

## Mitochondrial Neurogastrointestinal Encephalomyopathy Syndrome Maps to Chromosome 22q13.32-qter

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### Summary

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) syndrome is a rare, multisystem disorder characterized clinically by ptosis, progressive external ophthalmoplegia, gastrointestinal dysmotility, leukoencephalopathy, thin body habitus, and myopathy. Laboratory studies reveal defects of oxidative-phosphorylation and multiple mtDNA deletions frequently in skeletal muscle. We studied four ethnically distinct families affected with this apparently autosomal recessive disorder. Probands from each family were shown, by Southern blot, to have multiple mtDNA deletions in skeletal muscle. We mapped the MNGIE locus to 22q13.32-qter, distal to D22S1161, with a maximum two-point LOD score of 6.80 at locus D22S526. Cosegregation of MNGIE with a single chromosomal region in families with diverse ethnic backgrounds suggests that we have mapped an important locus for this disorder. We found no evidence to implicate three candidate genes in this region, by using direct sequence analysis for DNA helicase II and by assaying enzyme activities for arylsulfatase A and carnitine palmitoyltransferase.

### Introduction

Mitochondrial encephalomyopathies encompass a growing number of distinct clinical syndromes that are the result of defects in mitochondrial oxidative-phosphor-

ylation enzymes (DiMauro and Bonilla 1997). Those enzymes are assembled from subunits encoded in both mtDNA and nuclear DNA (nDNA). mtDNA is a double-stranded, circular molecule comprising 16,569 base pairs that encode 22 tRNA, 2 rRNA, and 13 polypeptide genes (Anderson et al. 1981). Of the 13 mtDNA-encoded polypeptides, 7 are subunits of complex I (NADH-coenzyme [Co] Q oxidoreductase), 1 of complex III (CoQ-cytochrome c oxidoreductase), 3 of complex IV (cytochrome c oxidase [COX]), and 2 of complex V (ATP synthase) (Attardi and Schatz 1988). Although the mtDNA-encoded subunits are vastly outnumbered by the ~1,000 mitochondrial polypeptides encoded in nDNA, to date the majority of mitochondrial encephalomyopathies have been associated with mtDNA mutations (Schon et al. 1997). Mutations in nDNA constitute a second cause of mitochondrial dysfunction; however, to date, only two nDNA mutations have been associated with defects in oxidative phosphorylation: one in the flavoprotein subunit of succinate dehydrogenase (complex II) (Bourgeron et al. 1995) and the other in the 18-kD (AQDQ) subunit of complex I (van den Heuvel et al. 1998). A third group of genetic mitochondrial disorders includes defects of intergenomic communication, presumably due to mutations of nDNA genes controlling replication and expression of the mitochondrial genome. The first intergenomic communication defect to be identified was autosomal dominant progressive external ophthalmoplegia (PEO), which was associated with multiple mtDNA deletions (Zeviani et al. 1989). This disorder has been mapped to two chromosomal regions, 10q23.3–24.3 and 3p14.1–21.2 (Suomalainen et al. 1995; Kaukonen et al. 1996). Wolf-ram syndrome, a second disorder associated with multiple mtDNA deletions in two families, is an autosomal recessive neurodegenerative condition clinically characterized by diabetes insipidus, insulin-dependent diabetes mellitus, optic atrophy, and deafness, and it has been linked to chromosomal markers at 4p16 (Barrientos et al. 1996).

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Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), or myoneurogastrointestinal encephalopathy syndrome (MIM 550900; Genome Database accession number 9835128), is a multisystem disorder clinically characterized by onset between the 2d and 5th decades of life, ptosis, PEO, gastrointestinal dysmotility (often pseudo-obstruction), diffuse leukoencephalopathy, thin body habitus, peripheral neuropathy, and myopathy, with a presumed autosomal recessive inheritance pattern (Bardosi et al. 1987; Hirano et al. 1994). Autosomal recessive inheritance has been inferred because of (1) the high recurrence rate among siblings (14 of 49 siblings of probands), (2) the lack of affected parents and progeny, and (3) the relatively high rate of consanguinity (4/19 families) (Hirano et al. 1994; this report). To date, fewer than 50 MNGIE patients, of various ethnic backgrounds, have been identified (Hirano et al. 1994). Laboratory studies demonstrate oxidative-phosphorylation defects, including lactic acidosis, ragged-red fibers in skeletal muscle biopsies, ultrastructurally abnormal mitochondria, and decreased activities of the mitochondrial electron-transport enzymes. The underlying nDNA defect in MNGIE is unknown; however, MNGIE often is associated with multiple mtDNA deletions. The observation of multiple mtDNA deletions in this disorder suggests that the autosomal defect may disrupt intergenomic communication.

In order to identify the chromosomal locus responsible for MNGIE, we screened the entire autosomal genome in two families, using simple-sequence-repeat (SSR) markers and linkage analysis. We then confirmed our finding by analyzing two additional families.

## Subjects and Methods

### *Clinical Characterization*

All individuals who were genotyped were examined by at least one neurologist. The diagnosis of MNGIE was made only for individuals >20 years of age who had the following four clinical features: (1) thin body habitus; (2) ptosis, ophthalmoparesis, or both; (3) gastrointestinal dysmotility (chronic diarrhea or pseudo-obstruction); and (4) sensorimotor peripheral neuropathy (stocking-glove sensory loss, absent tendon reflexes, distal limb weakness, and wasting) (Hirano et al. 1994). Laboratory studies of at least one affected individual in each family showed diffuse leukodystrophy (increased T2 signal on brain magnetic-resonance imaging); nerve conduction abnormalities with features consistent with demyelination, axonal degeneration, or both; skeletal muscle biopsy revealing neurogenic changes, ragged-red fibers, and COX-deficient fibers; and multiple mtDNA deletions.

Individuals V-1 and V-2 in family 3 died prior to the

start of this study. Photographic and medical records confirmed the information, obtained from other family members, that both sisters suffered from the same disorder as their cousin, individual V-4 (fig. 1). Photographs of the sisters when they were in their 20s confirmed that both were very thin and had ptosis. Both had gastrointestinal dysmotility and died several days after gastrointestinal surgery.

### *Genotyping*

Total leukocyte DNA was extracted by use of standard proteinase K, phenol-chloroform, and ethanol precipitation techniques (Zeviani et al. 1988). The DNA was genotyped by use of PCR-amplified fragments containing mapped SSR polymorphisms. For each reaction, 85 ng template DNA was amplified in microtiter wells in a total volume of 10  $\mu$ l containing 1–2 pmol of each primer, 0.02 mM of each dNTP, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 0.5 U *Taq* polymerase. One primer was 5'-labeled by use of  $\gamma$ [<sup>32</sup>P]-dATP and T4 polynucleotide kinase. The radiolabeled DNA fragments were electrophoresed on a 5% denaturing polyacrylamide gel (Long Ranger, FMC BioProducts), which was exposed to Kodak BioMax film.

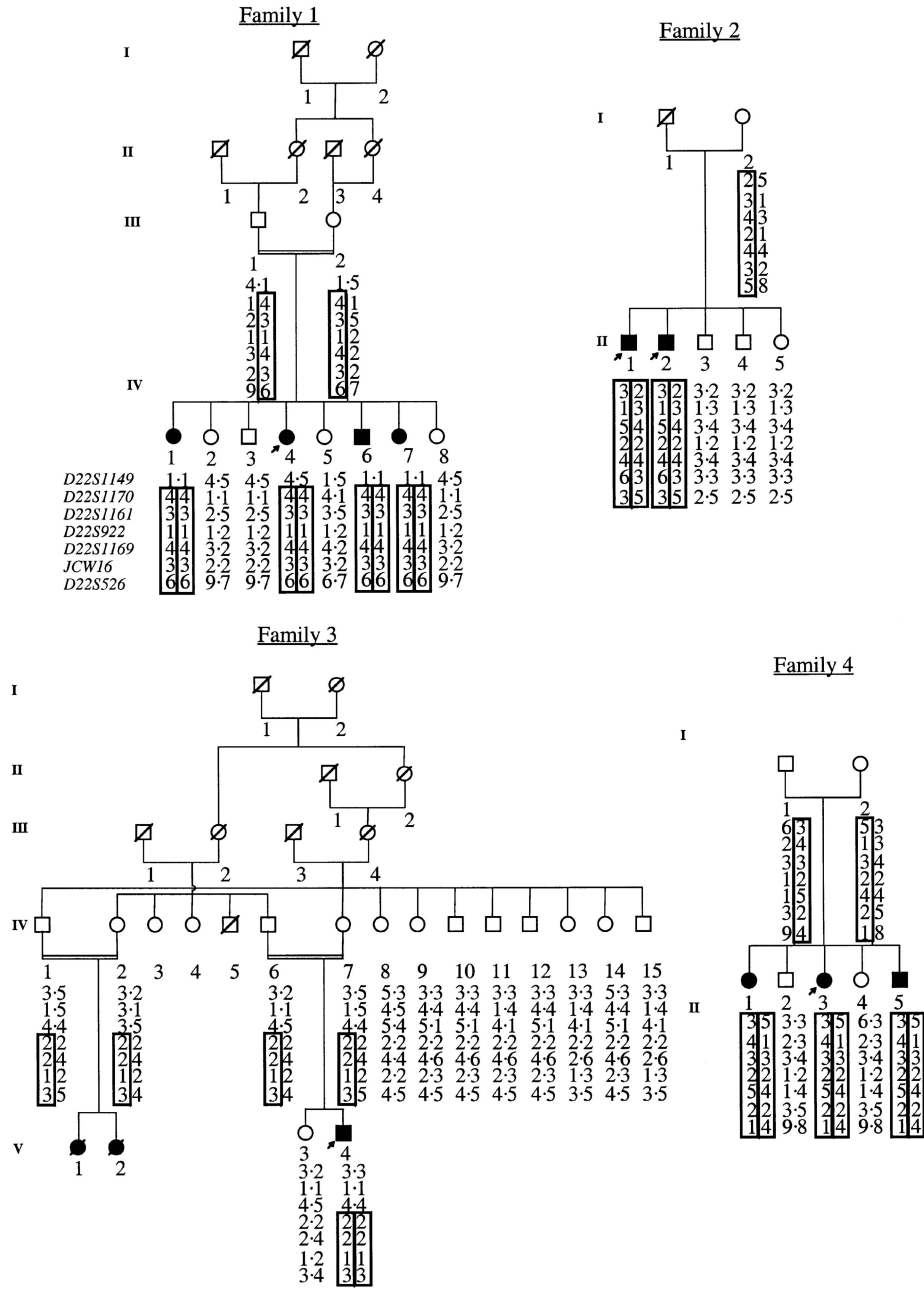
For families 1 and 2, we tested 398 SSR markers distributed across the entire human autosomal genome and spaced, on average, at 10-cM intervals. For the final analysis, we tested 21 SSR markers on chromosome 22, in all four families.

### *Linkage Analyses*

Two-point (marker to disease) analyses were performed by use of the MLINK option of FASTLINK 4.0 (Cottingham et al. 1993; Becker et al. 1998). The data were read by at least two individuals blind to subject identity and disease status. Data not consistent with Mendelian inheritance were reinspected and repeated, if necessary. In addition, data for entire chromosomes were analyzed by use of SIMWALK2, which uses simulated annealing to derive individual haplotypes with a minimum of recombination events (Sobel et al. 1995; Sobel and Lange 1996). Data for individuals or markers that caused apparent high-order recombination events were also reinspected, repeated, or both. Multipoint (at least two markers to disease) analyses were performed by use of the LINKMAP option. We used an autosomal recessive model with a disease penetrance of 95%, a disease frequency of 1 per 4 million, and a phenocopy rate of 1 per 4 billion. Marker allele frequencies were estimated by allele counting with all genotyped subjects.

### *Marker Mapping*

We genotyped 61 families of the CEPH reference collection (Dausset et al. 1990), using the methodology de-



**Figure 1** Pedigrees for MNGIE families. Proband is indicated by the arrows. Blackened symbols indicate affected individuals. Genotypes and inferred haplotypes are listed below the symbol for an individual. Haplotypes shared among the affected individuals and their parents are boxed.

scribed above. Map construction was performed by use of Multimap (Matisse et al. 1994), with error checking following the protocol we used previously (Weeks et al. 1995). Radiation-hybrid (RH) mapping was performed by use of the Stanford G3 and the Stanford TNG3 RH panels (Research Genetics).

*DNA Helicase II Analysis*

Using the cDNA sequence for human DNA helicase II (Ku protein p70 subunit) (Chan et al. 1989; Reeves and Stoeber 1989), we searched the databases, using the National Center for Biotechnology Information Ba-

sic Local Alignment Search Tool. We found DNA sequence fragments within the genomic clone 216E10 (submitted by the Wellcome Trust Genome Campus) that were identical to helicase II p70 subunit cDNA sequences. The complete cDNA sequence was arranged in 13 exons with corresponding intron splice sequences. In addition, we found a 3' UTR with the polyadenylation signal and promoter regions with one CCAAT and three GC-box sequences (M. Hirano and P. Magalhaes, unpublished data). We designed 12 primer pairs flanking the 13 exons and 4 additional primers, to amplify the gene promoter region (M. Hirano and P. Magalhaes, unpublished data). We amplified all 13 exons and the promoter regions of an affected and an unaffected sibling in family 1 (individuals IV-1 and IV-5, respectively) and of the probands from the other three families. The PCR products were electrophoresed in 2% low-melting-point agarose gels, were excised by cutting the gel containing the band, and were isolated by use of the Qiaex II Gel Purification kit (Qiagen).

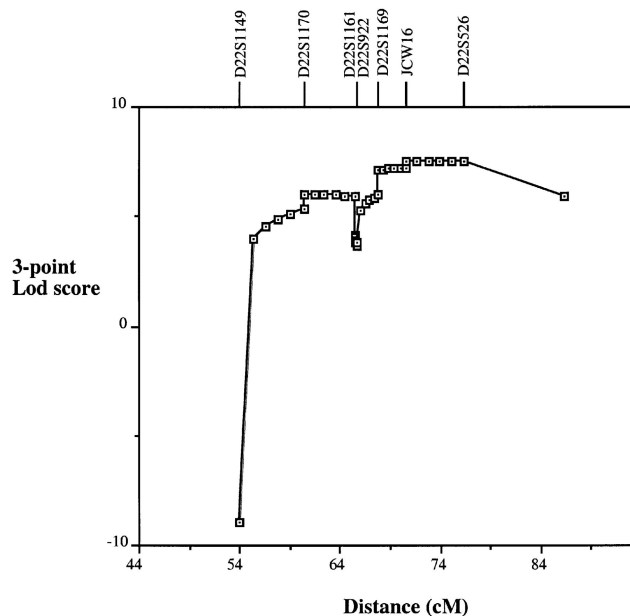
We identified three tandem-repeat sequences in the 216E10 genomic clone containing the helicase II gene—namely, two dinucleotide-repeat elements and one trinucleotide-repeat element. Two were previously identified SSRs, D22S1267 (ATA5F05) and D22S276 (AFM165za5). Genotyping and linkage analyses of all four MNGIE families were performed by use of the methods described above.

## Results

### Clinical Description

The four families have distinct ethnic origins: family 1 is Ashkenazi Jewish; family 2 is German American; family 3 is Puerto Rican; and family 4 is African American. In two families, affected individuals were offspring of consanguinous marriages (fig. 1). Table 1 lists the major clinical features of the affected family members. The proband in each family had the following clinical features: ptosis, ophthalmoparesis, peripheral neuropathy, myopathy, gastrointestinal dysmotility, and thin body habitus. Ten members of these families fulfilled the four diagnostic criteria for MNGIE (see Subjects and Methods), and 25 members were completely unaffected. Two deceased members in family 3 also fulfilled the diagnostic criteria for MNGIE, on the basis of a review of their medical histories and records. The affected brothers in family 2 were reported elsewhere as patients 4 and 5 (Hirano et al. 1994).

In each proband, skeletal muscle biopsy revealed ragged-red fibers and COX-deficient fibers. Southern blot analyses of the skeletal muscle biopsies, by use of restriction enzymes *Bam*HI and *Pvu*II, revealed multiple



**Figure 2** Three-point LOD scores based on genotype data from all four MNGIE families, for markers D22S1149, D22S1170, D22S1161, D22S922, D22S1169, JCW16, and D22S526, which were mapped across a 22.1-cM region on 22q13.32-qter. The maximum LOD score was 7.51 at D22S526. A LOD score of  $-12.4$  at D22S1149 indicates a recombination between this locus and the gene for MNGIE.

mtDNA deletions. Further analyses of skeletal muscle biopsies of two patients (IV-4 in family 1 and V-4 in family 3) have been reported (Carrozzo et al. 1998). Following digestion with each of three restriction enzymes—*Pvu*II, *Bam*HI, and *Sna*BI—Southern blot analysis of skeletal muscle showed multiple mtDNA deletions without duplications; the deletions represented 6% (patient IV-4 in family 1) and 20% (patient V-4 in family 3) of the total mtDNA (Carrozzo et al. 1998).

### Linkage Studies

For our initial screening, we genotyped 10 members of family 1 and 6 members of family 2, using 398 polymorphic SSR markers distributed across the human autosomal genome, at  $\sim 10$ -cM intervals. By multipoint analysis, we excluded  $>90\%$  of the autosomal genome (LOD scores  $\leq -2.0$ ) as the potential locus for MNGIE in families 1 and 2. The largest chromosomal region that was not excluded was 17 cM (except for the chromosomal region linked to the disease). We identified five loci yielding two-point LOD scores  $\geq 1.0$ . Subsequent evaluation of these regions, with additional markers and multipoint analyses, excluded linkage to all but one region, at the telomere of chromosome 22q.

For the final analyses, we genotyped all four families,

**Table 1****Clinical Features of MNGIE Patients**

Family (Ethnic Origin) and Patient	Age (years)	Age at Onset (years)	Ophthalmoparesis	Peripheral Neuropathy	Gastrointestinal Dysmotility	Multiple mtDNA Deletions
1 (Ashkenazi Jewish):						
IV-1	49	32	+	+	+	Not tested
IV-3 <sup>a</sup>	45	43	+	+	+	
IV-6	40 <sup>b</sup>	35	+	+	+	Not tested
IV-7	39	29	+	+	+	Not tested
2 (German American):						
II-1 <sup>a</sup>	26	19	+	+	+	+
II-2 <sup>a</sup>	25	20	+	+	+	+
3 (Puerto Rican):						
V-1	25 <sup>b</sup>	18	+	+	+	Not tested
V-2	Unknown <sup>b</sup>	Unknown	+	+	+	Not tested
V-4 <sup>a</sup>	34	24	+	+	+	+
4 (African American):						
II-1	27	Unknown	+	+	+	Not tested
II-3 <sup>a</sup>	23	Unknown	+	+	+	+
II-5	13	13	+	+	+	Not tested

NOTE.—A plus sign (+) indicates presence of the clinical feature.

<sup>a</sup> Proband.

<sup>b</sup> Patient died at age indicated.

with 21 markers on chromosome 22. The maximum two-point LOD score was 6.80 with marker D22S526, with no recombinations (table 2). Three-point analysis of the genotype data resulted in a maximum LOD score of 7.51 at D22S526 (fig. 2). The disease region was flanked proximally by an obligate recombination event in family 3, at D22S1161 (table 2).

The genetic map of the telomeric region of 22q is poorly defined. To determine the order of the seven markers across the linked region and to define its boundaries, we used these markers to create a high-resolution genetic map, by genotyping the extended CEPH reference collection (61 families; 1,032 meioses) (table 3 in Dausset et al. 1990). This order was supported by odds  $>10^{20}$  over any other order and places D22S526 distal to JCW16. The observed paternal recombination rate for the distal two markers was more than sixfold the maternal rate, which is consistent with observations of other telomeric regions. The genetic map order was also supported by the RH-mapping data, with odds  $>200:1$ , by use of the Stanford G3 and the Stanford TNG3 RH panels. The detailed genetic map then was used in multipoint analyses. Our results suggest that MNGIE is located in the telomeric region of chromosome 22q, distal to D22S1161.

#### Candidate-Gene Evaluation

Of 34 cDNA markers within our candidate region (D22S1149 to 22qter) (Schuler et al. 1996), we identified three human genes of known functions: arylsulfatase A, carnitine palmitoyltransferase I (CPTI), and ATP-depen-

dent DNA helicase II. We considered all three as potential candidate genes for MNGIE.

To examine the gene for carnitine palmitoyltransferase, we measured enzyme activity in total muscle homogenates from probands IV-4 in family 1 and V-4 in family 3; both were normal (data not shown). Leukocyte arylsulfatase activity was normal in patients IV-1 in family 1, II-1 and II-2 in family 2, and V-4 in family 3 (data not shown).

In our efforts to identify the genomic sequence of helicase II, we detected two previously identified tandem-repeat sequences, D22S1267 (ATA5F05) and D22S276 (AFM165za5), adjacent to the gene. We had genotyped these SSR markers as part of our MNGIE linkage efforts and were able to exclude the helicase gene in two families (1 and 3) by linkage alone. In the index patients of all four families, we sequenced the 13 exons of the helicase gene, all intron/exon (GT/AT) splice junctions, 1,609 nucleotides 5' of the first exon (which included one CCAAT and three GC boxes), and 24 nucleotides 3' of the polyadenylation signal. We found no mutations in the genomic DNA sequence.

#### Discussion

To date, no human gene has been identified as a cause of multiple mtDNA deletions. Our study demonstrates a chromosomal locus for MNGIE located in the 10.6-cM telomeric region of the long arm of chromosome 22 (22 q13.32-qter), distal to locus D22S1161. Our identification of a single chromosomal region that cosegre-

**Table 2**

**Two-Point LOD Scores for Chromosome 22q Markers and MNGIE Region, with Sex-Averaged Relative Map Locations Based on the Extended (61 Families) CEPH Reference Collection**

MARKER (RELATIVE DISTANCE [IN cM]) AND FAMILY	LOD SCORE AT RECOMBINATION FRACTION OF						
	.00	.01	.05	.10	.20	.30	.40
D22S532:	<u>-10.70</u>	-.36	.78	1.05	.98	.64	.25
1	-11.81	-1.44	-.20	.20	.39	.31	.13
2	-.19	-.19	-.16	-.13	-.07	-.03	-.01
3	.46	.45	.39	.32	.19	.10	.04
4	.84	.82	.75	.66	.46	.26	.08
D22S1160:	<u>-.81</u>	.29	.77	.82	.65	.38	.12
1	.18	.18	.15	.11	.06	.02	.01
2	-.01	-.01	-.01	-.01	.00	.00	.00
3	-1.82	-.70	-.12	.06	.13	.10	.04
4	.84	.82	.75	.66	.46	.26	.08
D22S1149 (0):	<u>-9.50</u>	.81	1.82	1.94	1.59	1.01	.41
1	-11.33	-.96	.25	.61	.70	.53	.26
2	-.22	-.21	-.19	-.16	-.10	-.04	-.01
3	.60	.58	.48	.38	.21	.10	.03
4	1.44	1.41	1.28	1.12	.78	.43	.13
D22S1170 (6.4):	<u>4.59</u>	4.50	4.14	3.68	2.72	1.69	.66
1	2.89	2.84	2.62	2.34	1.75	1.11	.46
2	-.21	-.20	-.18	-.14	-.08	-.03	-.01
3	.46	.45	.39	.32	.21	.13	.06
4	1.44	1.41	1.30	1.16	.83	.48	.15
D22S1161 (11.5):	<u>2.62</u>	3.72	3.93	3.64	2.72	1.66	.62
1	2.76	2.71	2.51	2.24	1.68	1.07	.44
2	.96	.93	.84	.71	.48	.25	.07
3	-1.94	-.75	-.16	.03	.11	.08	.03
4	.84	.82	.75	.66	.46	.26	.08
D22S922 (11.5):	<u>3.56</u>	3.48	3.15	2.75	1.93	1.13	.42
1	1.84	1.80	1.65	1.46	1.07	.67	.29
2	.72	.70	.63	.53	.35	.18	.05
3	.16	.15	.13	.10	.05	.02	.01
4	.84	.82	.75	.66	.46	.26	.08
D22S1169 (13.7):	<u>5.38</u>	5.26	4.78	4.17	2.93	1.71	.62
1	2.62	2.57	2.38	2.13	1.59	1.01	.40
2	.65	.64	.58	.50	.34	.18	.05
3	1.27	1.23	1.08	.89	.54	.26	.08
4	.84	.82	.75	.66	.46	.26	.08
JCW16 (16.4):	<u>6.50</u>	6.35	5.76	5.01	3.47	1.98	.68
1	2.66	2.61	2.41	2.15	1.59	.99	.37
2	.96	.93	.81	.66	.39	.18	.05
3	1.44	1.40	1.24	1.04	.65	.33	.11
4	1.44	1.41	1.30	1.16	.83	.48	.15
D22S526 (22.1):	<u>6.80</u>	6.65	6.04	5.27	3.68	2.15	.82
1	3.03	2.98	2.76	2.47	1.86	1.22	.54
2	.96	.93	.81	.66	.39	.18	.05
3	1.37	1.33	1.17	.97	.59	.27	.08
4	1.44	1.41	1.30	1.16	.83	.48	.15

NOTE.—Underlined numbers indicate LOD scores derived from all four families with no recombinations.

gates with MNGIE in four families of different ethnic origins strongly supports the concept that one nDNA gene is responsible for this disorder. The localization of this gene is distinct from those for autosomal dominant PEO and autosomal recessive Wolfram syndrome with multiple mtDNA deletions (Suomalainen et al. 1995; Barrientos et al. 1996; Kaukonen et al. 1996).

We considered each of the three identified human

genes within 22q13.32-qter as candidates. Arylsulfatase A deficiency is the cause of metachromatic leukodystrophy. Because cerebral leukodystrophy and peripheral neuropathy with demyelination are prominent features of MNGIE, arylsulfatase A deficiency could account for these features of the disease. However, normal arylsulfatase A activity in leukocytes and absence of metachromatic deposits in peripheral-nerve biopsies from

MNGIE patients indicate that arylsulfatase A is unlikely to cause MNGIE (Hirano et al. 1994).

CPTI is located in the inner side of the outer mitochondrial membrane and is crucial for lipid metabolism. Specifically, CPTI catalyzes the transport of long-chain and very-long-chain acyl-CoA molecules across the inner mitochondrial membrane, by esterization of those molecules to acylcarnitine and with the release of free CoA. Patients with CPTI deficiency have presented with infantile hypoketotic hypoglycemia or, less commonly, with myopathy (Hirano and DiMauro, in press). Skeletal muscle from MNGIE patients showed normal carnitine palmitoyltransferase activity, indicating that this gene product probably is not the cause of the disorder. Moreover, because the CPTI isoform located on chromosome 22q13.3 is expressed predominantly in skeletal and heart muscle, a mutation in the CPTI gene is unlikely to cause a multisystem disorder such as MNGIE (Yamazaki et al. 1996).

The 70-kD subunit of ATP-dependent DNA helicase II potentially is the strongest candidate, because it is a DNA-binding protein that is involved in nDNA replication, transcription, repair, and recombination (Vishwanatha et al. 1995). According to one report, helicase II may be found in cytosol and the nucleus; therefore, it could have a role in intergenomic communication (Vishwanatha et al. 1995). However, by both linkage analysis of two of the families and by direct sequence analysis of all four families, we excluded this gene.

Although our investigation of candidate genes has not revealed the underlying nDNA defect in MNGIE, we have restricted the search to the portion of 22q distal to D22S1161 and have provided a detailed genetic framework for future studies. Examination of the inferred haplotypes for family 3 (fig. 1) reveals that the proband (V-4) and his paternal aunt (IV-2), who is an obligate carrier, share a haplotype only for markers distal to D22S1161. This obligate recombination event defines the proximal boundary of the MNGIE region.

The localization of MNGIE contributes to our nascent but growing understanding of the intergenomic communication between the nDNA and mtDNA. Ultimately, identification of the gene responsible for MNGIE and other autosomally inherited multiple mtDNA-deletion syndromes will provide much insight into the interactions between these genomes.

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## Electronic-Database Information

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

Genome Database, <http://www.gdb.org/> (for MNGIE [9835128])

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim> (for MNGIE [MIM 550900])

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