9. Phylum-level signatures occurred at the following positions (*Escherichia coli* numbering): 358 (C), 359 (U), 361 (A), 396 (G), and 555 (U). *Treponema* genus-level signatures occurred at 108 (G) and 111 (U) [B. J. Paster *et al.*, *J. Bacteriol.* **173**, 6101 (1991)].
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11. RFS clones [from *Reticulitermes flavipes* (Kollar) (Rhinotermitidae)] and clone ZAS89 were from T. G. Lilburn, T. M. Schmidt, and J. A. Breznak (in preparation); sp40 clones were from *Mastotermes darwiniensis* Froggatt (Mastotermitidae) (*5*); and clone NL1 was from *Nasutitermes lujae* (Wasmann) (Nasutitermitinae) (*2*).
11. YA was prepared by allowing 56 g of dry baker's yeast (Red Star Yeast and Products, Milwaukee, WI) to autolyze in 200 ml of distilled water for 24 hours at 56°C. Cell debris was removed by centrifugation, and the supernatant was neutralized with 5N NaOH and filter sterilized. Cofactor solution contained pyridoxal HCl and pyridoxal phosphate (250 μg/ml each); calcium folinic acid, β-NAD, coenzyme A, and FAD (50 μg/ml each); nicotinamide (25 μg/ml); folic acid (2.5 μg/ml); riboflavin (0.5 μg/ml); hemin (in 10 mM NaOH) (65 μg/ml); and thiamine pyrophosphate (2500 μg/ml); and was filter sterilized. Replacement of rumen fluid and nutrient broth in modified JM-4 medium (*6*) with 2% (by volume) each of YA and cofactor solution yielded 2YACo medium; increasing YA to 4% yielded 4YACo medium.
13. Commercial preparations tested included yeast extract (Difco Laboratories, Detroit, MI; Sigma, St. Louis, MO; and ICN Nutritional Biochemicals, Cleveland, OH); yeast hydrolyzate enzymatic (USB, Cleveland, OH); TC yeastolate (Difco); and yeast extract solution (Gibco-BRL Life Technologies, Grand Island, NY).
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28. The nucleotide sequences of 16*S* rRNAs were inferred from 16*S* rDNAs that were amplified from genomic DNA by PCR with primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and either 1492R (5′-GGTTACCTTGTTACGACTT-3′; for ZAS-1) or 1400R [5′-ACTC-(KC)GKTGGPGTGACGGGC-3′, where P is 6H, 8H-3,4-dihydropyrimido(4,5c)(1,2)oxazin-7-one, K is 2- amino-6-methoxyamine purine, and KC is a degenerate position; for ZAS-2], cloned into pCR2.1, and sequenced (both strands, at a mean redundancy of 2.5 nucleotides per position). PCR consisted of 30 cycles, each of 94°C for 15 s, 57°C for 30 s, and 72°C for 60 s. The last cycle was followed by incubation at 70°C for an additional 10 min. Other procedures were previously described [K. S. Kim, T. G. Lilburn, M. J. Renner, J. A. Breznak, *Appl. Environ. Microbiol.* **64**, 1919 (1998)]. The sequences of each 16*S* rDNA (1464 and 1367 unambiguous nucleotides for ZAS-1 and ZAS-2, respectively) were aligned within ARB [O. Strunk and W. Ludwig, "ARB: Software for phylogenetic analysis" (Technical University of Munich, Germany, 1997)] and analyzed by maximum likelihood [fastDNAml; G. J. Olsen, H. Matsuda, R. Hagstrom, R. Overbeek, *CABIOS* **10**, 41 (1994)] and by parsimony [D. L. Swofford, "PAUP: Phylogenetic analysis using parsimony," version 3.1.1 (Smithsonian Institution, Washington, DC, 1993)]. The 16*S* rDNA sequences of ZAS-1 (accession number AF093251) and ZAS-2 (accession number AF093252) have been deposited in GenBank. The accession numbers and alignments of all sequences used in this study and the designation of the nucleotide positions used to generate Fig. 1D are available from the Ribosomal Database Project at www.cme.msu.edu/RDP. A distance matrix constructed from the data is available on request from the corresponding author.
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30. Two to four × $10^5$ becquerels of $Na_2^{14}CO_3$ (American Radiochemicals, St. Louis, MO) were incorporated into rubber-stoppered culture tubes containing 5 ml of 2YACo medium (for ZAS-1) or 4YACo medium (for ZAS-2) under 80% $H_2$ or $N_2$ (the balance was $CO_2$). Samples of the gas and liquid phases were removed for determination of initial radioactivity. The specific activity of $^{14}CO_2$ was estimated from the $CO_2$ content of the gas phase (determined by gas chromatography) and the amount of $HCO_3^-$ calculated to exist in the medium at the (measured) initial pH of 7.15 (*17*, *26*). Products were purified for determination of radioactivity during high-performance liquid chromatography (HPLC) analysis (*17*). The percent of product derived from $^{14}CO_2$ equals [the specific activity of the product/(number of C atoms in the product × the specific activity of $^{14}CO_2$)] × 100. The amount of $^{14}CO_2$ assimilated into cell material was 1.0 to 1.2% (ZAS-1) and 0.3% (ZAS-2). Other products included $^{14}C$ compounds whose peaks were masked by other medium components during HPLC or were present in amounts too low to elicit a significant detector response.
31. This paper is dedicated to Professor Ercole Canale-Parola, who introduced one of us (J.A.B.) to the study of spirochetes many years ago, who inspired the current work, and who recently retired after more than three decades of making important contributions to microbiology. We thank T. G. Lilburn for helpful discussions, J. Shellman-Reeve for samples of *Z. angusticollis*, H. S. Pankratz for electron microscopy, K. S. Kim for technical assistance, and P. Lamoureux for production assistance. This work was supported by NSF grants IBN97-09000 (J.A.B.) and BIR91-20006 (the Center for Microbial Ecology).


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# Thymidine Phosphorylase Gene Mutations in MNGIE, a Human Mitochondrial Disorder

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**Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive human disease associated with multiple deletions of skeletal muscle mitochondrial DNA (mtDNA), which have been ascribed to a defect in communication between the nuclear and mitochondrial genomes. Examination of 12 MNGIE probands revealed homozygous or compound-heterozygous mutations in the gene specifying thymidine phosphorylase (TP), located on chromosome 22q13.32-qter. TP activity in leukocytes from MNGIE patients was less than 5 percent of controls, indicating that loss-of-function mutations in *TP* cause the disease. The pathogenic mechanism may be related to aberrant thymidine metabolism, leading to impaired replication or maintenance of mtDNA, or both.**

Mutations in mtDNA have been associated with a wide spectrum of mitochondrial diseases (*1*), and more than 50 pathogenic mtDNA point mutations have been identified as causes of maternally inherited mitochondrial encephalomyopathies. Another group of diseases are typically associated with multiple deletions of mtDNA, but show autosomal transmission and thus have been attributed to defective communication between the nuclear and mitochondrial genomes. Among these diseases are autosomal dominant progressive external ophthalmoplegia, which has been linked to two chromosomal loci, 10q23.3-q24.3 and 3p14.1-p21.2 (*2*), and an autosomal recessive disease associated with multiple deletions, mitochondrial neurogastrointestinal

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encephalomyopathy (MNGIE; Mendelian Inheritance in Man number 550900, Genome Database accession number 9835128), linked to chromosome 22q13.32-qter (*3*).

MNGIE is characterized clinically by onset between the second to fifth decades, ptosis, progressive external ophthalmoplegia, gastrointestinal dysmotility, thin body habitus, peripheral neuropathy, myopathy, leukoencephalopathy, and lactic acidosis (*4*). Laboratory studies show various mitochondrial abnormalities in skeletal muscle, including ragged-red fibers with ultrastructurally abnormal mitochondria, decreased activities of respiratory chain enzymes, and multiple mtDNA deletions or mtDNA depletion, or both (*4, 5*).

Linkage to chromosome 22q13.32-qter was confirmed in a total of seven families with MNGIE (*3, 6*). In this region (*7*), the gene encoding thymidine phosphorylase (TP; E.C. 2.4.2.4) appeared particularly interesting. TP catalyzes the reversible phosphorolysis of thymidine (thymidine + phosphate ↔ thymine + 2-deoxy D-ribose 1-phosphate) and is likely to have an important role in nucleoside metabolism by regulating the availability of thymidine for DNA synthesis (*8*).

To screen for *TP* mutations, we designed primers to amplify and sequence all the TP coding exons and the flanking regions (*9*). We sequenced DNA from 12 unrelated MNGIE probands and one control, and compared the data to a reference sequence (*10*). All the probands had homozygous or compound-heterozygous mutations (Table 1). Southern blot analysis of skeletal muscle DNA revealed that seven of nine patients had multiple mtDNA deletions. We were unable to study skeletal muscle DNA in three probands.

We identified 10 different mutations: four missense, three splice-site, two deletions, and a single-nucleotide insertion (Table 1 and Fig. 1). We confirmed segregation of these point mutations with the disease in families 1 to 7. Three of the missense mutations, G1419A, G1443A, and A3371C, were found in multiple families.

Two missense mutations, G1419A and G1443A, change the second and tenth encoded amino acids within the thymidine/pyrimidine-nucleoside phosphorylase consensus sequence [$Gly^{145}$ → $Arg^{145}$ (G145R) and G153S (*11*), respectively] (*12*). The other two missense mutations, A2744G and A3371C, change amino acids K222S and E289A, respectively (Table 1 and Fig. 2). Lysine-222 in human TP corresponds to K191 in the *Escherichia coli* protein, which forms a hydrogen bond with a phosphate (*13*). Because serine has an uncharged alkyl group, the K222S mutant presumably cannot create a hydrogen bond with a phosphate. Although the functional importance of E289 is unknown, this residue is well conserved (Fig. 2). The four TP missense mutations were not present in DNA from 63 control individuals (*14*).

Splice-site mutations in TP caused aberrant mRNA splicing. By reverse transcription–polymerase chain reaction (RT-PCR) analysis, we confirmed exon skipping in blood samples from two patients (*15*). The T1504C mutation disrupted the splice donor site sequence (GT) in intron 4, resulting in skipping of exon 4 (Fig. 1). The G3867C mutation disrupted the splice acceptor site sequence (AG) in intron 8, resulting in exon 9 skipping. Because a "leucine zipper pattern" sequence (*12*) (amino acids 417 to 438) spans the junction between exons 9 and 10 (Fig. 2), this mu-

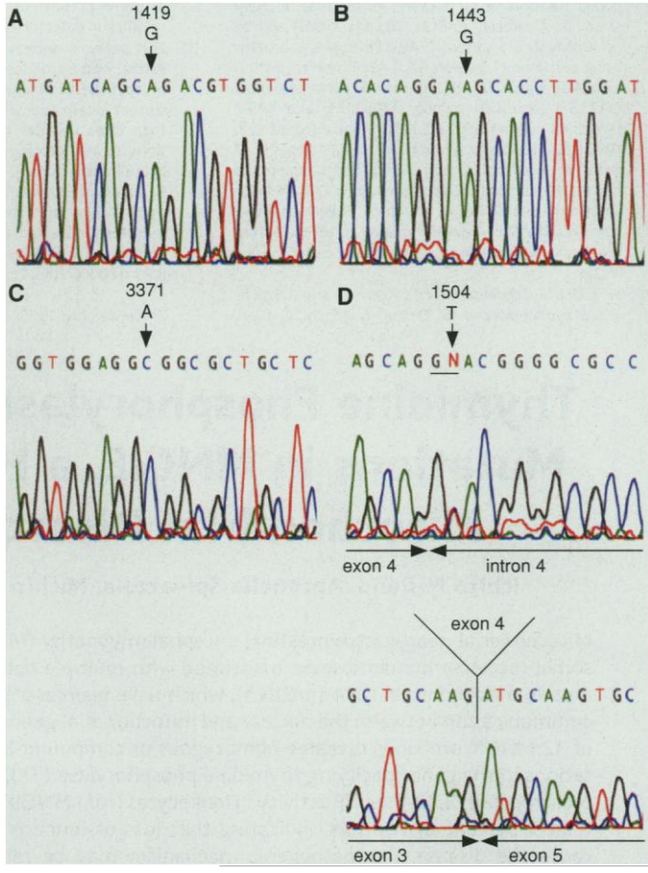


**Fig. 1.** Representative electropherograms showing *TP* mutations in patients with MNGIE. (**A**) Homozygous G1419A in patient 6. (**B**) Homozygous G1443A in patient 12. (**C**) Homozygous A3371C in patient 1. (**D**) Heterozygous T1504C in patient 2 (top), which is designated "N" by Sequence Analysis version 3.0 software (Perkin-Elmer). The mutated splice-donor site (GN) is underlined. RT-PCR produced two bands, which were excised separately from the gel. Direct sequencing of the shorter fragment revealed exon 4 skipping (bottom).

| Mutation | 145 R | 153 S | 222 S | 289 A |
|---|---|---|---|---|
| Human | VPMISGRGLGHTGGTLDKLESIP | PLITASILSKKLVEGLSALVV | GRCVGHALEVEEALLCMDGAG | |
| *Methanococcus jannaschii* | IPKTSSRAITSAAGTADVVEVL- | PLLLSSVMAKKLAMGVNKLLI | GRAIGPALEAKEALLALE-DY | |
| *Mycoplasma genitalium* | VAKLSGRGLGYTGGTIDKLEAV- | PLIASSIMSKKLAVMNEYIFI | GKAVGNVIEVNEAVNFLKQDL | |
| *Mycoplasma pneumoniae* | VAKLSGRGLGYTGGTIDKLEAV- | PLIASSVMSKKLAINNDYIFI | GKTIGNALEVLEVVHFLKRNW | |
| *Mycoplasma pirum* | VAKLSGRGLGFTGGTIDKLESI- | PLIAASILSKKFALESDYIFI | GNTVGNAIEVKEAIDFLKNN- | |
| *Mycobacterium tuberculosis* | VPQASGRGLGHTGGTLDKLESIT | PLIASSIMSKKLAEGAGALVL | GRTVGNALEVAEALEVLAGGG | |
| *Escherichia coli* | IPMISGRGLGHTGGTLDKLESIP | PLITASILAKKLAEGLDALVM | ASSAGNAVEVREAVQFLTGEY | |
| *Bacillus stearothermophilus* | VAKMSGRGLGHTGGTIDKLESVP | PLIASSIMSKKIAAGADAIVL | GYAIGNALEVKEAIDTLKGEG | |
| *Bacillus subtilis* | VAKMSGRGLGHTGGTIDKLEAIM | PLIASSIMSKKIAAGADAIVL | GFAIGNALEVKEAIDTLKGEG | |

**Fig. 2.** Amino acid conservation through evolution (*11*). All of the identified missense mutations replace the well-conserved amino acids (shaded residues). The thymidine/pyrimidine-nucleoside consensus signature (*12*) is underlined. Sequences of *Bacillus stearothermophilus* and *B. subtilis* encode portions of pyrimidine-nucleoside phosphorylase, which also has uridine phosphorylase activity in addition to thymidine phosphorylase activity, while other sequences are for thymidine phosphorylase. Multiple alignments and consensus sequences were obtained from the ProDom database (*25*). We also added the amino acid sequence of *Mycobacterium tuberculosis* TP predicted from the DNA sequence of *M. tuberculosis* H37Rv complete genome, segment 143/162 (European Molecular Biology Laboratory accession AL021841).

tation is predicted to delete an important segment of the protein.

The 6–base pair (bp) deletion in exon 9 would result in the deletion of L397 and A398. Although these amino acids are not strictly conserved, their loss could alter the structure and enzymatic activity of the protein. Insertion of a C at nucleotide (nt) 4196 shifts the reading frame from amino acid 471, which would remove an in-frame stop codon in the cDNA sequence.

We found a heterozygous 4-bp deletion in intron 7 of patient 8, who had the G1419A transition in the other allele. This microdeletion may be a neutral polymorphism or it may affect splicing. Alternatively, the second pathogenic mutation in patient 8 may be present in exon 1; or in another intron, resulting in alteration of the mRNA; or a regulatory sequence.

All of the patients harbored a homozygous A3673G polymorphism relative to the reference sequence. We also identified a T3576C polymorphism, which was homozygous in patients 3 to 6 and 11 and 12, and heterozygous in patients 8 and 10. Both polymorphisms are unlikely to be pathogenic, because a healthy control was also homozygous for the A3673G transition; the T3576C polymorphism is silent.

To investigate the functional effects of the TP mutations, we assayed TP activity in peripheral leukocytes from six probands and 19 normal controls. Patients 1, 2, and 4 had no detectable TP activity, and all others had activity that was <5% of that in controls (Fig. 3). This finding, and the fact that TP mutations segregated with MNGIE, lead us to conclude that the disease is caused by loss-of-function mutations in *TP*.

Human TP has been studied extensively and shown to possess at least three functions: catalysis (as thymidine phosphorylase), angiogenesis, and cell trophism (*8*). TP is also called platelet-derived endothelial cell growth factor (PD-ECGF) or endothelial cell growth factor 1 (ECGF1), because of its angiogenic properties (*16*), or gliostatin, to denote its inhibitory effects on glial cell proliferation. TP is also present in prokaryotes, and its sequence is highly conserved (*16*). Human TP has been investigated extensively by cancer researchers, because TP expression and activity are increased in some tumors, presumably reflecting their neovascularization (*9*). Our MNGIE patients did not have vascular abnormalities, suggesting that the absence of

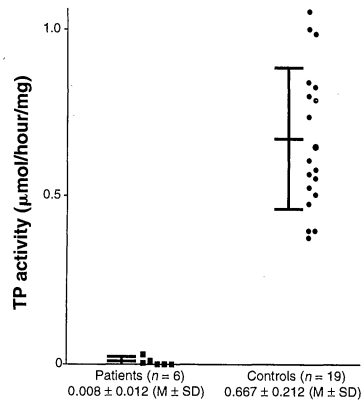


**Fig. 3.** Assay of TP activity in leukocytes from patients with MNGIE. Patients 1, 2, 3, 4, 6, and 7 and 19 controls were studied. The rate of conversion of thymidine to thymine was measured spectrophotometrically as in (*26*). Peripheral leukocytes were homogenized in lysis buffer [50 mM tris-HCl (pH 7.2) containing 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 0.02% 2-mercaptoethanol] and subjected to brief sonication. Samples were centrifuged at 20,000*g* for 30 min at 4°C, and the supernatants were used for the enzyme assay. From each supernatant, 100 to 250 μg of protein was incubated with 0.1 M tris-arsenate buffer (pH 6.5) and 10 mM thymidine in a total volume of 0.1 ml. After 1 hour of incubation at 37°C, the reaction was stopped by adding 1 ml of 0.3 M NaOH. The amount of thymine formed was measured at 300 nm wavelength, based on a $3.4 \times 10^3$ difference in the molar extinction coefficient between thymidine and thymine at alkaline pH. Enzyme activity was expressed as micromoles of thymine generated per hour per milligram of protein. The protein content was determined as in (*27*). Results were obtained from two independent experiments performed in duplicate. Patients 1, 2, and 4 showed no detectable activity. The difference between patients and controls were statistically significant, as assessed by the two-tailed Student's *t*-test ($P < 10^{-10}$). Bars indicate the mean value (M) and standard deviation (SD).

**Table 1.** *TP* mutations with MNGIE. Exons 2 through 10 from 12 probands were sequenced. Patients 1 to 4 are the original families used for linkage analysis and correspond to individuals IV-3, II-1, V-3, and II-1, respectively in (*3*). Southern blot analyses of skeletal muscle were performed with Bam HI and Pvu II (*24*). Multiple deletions of mtDNA were not found in patients 8 and 9; this might be due to partial mtDNA depletion (*6*). 4-bp del, deletion of nts 3527–3530; 6-bp del, deletion of nts 3895–3900; ins4196C, insertion of C at nt 4196; Pi, phosphate; aa, amino acid; ND, not determined.

| Patient number | Ethnic origin | Mutation* | Exon/ intron | mRNA alteration | Predicted aa alteration | Protein motif | Multiple mtDNA deletions† |
|---|---|---|---|---|---|---|---|
| 1 | Ashkenazi Jewish | A3371C | E7 | ND | E289A | – | + |
| | | A3371C | E7 | ND | E289A | – | |
| 2 | German American | t1504c | I4 | E4 skipping | Loss of 33 aa | TP consensus | + |
| | | A3371C | E7 | ND | E289A | – | |
| 3 | Puerto Rican | G1419A | E4 | ND | G145R | TP consensus | + |
| | | G1419A | E4 | ND | G145R | TP consensus | |
| 4 | Jamaican | A2744G | E6 | ND | K222S | Pi binding site | + |
| | | ins4196C | E10 | ND | Frame shift | – | |
| 5 | Israeli | G1419A | E4 | ND | G145R | TP consensus | ND |
| | | G1419A | E4 | ND | G145R | TP consensus | |
| 6 | Puerto Rican | G1419A | E4 | ND | G145R | TP consensus | + |
| | | G1419A | E4 | ND | G145R | TP consensus | |
| 7 | German | A3371C | E7 | ND | E289A | – | + |
| | | g3867c | I8 | E9 skipping | Loss of 47 aa | Leucine zipper | |
| 8 | German American | G1419A | E4 | ND | G145R | TP consensus | – |
| | | 4-bp del | I7 | ND | ? | – | |
| 9 | German Canadian | 6-bp del | E9 | ND | Loss of 2 aa | – | – |
| | | g4090a | I9 | ND | ? | Leucine zipper? | |
| 10 | European American | G1443A | E4 | ND | G153S | TP consensus | + |
| | | A3371C | E7 | ND | E289A | – | |
| 11 | English | G1443A | E4 | ND | G153S | TP consensus | ND |
| | | A3371C | E7 | ND | E289A | – | |
| 12 | English | G1443A | E4 | ND | G153S | TP consensus | ND |
| | | G1443A | E4 | ND | G153S | TP consensus | |

*Mutations in exons (E) are denoted by capital letters and in introns (I) are identified by lower case. †Symbols indicate presence (+) or lack (–) of multiple deletions.

TP activity does not interfere with normal angiogenesis.

TP is widely expressed in human tissues, including the gastrointestinal system, brain, peripheral nerves, spleen, bladder, and lung, but is not expressed in muscle, kidney, gall bladder, aorta, and fat (*17*). This expression pattern is consistent with the major clinical features of MNGIE: neuropathy, gastrointestinal dysmotility, and leukoencephalopathy. Because TP is also expressed in autonomic nerves, gastrointestinal symptoms may be partly due to autonomic neuropathy. TP has neurotrophic effects on cortical neurons and inhibits glial-cell proliferation (*18*). Therefore, encephalopathy in MNGIE may be caused by the loss of gliostatin function, rather than by decreased enzymatic activity. Paradoxically, skeletal muscle has no TP activity, although it is usually affected in MNGIE and harbors multiple mtDNA deletions. This paradox suggests that the mtDNA abnormalities in MNGIE might be an epi-phenomenon. A possible explanation for muscle involvement is that low levels (that is, undetectable levels) of TP might be vital for mtDNA maintenance (*19*). A second and perhaps more plausible possibility is that muscle mtDNA may be adversely affected by abnormal extracellular thymidine pools due to TP dysfunction (*20*). Therefore, although TP is not expressed in some aerobic tissues, such as muscle and kidney, TP activity may be indirectly essential for mtDNA maintenance in these tissues.

Thymidine is either degraded to thymine by TP catabolism or salvaged to deoxythymidine monophosphate (dTMP) by thymidine kinase (TK). Because mtDNA is constantly replicating, even in quiescent cells, a constant supply of thymidine and other nucleotides is likely to be vital for the maintenance of the mitochondrial genome. Mitochondria have physically separate deoxynucleotide triphosphate (dNTP) pools and may also have an independent thymidine salvage pathway (*21*). In support of this notion, at least two different forms of TK exist in eukaryotic cells. One (cytosolic, TK1) is highly active only in dividing cells, whereas the other (mitochondrial, TK2) is constitutively expressed (*22*), suggesting that the thymidine salvage pathway is important for mtDNA maintenance. Conceivably, the imbalance of dNTP pools caused by elevated thymidine levels might affect mtDNA more adversely than nuclear DNA, resulting in pathogenic multiple mtDNA deletion or partial mtDNA depletion, or both (*23*).

Finally, our results may have clinical implications for diagnosis and therapy of patients with MNGIE for which there is no effective treatment. For example, administration of parenteral TP or restoration of the normal nucleotide pools, or both, warrant investigation as potential therapies.

### References and Notes


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5. A. Papadimitriou *et al.*, *Neurology* **51**, 1086 (1998). Depletion of mtDNA was first described in patients with infantile mitochondrial myopathy and hepatopathy by C. T. Moraes *et al.* [*Am. J. Hum. Genet.* **48**, 492 (1991)].
6. The diagnosis of MNGIE was based on criteria described previously (*3, 4*). We genotyped families 5 to 7 and confirmed linkage to the MNGIE locus that we reported earlier with families 1 to 4 (*3*). Patient 10 was previously described by A. B. Threlkeld *et al.* [*Am. J. Ophthalmol.* **114**, 322 (1992)] and D. R. Johns, A. B. Threlkeld, N. R. Miller, and O. Hurko [*ibid.* **115**, 108 (1993)].
7. The Human Chromosome 22 Sequencing Group, Sanger Centre, Cambridge, UK (www.sanger.ac.uk/HGP/Chr22) and the Advanced Center for Genome Technology at the University of Oklahoma, Norman (www.genome.ou.edu/maps/ch22.html). Clone bK384D8 (GenBank accession U62317), which contains *TP*, was mapped, identified, and characterized by U.-G. Kim *et al.* [*Proc. Natl. Acad. Sci. U.S.A.* **93**, 6297 (1996)].
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9. Informed consent for blood samples was obtained from each study participant under a Columbia University Institutional Review Board approval protocol. *TP* has 10 exons and an open reading frame spanning from exons 2 to 10 (*12*). We amplified four fragments from genomic DNA, which encompassed exons 2 and 3, 4, 5 and 6, and 7 to 10. All primers were 20 nucleotides in length. Their 5′ positions and direction (F indicates forward, and R reverse) are: 267F and 952R for exons 2 and 3; 1283F and 1540R for exon 4; 2258F and 2959R for exons 5 and 6; and 3197F and 4254R for exons 7 to 10. We sequenced the fragments directly, using the BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA), and then electrophoresed the samples using an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer).
10. We used the sequence and numbering system of K. Hagiwara *et al.* [*Mol. Cell. Biol.* **11**, 2125 (1991)]. However, we found that exon 3 ended at nt 878, as illustrated in their figure 1, rather than at nt 833, as in their figure 2.
11. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
12. PROSITE at the Swiss Institute of Bioinformatics. Accession numbers are PS 00647 (thymidine/pyrimidine-nucleoside phosphorylase consensus) and PS 00029 (leucine zipper pattern). We used the "MOTIF" search program (www.motif.genome.ad.jp/).
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14. To screen for the mutations G1419A, G1443A, and A3371C, we used mismatch primers that would create new recognition sites for restriction enzymes when the template DNA contained the mutations. For G1419A, we first performed PCR with the mismatch primer, 5′-TGACCACCAGGTGCCAATGATCTGC-3′ (mismatch sites are underlined), corresponding to nts 1394–1418, and the reverse primer corresponding to nts 1540–1521. The amplified fragments were then digested overnight with Pst I. The products were subjected to electrophoresis in a 4% agarose gel. Similarly, fragments were amplified with the mismatch primers 5′-TAGACTCCAGCTTATCCAAGAAGC-3′ corresponding to nts 1467-1444 and a primer corresponding to nts 1283–1302 for G1443A and the mismatch primer 5′-TGCGCCGTCCATGCAGAGCAGCGGC-3′ corresponding to nts 3396–3372 and a primer corresponding to nts 3197–3216 for A3371C. The PCR products were digested with Hind III and Nar I, respectively, and subjected to electrophoresis in a 4% agarose gel. The A2744G mutation creates a new recognition site for Hinf III. The flanking region was amplified with primers corresponding to nts 2666–2685 and 2950–2831, digested with Hinf III, and subjected to electrophoresis in a 2% agarose gel.
15. Total RNA was extracted from fresh blood samples using RNAqueous Kit and RNAqueous Blood Module (Ambion, Austin, TX) and was reverse-transcribed into cDNA with oligo$(dT)_{12-18}$ primer using the SuperScript Preamplification System (Life Technologies, Gaithersburg, MD). Using PCR, we amplified the cDNA fragments with primers corresponding to nts 473–492 in exon 2 and nts 3291–3272 in exon 7, for the T1504C mutation; and nts 3405–3424 in exon 7 and nts 4254–4235 in exon 10, for the G3867C mutation. Amplified fragments were gel purified and sequenced (*9*). RNA from one healthy individual was simultaneously amplified and sequenced to verify all of the exon junctions and confirm that no exon was skipped.
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19. Using RT-PCR, we amplified TP cDNA from skeletal muscle, which indicates that TP is expressed at low levels in this tissue.
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