (fig. 2*b*).

BLAST search of the deleted sequence against the nr and dbEST databases revealed the presence of three genes and 20 expressed sequence tags (ESTs) (fig. 3*a*). These three genes—*SLC3A1,* protein phosphatase 2Cβ (*PP2Cβ* [MIM 603770]; Marley et al. 1998), and

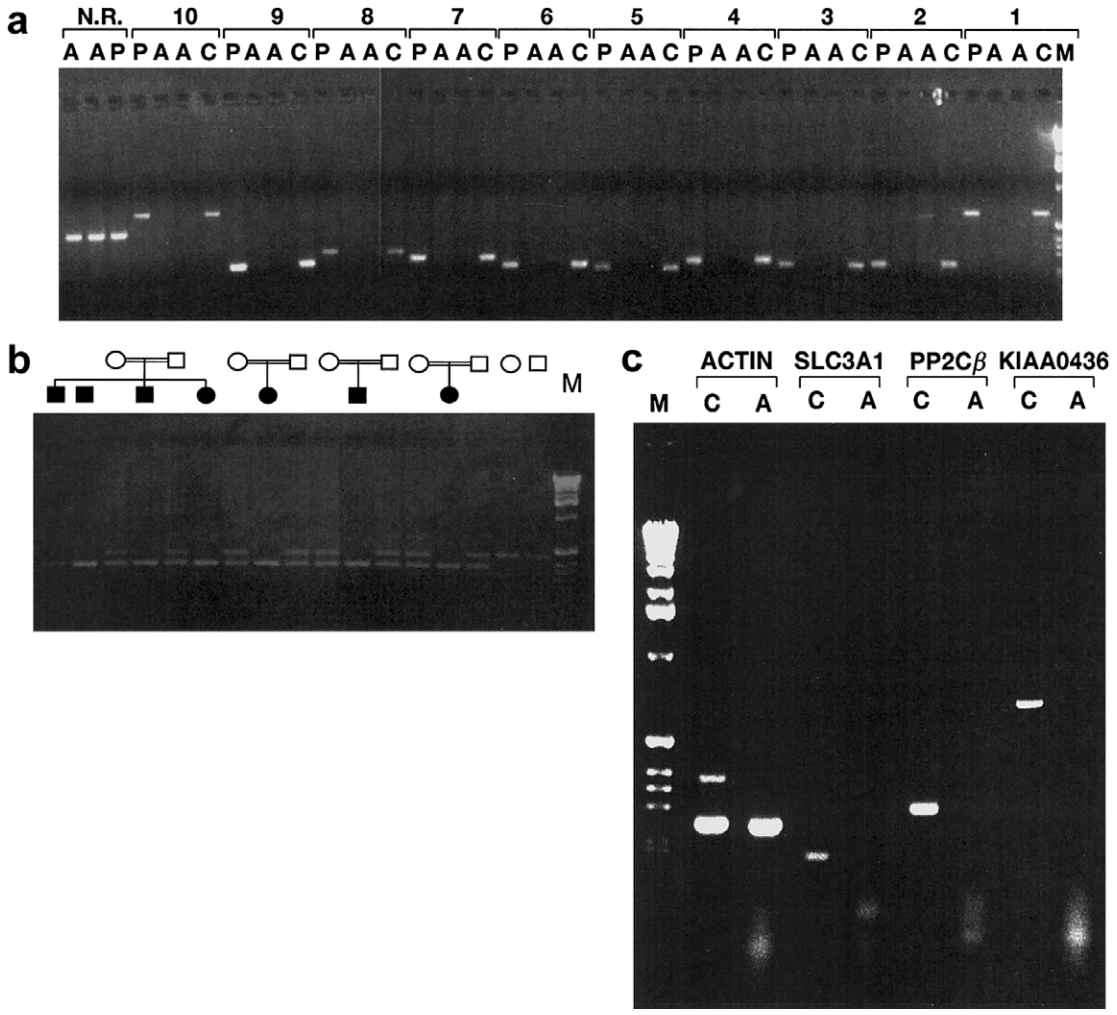

Figure 2 *a,* Results of PCR amplifications of the *SLC3A1* exons (1–10) and an unrelated gene (N.R.). The patients' DNA (V10 and V15 in fig. 1*a*) failed to amplify the *SLC3A1* exons, whereas good amplification was observed for DNA from a parent (IV1 in fig. 1*a*) and a control. The quality of the patients' DNA was assessed by its ability to amplify an unrelated gene. A = affected, P = parent, C = unrelated control individual, and M = molecular weight marker X of Amersham. *b,* Multiplex PCR assay defining the deletion borders. Affected children are indicated by blackened symbols. The PCR reaction included primers a, b, and c, as indicated in fig. 3*a*. Primers' concentrations were 1 mM for primers b and c and 0.5 mM for primer a; annealing temperature was 57°C. The 439-bp product was obtained only if a normal allele was present; the 376-bp product was obtained only if a deletion allele was present. All parents are therefore heterozygotes for the deletion and the wild-type allele. M = the molecular weight marker X of Amersham. *c,* Results of RT-PCR from lymphoblastoid RNA from an affected individual (A; V10 in fig. 1*a*) compared with control (C) of the *SLC3A1, PP2Cβ,* and *KIAA0436* genes. None of these transcripts are PCR-amplified in the affected individual. The actin gene was included to assess RNA quality. Primers for the PCR were selected on different exons, to avoid amplification from residual DNA in the RNA preparation.

KIAA0436 (Ishikawa et al. 1997)—were not transcribed in the patients (fig. 2*c*). RNA was extracted, using Trizol (Gibco), from lymphoblastoid cell lines of two patients. RT-PCR amplification of *SLC3A1, PP2Cβ,* and *KIAA0436* was performed in one step, using the Ready to Go kit (Amersham Pharmacia). For better visualization of the *KIAA0436* product, the RT-PCR was PCR-reamplified. Primers used were as follows: for *SLC3A1,* forward: 5′-gcaaatctcaatgaaagctatg-3′, and reverse: 5′-cttgatataacttcaaagccg-3′; for *KIAA0436,* forward: 5′-actgactctgaggacttgcag–3′, and reverse: 5′-ggtgctgtcagtccaagttc–3′; and for *PP2Cβ,* forward: 5′-gggaagtcgagataacatgag-3′, and reverse: 5′-ccccatcactttctctatgtg-3′. Annealing temperature was 55°C.

The absence of the *SLC3A1* gene is compatible with the early age at onset of renal calculi in all our patients; in contrast, in a large series of cystinuric patients, only 25% reported their first stone during the first decade of life (Stephens 1989). However, the concomitant occurrence of dysmorphism, neonatal seizures, hypotonia, and

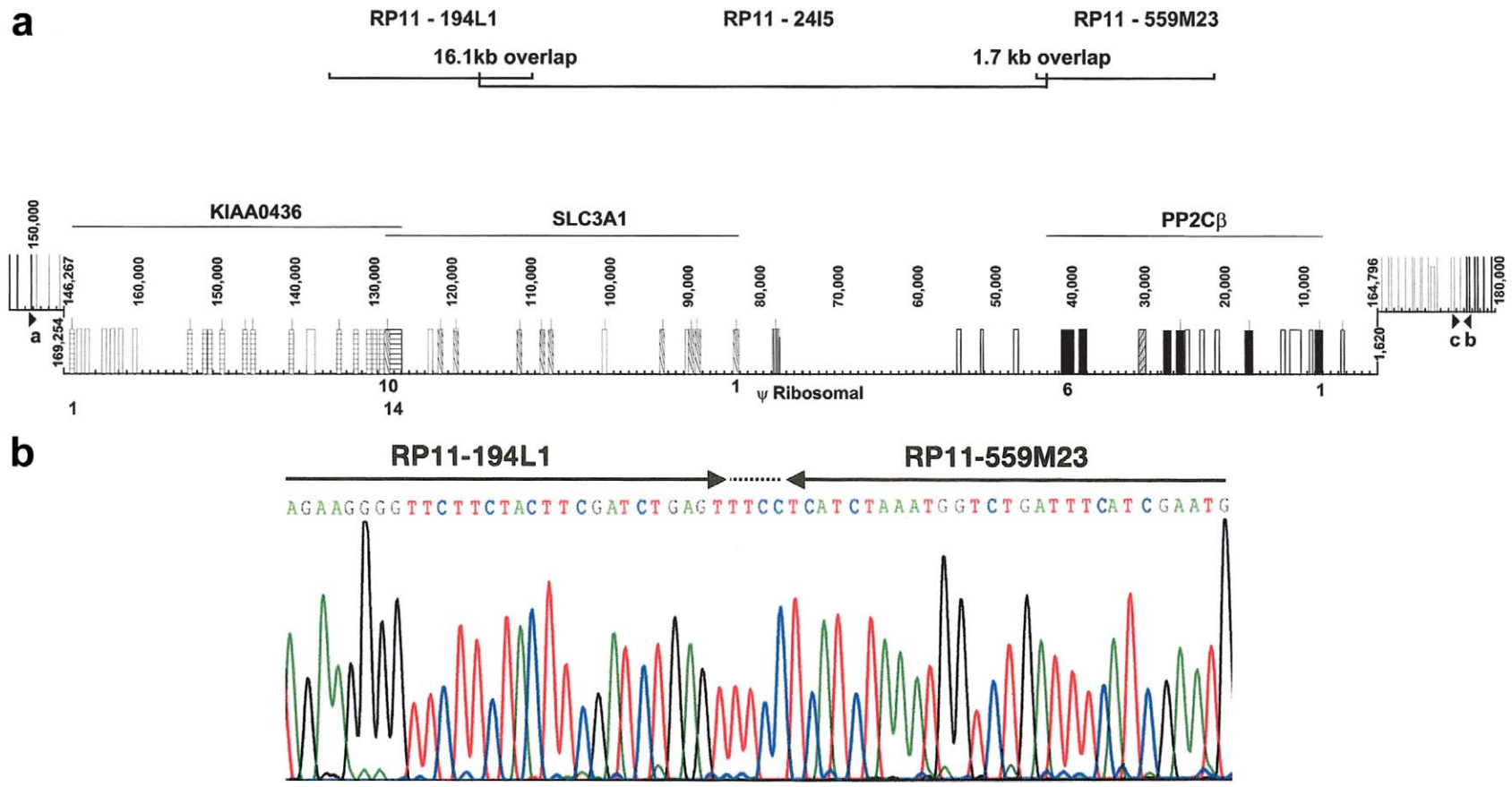

Figure 3 *a,* Schematic representation of the deleted region. *Upper part,* The published sequence of the clones complementary to the sequence reported here. *Lower part,* Details of the clones. The genes contained in the region are indicated by horizontal lines above them. Alignment of each exon was performed by means of the BLAST program of the genomic sequence to the nr database. The 10 exons of the *SLC3A1* (*gray squares*) have been published elsewhere. The genomic organization of the *PP2Cβ* and *KIAA0436* exons (*black squares* and *squares marked with horizontal lines,* respectively) was derived from comparison of the mRNA sequence with the genomic sequence. The numbers of the first and last exons of these genes are indicated below the ruler line. Note that exon 10 of *SLC3A1* and exon 14 of *KIAA0436* overlap; this overlap is in the 3′ untranslated regions of both genes. ESTs are represented by white squares. A hatched square between exons 4 and 5 of the *PP2Cβ* gene represents a clone, AL11753, associated with a UniGene cluster that demonstrates no open reading frame. A pseudoribosomal gene in the interval between *SLC3A1* and *PP2Cβ* is shown by a gray square, and "ψ Ribosomal" appears below it. The positions of the primers used for the definition of the deletion (primers a and b) and for the detection of the wild-type allele (primer c) are marked by arrowheads. Light vertical lines represent the positions of STSs that were not amplified in the affected children. Bold vertical lines represent the positions of STSs that were amplified in the affected children. The numbers on the ruler line are the original numbers in the published sequence of the clones (as appearing in May 1, 2001). The numbers at the ends of clone RP11-24I5 that overlap the numbers of the overlapping clones are indicated. *b,* The sequence at the joining of the deletion ends. The PCR product derived from patient's DNA with primers a and b (as indicated in *a*) was subjected to sequencing. The regions contained in the adjacent clones RP11-194L1 and RP11-559M23 are represented by the upper lines. The four bases TTCC could be derived from either clone, as they are present in both.

severe somatic growth and psychomotor retardation is not explained by the deletion of the *SLC3A1* gene (Gold et al. 1977). These features could be attributed to the MRC defect identified in the muscle biopsy specimens of three of the patients. Hypotonia, developmental delay, and lactic acidemia are well-recognized signs of MRC disorders. The findings of ragged red fibers in the muscle biopsy specimen of one patient, the reduced activity of the mitochondrial encoded enzymatic complexes in two other patients, and the finding of mtDNA depletion in the only patient whose muscle DNA was available for analysis are all consistent with MRC disease.

mtDNA depletion is a heterogeneous group of autosomal disorders presenting in infancy, either with multisystem, fatal disease or isolated liver failure or with a devastating myopathy simulating Duchenne muscular dystrophy (Vu et al. 1998). The underlying pathology may involve a component of the mtDNA replication machinery that is encoded by an as-yet-unidentified gene within the deleted region.

At present, we are unable to exclude the potential contribution of the other genes, within the deleted region, to the phenotype of our patients. The protein serine/threonine phosphatase *PP2Cβ* is widely expressed but is most abundant in heart and skeletal muscle (Marley et al. 1998), and thus its absence may be the cause of the muscle hypotonia. This phosphatase has two alternative splicing variants at its 3′ end (see GenBank accession numbers), and their tissue distribution and functions are unknown. The cyclin-dependent kinases Cdk2 and Cdk6 were reported to be the substrates of one isoform, *PP2Cβ2*, but not substrates of the other *PP2Cβ* isoform (Cheng et al. 2000). Thus the *PP2Cβ* gene with its different isoforms and ubiquitous tissue distribution may have pleiotrophic effects that could contribute to the patients' phenotype. *KIAA0436* represents a clone with weak homology to proteinase II (Ishikawa et al. 1997). It is ubiquitously expressed, and its function awaits further study.

Analyses using the programs Prediction of Protein Localization Sites (PSORT) and Prediction of Mitochondrial Targeting Sequences (MitoProt) indicate that neither *KIAA0436* nor *PP2Cβ* has a signal or mitochondrial targeting peptide.

Thus we have delineated a new syndrome caused by a homozygous deletion of 179,311 bp on chromosome 2p16 and have identified part of the genes contained in this region. In contrast to most deletion syndromes, transmitted as dominant traits, the 2p16 deletion is recessively inherited, implying that only half the product of all genes within the deletion is sufficient for normal life as observed in the parents and siblings of our patients. Further studies of these genes will contribute to the understanding of their function and their relation to the disease phenotype. Because the product of one of these genes, at present probably represented only as an EST, likely participates in the synthesis of the mitochondrial encoded protein, we suggest that families with similar combinations of enzymatic defects should be investigated for linkage to this chromosomal region.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

BLAST (National Center for Biotechnology Information), http://www.ncbi.nlm.nih.gov/BLAST/

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html (for clone RP11-559M23, accession number AC012919; clone RP11-24I5, AC013717; clone RP11-194L1, AC016703; protein phosphatase 2C*β* isoforms, NM_002706 (same isoform as AJ271832), AF294792 (same isoform as AJ271835); *KIAA0436*, AB007896

GeneMap'99,http://www.ncbi.nlm.nih.gov/genemap99/

Genome Database, http://www.gdb.org/

MITOPROT: Prediction of Mitochondrial Targeting Sequences, http://www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for *ABCC8* gene [MIM 600509], USHER Syndrome type IC gene *USH1C* [MIM 276904], Cystinuria [MIM 220100], *SLC3A1* gene [MIM 104614], *SLC7A9* gene [MIM 604144], *MDH1* [MIM 154200], *SFRS7* [MIM 600572], *ATP6H* [MIM 603931], *MTIF2* [MIM 603766], *DGUOK* [MIM 601465], and *PP2Cβ* [MIM 603770]).

PSORT: Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences, http://psort.nibb.ac.jp/

RepeatMasker, http://ftp.genome.washington.edu/cgi-bin/RepeatMasker

Unified Database for Human Genome Mapping, The, http://bioinformatics.weizmann.ac.il/cgi-bin/udb/aliases.pl

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