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A Disease-Associated G5703A Mutation in Human Mitochondrial DNA Causes a Conformational Change and a Marked Decrease in Steady-State Levels of Mitochondrial tRNA^{Asn}

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We introduced mitochondrial DNA (mtDNA) from a patient with a mitochondrial myopathy into established mtDNA-less human osteosarcoma cells. The resulting transmitochondrial cybrid lines, containing either exclusively wild-type or mutated (G5703A transition in the tRNA^{Asn} gene) mtDNA, were characterized and analyzed for oxidative phosphorylation function and steady-state levels of different RNA species. Functional studies showed that the G5703A mutation severely impairs oxidative phosphorylation function and mitochondrial protein synthesis. We detected a marked reduction in tRNA^{Asn} steady-state levels which was not associated with an accumulation of intermediate transcripts containing tRNA^{Asn} sequences or decreased transcription. Native polyacrylamide gel electrophoresis showed that the residual tRNA^{Asn} fraction in mutant cybrids had an altered conformation, suggesting that the mutation destabilized the tRNA^{Asn} secondary or tertiary structure. Our results suggest that the G5703 mutation causes a conformational change in the tRNA^{Asn} which may impair aminoacylation. This alteration leads to a severe reduction in the functional tRNA^{Asn} pool by increasing its in vivo degradation by mitochondrial RNases.

Mutations in mitochondrial DNA (mtDNA) have been implicated in the pathogenesis of different clinical syndromes (19, 31). In the past 9 years, pathogenic large-scale rearrangements as well as several point mutations in the human mtDNA, most of them heteroplasmic (i.e., mutated mtDNA coexisted with the wild-type mtDNA), have been described. Point mutations in mitochondrial tRNA genes seem to be particularly frequent in neuromuscular disorders, possibly because of their generalized effect on mitochondrial protein synthesis and consequent impairment of multiple oxidative phosphorylation enzyme complexes (26). The molecular pathogenesis of these mutations however, is poorly understood. Two mutations in mitochondrial tRNA genes have been extensively studied: a A3243G transition in the tRNA$^{Leu(UUR)}$ gene and a A8344G transition in the tRNA^{Lys} gene (mtDNA positions according to reference 1). Although their mechanisms for pathogenicity are also not fully understood, these two mutations seem to affect mitochondrial protein synthesis by different mechanisms. The A3243G mutation causes a decrease in mitochondrial protein synthesis and an impairment in the processing of an intermediate transcript containing the tRNA$^{Leu(UUR)}$ (5, 14) whereas the A8344G mutation was associated with a mild decrease in steady-state levels of tRNA^{Lys} and also a partial decrease in the aminoacylation capacity of the mutated tRNA (8).

We have studied the molecular pathogenesis of a G5703A transition in the mitochondrial tRNA^{Asn} gene and found molecular abnormalities which were different from the ones described above for other mitochondrial tRNA mutations.

MATERIALS AND METHODS

Cell lines and medium. The human cell line 143B (TK−) and its mtDNA-less derivative 143B/206 (13) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). A fibroblast line from a patient harboring a heteroplasmic (6% mutated) G→A transition at position 5703 within the mitochondrial tRNA^{Asn} was obtained and characterized previously (25). W16, W20, and W60 were transmitochondrial cybrid lines, essentially homoplasmic for the wild-type tRNA^{Asn} gene. W49 and W72 were transmitochondrial lines essentially homoplasmic for the G5703A mutation in the tRNA^{Asn} gene. All transmitochondrial cybrids described above were isolated from the same fusion of patient fibroblasts with the 143B/206 line. Transmitochondrial cybrids were grown in high-glucose DMEM supplemented with 10% FBS.

Establishment and genetic characterization of transmitochondrial cybrids. Enucleated fibroblasts from the patient were fused to the mtDNA-less 143B/206 cell line as previously described (13). Individual cybrid clones were isolated and analyzed for the mtDNA G5703A transition by *Dde*I restriction fragment length polymorphism (RFLP) of PCR-amplified fragments (25). Two heteroplasmic clones (W78 and W55) were treated with ethidium bromide (50 ng/ml) for 15 or 24 days to deplete their mtDNA and then grown in complete medium for 24 days to restore normal mtDNA levels. Individual clones were analyzed by PCR-RFLP. The percentage of wild-type molecules was determined by "last-cycle hot" PCR as previously described (24). PCR fragments corresponding to mtDNA positions 5472 to 5798 from a wild-type and a mutant transmitochondrial cybrid were subcloned into a plasmid vector (TA cloning/pCR II kit; Invitrogen). Individual bacterial clones were isolated, and their plasmids were tested for the presence or absence of the 5703 mutation. Pure mutated or wild-type plasmids were used to determine the sensitivity of the RFLP method (12).

To confirm that the wild-type mtDNA present in transmitochondrial cybrids was patient derived, a non-disease-related *Dra*I polymorphism (C→T at nucleotide position 4312) previously identified in the patient was analyzed by RFLP (25).

To determine the ratio of mtDNA to nuclear DNA, 5 μg of total DNA were digested with *Pvu*II, electrophoresed through a 0.8% agarose gel, transferred to a nylon membrane, and hybridized simultaneously with two specific probes. One probe was a [α-^{32}P]dCTP-labeled 2.8-kb PCR fragment encompassing the mtDNA D-loop region (nucleotide positions 13956 to 175). The second probe was a [α-^{32}P]dCTP random primer-labeled 5.8-kb *Eco*RI insert from a plasmid containing the nucleus-encoded 18S rRNA gene (32).

Respiratory function assays. Oxygen consumption was measured in a Biological Oxygen Monitor (YSI model 5300). Measurements were carried out with 3×10^6 exponentially growing cells, resuspended in 1.8 ml of DMEM without glucose–5% dialyzed FBS as previously described (13). Lactate released to the cultured medium was measured with a commercial testing kit (Sigma Co.) and normalized to the number of the cells present at the end of the experiment.

Analysis of mitochondrial translation products. Exponentially growing transmitochondrial cybrids and 143B and 143B/206 cells ($\sim 2 \times 10^6$) were labeled with [^{35}S]methionine (1,000/mmol, 250 μCi/ml) for 30 min in the presence of emetine (100 μg/ml) as described previously (6). After labeling, cells were washed with

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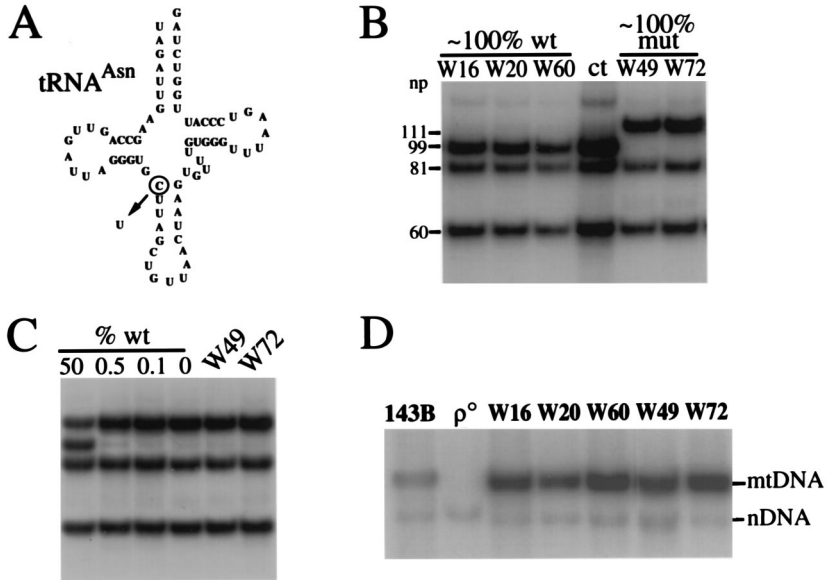

FIG. 1. Genetic characterization of transmitochondrial cybrid lines. Three cell lines containing essentially 100% wild-type mtDNA and two cell lines containing essentially 100% mutated mtDNA (at mtDNA position 5703) were characterized in detail and used in the subsequent studies. (A) Position of the mutation in the mitochondrial $tRNA^{Asn}$ cloverleaf structure; (B) PCR-RFLP analysis of the 5703 mutation in selected clones. PCR products originated from wild-type genomes have an additional *Dde*I site, creating a diagnostic size difference in a nondenaturing polyacrylamide gel after digestion of PCR products (25). The sensitivity of the assay was determined by PCR amplifying mixtures of plasmids containing the wild-type (wt) or mutated (mut) region (C). We were able to distinguish up to 0.5% wild type from 0% wild type, indicating that the mutant clones had >99.5% mutated mtDNA. mtDNA levels were estimated by Southern blot analysis using mtDNA-specific and nuclear (18S rRNA gene) DNA-specific probes (D). NP, nucleotide position.

DMEM and cold phosphate-buffered saline and lysed with 1% sodium dodecyl sulfate (SDS). Equal amounts of total protein (determined by the Bio-Rad protein assay method) were resolved on a 15 to 20% exponential gradient polyacrylamide gel, subjected to fluorography after electrophoresis, and exposed to X-ray film for 16 h at −80°C (6).

Northern blotting. Total RNA samples (20 μg) extracted by acid guanidinium thiocyanate-phenol-chloroform extraction (3) from each cybrid line were separated on a 1.2% agarose–18% formaldehyde gel and transferred to a nylon membrane. For detection of $tRNA^{Asn}$, we used a synthetic 39-nucleotide oligonucleotide (corresponding to mtDNA positions 5660 to 5698) end labeled with [γ-^{32}P]ATP. The mitochondrial $tRNA^{Leu(UUR)}$ was detected with a γ-^{32}P 5′-end-labeled 48-nucleotide oligonucleotide (corresponding to mtDNA positions 3304 to 3257). High-resolution Northern blotting was performed as previously described (18). For detection of the $tRNA^{Asn}$, the probe described above was used. The mitochondrial $tRNA^{Val}$ probe was a 316-bp PCR fragment of mtDNA (nucleotides 1460 to 1776) obtained by the random primer labeling with [α-^{32}P]dCTP. A 38-mer oligonucleotide complementary to the $tRNA^{Ala}$ (mtDNA positions 5617 to 5654) end labeled with [γ^{32}-P]ATP was also used as a hybridizing probe.

Analyses of mitochondrial tRNAs in vivo aminoacylation. Condition for isolation of mitochondrial and total RNA under acid conditions were as described previously (9); 4×10^7 exponentially growing cells were used to purify mitochondria. Total RNAs were obtained by phenol extraction from mitochondrial pellets under acid conditions (pH 4.1) to preserve aminoacylated tRNAs. Total RNA (3 μg) was fractionated on a 0.4-mm-thick 6.5% polyacrylamide (29:1)–8 M urea gel in 0.1 M sodium acetate (pH 5.0). Electrophoresis was carried out at 120 V for 18 h in a 20-cm gel and for 30 h in a 60-cm gel, with the buffer being continuously recirculated at 4°C. To remove the aminoacyl moiety, RNA samples were boiled for 10 min at pH 8 (10 mM Tris-HCl). The gel was then transferred to a Zeta-probe membrane in Tris-acetate-EDTA buffer at 25 V for 3 h and hybridized to different probes as described above. Detection of the mitochondrial $tRNA^{Lys}$ was performed with a γ-^{32}P 5′-end-labeled 39-mer oligonucleotide corresponding to mtDNA positions 8295 to 8330.

Conformational analysis. Conformational studies of tRNAs were based on established single-strand conformation polymorphism (SSCP) techniques (29). Total RNA (8 μg) was electrophoresed under nondenaturing conditions through a 6% polyacrylamide gel containing 1× Tris-borate-EDTA (TBE). Electrophoresis was carried out at 4°C and 80 V for 16 to 20 h. The gel was denatured by soaking in 0.2 N NaOH–0.5 M $NaCl_2$ for 10 min and then neutralized by washing two times (10 min each) in 5× TBE and once for 10 min in 0.5× TBE. The gel was then electroblotted to Zeta-probe membrane in 1× TBE buffer at 20 V for 3 h. For detection of $tRNA^{Asn}$, we used the probe described above. The $tRNA^{Phe}$ probe was a γ-^{32}P 5′-end-labeled 43-mer oligonucleotide (corresponding to mtDNA positions 577 to 619). Computer predictions of tRNA secondary structure were performed with MacDNAsis version 3.2 (Hitachi Ltd.).

Degradation of mitochondrial tRNAs. Mitochondrial RNase extracts were prepared by two different procedures as described below, but in both cases the starting material was mitochondria isolated from 143B cells by standard isolation procedure (11). (i) Purified mitochondria were resuspended in HEPES buffer (pH 7.4) supplemented with pepstatin A (0.05 μg/ml), leupeptin (0.05 μg/ml), and Triton X-100 (140 mg/ml). The suspension of mitochondria was dialyzed against HEPES buffer overnight at 4°C. (ii) Purified mitochondria were resuspended in 10 mM Tris-HCl (pH 7.1)–1.5 mM EDTA–0.25 M sucrose. The suspension was frozen and thawed to damage the mitochondrial membrane, thereby releasing matrix factors.

Total RNA samples were incubated in 1 M Tris-HCl (pH 8.0) at 37°C for 30 min for complete removal of amino acids from tRNAs (7). The RNA fraction was purified by ethanol precipitation and incubated with 1 μg of a mitochondrial enzyme extract in buffer containing 10 mM Tris-HCl (pH 7.1), 1.5 mM EDTA, and 0.25 M sucrose (or HEPES buffer containing 50 mM HEPES [pH 7.4], 16.5 mM potassium acetate, 3 mM $MgCl_2$, 0.4 mM phenylmethylsulfonyl fluoride, 7% glycerol, 0.2 mM EDTA, and 0.2 mM dithiothreitol). Samples were incubated at 37°C for 2, 4, and 8 h. Reactions were stopped by the addition of electrophoresis denaturing loading buffer. RNA fractions were analyzed by high-resolution Northern hybridization as described above.

RESULTS

Isolation and characterization of transmitochondrial cybrids. To investigate the pathogenetic mechanism of the G5703A mtDNA mutation in $tRNA^{Asn}$ gene (Fig. 1A), transmitochondrial cybrids were produced by introducing mtDNA from a fibroblast line containing 6% mutated mtDNA into human cell lines devoid of mtDNA (ρ^0 line 143B/206). Ninety-eight individual cybrid clones were isolated from the fusion. The vast majority of transmitochondrial cybrids were homoplasmic wild-type at mtDNA position 5703. Only one clone was a homoplasmic mutant, and six were heteroplasmic (ranging from 10 to 50% mutated mtDNA). Two heteroplasmic cybrids (W55 and W78) containing approximately 50% mutated mtDNA were chosen to attempt to obtain additional cell lines essentially homoplasmic for the mutated mtDNA. These

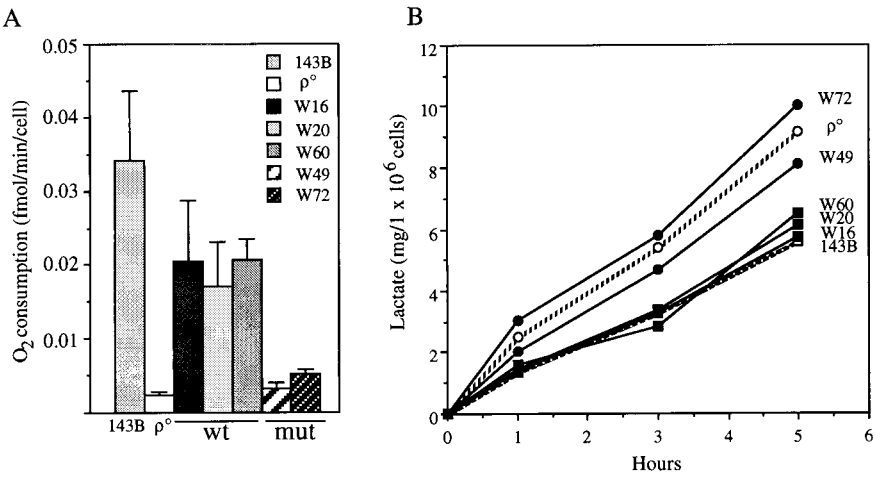

FIG. 2. Mitochondrial functional assays in transmitochondrial cybrid clones. (A) Rates of oxygen consumption per cell of 143B, 143B/206 (ρ°), and the indicated transmitochondrial cybrid lines, with error bars representing ± standard deviation of three independent determinations; time course release of lactate to the culture medium normalized to the number of cells.

two cell lines were exposed to 50 ng of ethidium bromide per ml (16) for 15 days. Clones derived from W55 became ρ^0 cell lines, whereas W78 re-clones remained heteroplasmic after 15 days of exposure to ethidium bromide. After 24 days of treatment with ethidium bromide, a fraction of reclones from W78 became homoplasmic mutants.

We selected three wild-type and two mutant clones (one obtained from the initial fusion and one reclone of ethidium bromide-treated W78) to perform subsequent experiments (Fig. 1B). To determine the sensitivity of the RFLP assay, we mixed known amounts of purified wild-type and mutant plasmid constructs corresponding to 50, 0.5, 0.1, and 0% wild-type mtDNA. These mixtures were PCR amplified in parallel with DNA from the two mutant clones (W72 and a W78-derived clone termed W49) used in our studies. By overexposing the gel, we were able to detect 0.5% wild-type mtDNA in the test mixture. In some experiments, lower percentages of plasmids harboring the wild-type sequence, including 0% wild type, showed a very light band migrating close to the wild-type band position. Even in these cases, 0.5% wild type was still detectable. A pattern similar to 0% wild type was observed with the two mutant clones, showing that they had >99.5% mutated mtDNA (Fig. 1C). Relative mtDNA levels were found to be similar for all selected mutant and wild-type clones, though cybrids had approximately 30% higher values than the parental 143B line (Fig. 1D). The identity of patient-derived mtDNA was confirmed by RFLP analysis of an unrelated *Dra*I mtDNA polymorphism previously identified in the patient (see Materials and Methods; not shown).

Respiratory function. To study the respiratory function of mitochondria, oxygen consumption and lactate production of intact cells were measured. As shown in Fig. 2A, the rate of oxygen consumption was significantly decreased in the mutant clones (an average reduction of 90% relative to control lines). Compatible results were obtained from the lactate production assays, which show a clear increase in the mutant clones (Fig. 2B), suggesting a severely defective oxidative phosphorylation function associated with the G5703A mtDNA mutation.

Mitochondrial translation. The influence of the mtDNA G5703A mutation on mitochondrial protein synthesis was investigated by labeling mutant and wild-type cybrids with [^{35}S]methionine in the presence of emetine (an inhibitor of cytoplasmic protein synthesis). The translation products were resolved in an SDS–15 to 20% exponential gradient polyacrylamide gel. Figure 3 shows that mutant cybrids have a marked reduction in all mitochondrial protein synthesis relative to the wild-type clones (<10%). No abnormal-sized bands were observed in overexposed X-ray films (not shown).

Mitochondrial tRNA steady-state levels and processing. To investigate the effect of the G5703A mutation in the steady-state levels and processing of tRNA^{Asn}, equal amounts of total RNAs from mutant and wild-type cybrids were electrophoresed through a 1.2% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with specific probes for tRNA^{Asn} and tRNA$^{Leu(UUR)}$. The levels of tRNA^{Asn} were significantly lower in mutant clones than in wild-type cybrids and the parental 143B (79% reduction of the mutant clone

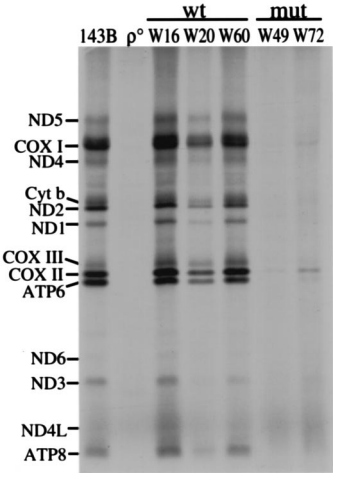

FIG. 3. Mitochondrial protein synthesis in transmitochondrial cybrid clones. A fluorogram of mitochondrial translation products labeled with [^{35}S]methionine after electrophoresis through an SDS–15 to 20% gradient polyacrylamide gel is shown for the different wild-type (wt) and mutant (mut) cybrid lines. After 30 min labeling in the presence of emetine, equal amounts of an SDS lysate of total cellular protein (50 μg) were loaded in each lane. Bands were assigned according to reference 6.

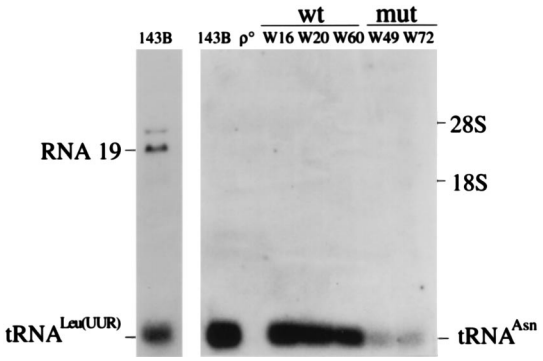

FIG. 4. Northern blot hybridization analyses. Total RNAs extracted from various cell lines were electrophoresed through a 1.2% agarose gel, blotted into a nylon membrane, and hybridized to two different probes, specific for $tRNA^{Leu(UUR)}$ (left) and $tRNAs^{Asn}$ (right). The ethidium bromide-determined positions of 28S and 18S rRNAs are shown on the right. Band assignments were based on molecular weight and probe specificity. RNA 19 corresponds to an intermediary transcript composed of 16S rRNA, $tRNA^{Leu(UUR)}$, and ND1 (14). wt, wild type; mut, mutant.

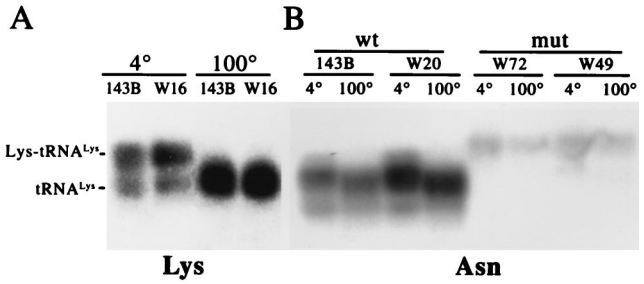

FIG. 6. In vivo amynoacylation assays. RNA was extracted under acid conditions from mitochondria isolated from the cell lines indicated above the lanes. The RNA was electrophoresed under acidic conditions and transferred to a nylon membrane as described in Materials and Methods. Samples separated in a 20-cm gel were hybridized with a ^{32}P-labeled oligonucleotide complementary to the mitochondrial $tRNA^{Lys}$ (A). Samples hybridized with a $tRNA^{Asn}$-specific probe were separated in a 60-cm gel (B). Although the assay was able to distinguish the aminoacylated form of the $tRNA^{Lys}$ (the aminoacyl moiety is preserved at 4°C but is removed by treatment at 100°C), it was not able to completely separate the two forms of $tRNA^{Asn}$. wt, wild type; mut, mutant.

mean value relative to the wild-type and 143B clone mean values), and no higher-molecular-weight intermediate transcripts were detected (Fig. 4). An intermediate unprocessed transcript encompassing the 16S rRNA, $tRNA^{Leu(UUR)}$, and ND1 (termed RNA19 [14]) was detected by hybridizing part of the same blot for the $tRNA^{Leu(UUR)}$ (Fig. 4). These results suggest that the decreased levels of $tRNA^{Asn}$ associated with the G5703A mutation are not a consequence of impaired processing of intermediate transcripts. The quantitative defect of $tRNA^{Asn}$ was also observed by high-resolution Northern blotting (Fig. 5). A marked reduction of $tRNA^{Asn}$ in mutant clones compared to wild-type cybrids was observed. In contrast, both mutant and wild-type cybrids had nearly identical levels of $tRNA^{Ala}$ (an L-strand transcript immediately downstream of $tRNA^{Asn}$ gene), $tRNA^{Val}$ (an H-strand transcript), or $tRNA^{Leu(UUR)}$ (also an H-strand transcript [not shown]). The average reduction in the levels of $tRNA^{Asn}$ in the two mutant clones relative to the mean of the wild-type and 143B lines was 70% when normalized to $tRNA^{Ala}$, 74% when normalized to $tRNA^{Val}$, and 73% when normalized to $tRNA^{Leu(UUR)}$.

Aminoacylation assay in vivo. We attempted to investigate for a potential deficiency in the aminoacylation capacity of the mutated $tRNA^{Asn}$. Total RNA was isolated from purified mitochondria under acidic conditions and electrophoresed through a 6.5% polyacrylamide–8 M urea gel in acid buffer to preserve the aminoacylated tRNAs. After electroblotting, the membranes were hybridized for specific mitochondrial tRNAs.

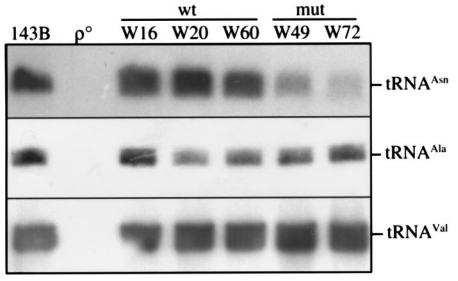

FIG. 5. High-resolution Northern hybridization. Total RNAs extracted from different cell lines (indicated above the lanes) were electrophoresed through a 20% polyacrylamide gel, electrotransferred to a nylon membrane, and hybridized to $tRNA^{Asn}$, $tRNA^{Ala}$, and $tRNA^{Val}$ probes. wt, wild type; mut, mutant.

To ensure that the extraction procedure preserved aminoacylated tRNAs, we also analyzed the mitochondrial $tRNA^{Lys}$. Lys-$tRNA^{Lys}$ and uncharged $tRNA^{Lys}$ were clearly distinguishable by this system (Fig. 6A). Aminoacylated and nonaminoacylated $tRNA^{Asn}$ could not be resolved under the same conditions (not shown). We performed the same experiment using a longer gel apparatus to attempt to increase the separation between aminoacylated and free $tRNA^{Asn}$. Under these modified conditions, the wild-type samples showed a slight mobility shift after removal of the amino acid (100°C [Fig. 6B]). This small shift was not enough to completely separate the two forms of the $tRNA^{Asn}$ and therefore did not allow us to determine the actual percentage of aminoacylated $tRNA^{Asn}$. In the case of the mutant samples, boiling did not alter the migration of the $tRNA^{Asn}$, suggesting that the residual mutated $tRNA^{Asn}$ was poorly acylated. Because the electrophoresis has to be performed at 4°C to preserve the labile amino acid moiety, mutated $tRNA^{Asn}$ molecules migrated more slowly than wild-type molecules. Fully denaturing gels (Fig. 5) abolished differences in migration between mutated and wild-type $tRNA^{Asn}$. This observation suggested that conformational changes could be implicated in the pathogenesis of the G5703A mutation.

tRNA conformational analysis. To look for potential conformational changes of the mutated $tRNA^{Asn}$, we subjected equal amounts of total RNAs to nondenaturing SSCP polyacrylamide gel electrophoresis, electroblotted the gel into a nylon membrane, and probed the membrane for mitochondrial $tRNA^{Asn}$ and $tRNA^{Phe}$. Figure 7 shows a change in the migration pattern of the residual $tRNA^{Asn}$ in mutant cybrids. The $tRNA^{Asn}$ band migrated more slowly and had a smeary appearance in RNA samples from mutant cybrids compared to wild-type clones. The content and migration pattern of $tRNA^{Phe}$ were similar for mutant and wild-type cybrids. The same abnormal migration pattern for the mutated $tRNA^{Asn}$ was observed in two independent experiments.

Degradation of $tRNA^{Asn}$ in vitro. The severe reduction in steady-state levels and the conformational changes of $tRNA^{Asn}$ associated with the G5703A mutation suggested that the mutated $tRNA^{Asn}$ was more susceptible to degradation by mitochondrial RNases in vivo. We tested this hypothesis in vitro by incubating RNA samples with mitochondrial extracts. The reaction products were analyzed by high-resolution Northern hybridization. Unexpectedly, the residual mitochondrial

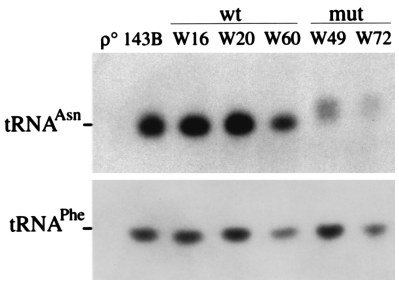

FIG. 7. Conformational changes in the mitochondrial tRNA^{Asn} associated with the G5703A mutation. Total RNAs extracted from the cell lines indicated above the lanes were electrophoresed under nondenaturing conditions through a 6% polyacrylamide gel commonly used for SSCP analysis. The gel was denatured and electrotransferred to a nylon membrane, and the membrane was hybridized to a ^{32}P-labeled oligonucleotide complementary to the mitochondrial tRNA^{Asn}. The same membrane was washed at high stringency to elute the tRNA^{Asn} probe and was reprobed with a ^{32}P-labeled oligonucleotide complementary to the mitochondrial tRNA^{Phe}. wt, wild type; mut, mutant.

tRNA^{Asn} from the mutant clones was not degraded faster than the wild-type tRNA^{Asn}, and they seemed to plateau at similar levels (Fig. 8). We repeated the experiment with mitochondrial extracts obtained by different procedures (see Materials and Methods), but the results were essentially identical to those shown in Fig. 8.

DISCUSSION

The molecular pathogenesis of the G5703A mtDNA mutation. Despite our increased awareness of the nature of many mtDNA mutations underlying mitochondrial diseases, not much is known about their molecular pathogenic mechanisms. The use of transmitochondrial cybrids (13) has facilitated detailed molecular studies by allowing the complete segregation of mutated and wild-type mtDNAs in established cell lines. We used this approach to investigate the molecular pathogenesis of a G5703A mutation in the mitochondrial tRNA^{Asn} gene

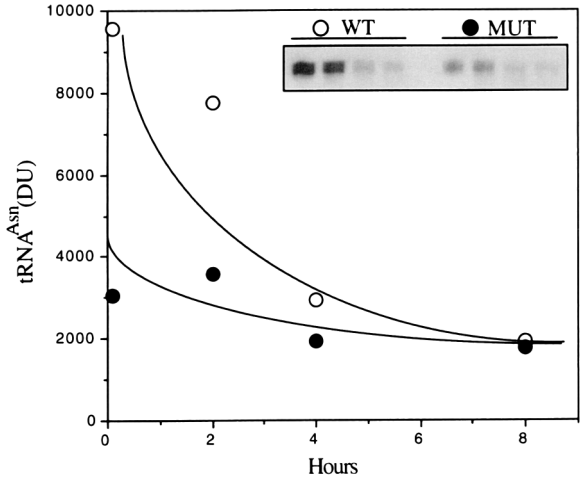

FIG. 8. In vitro degradation of mutated (mut) and wild-type (wt) tRNA^{Asn}. Total RNA extracted from different cell lines was incubated with a mitochondrial enzymatic extract prepared as described in Materials and Methods. Incubations proceeded for the indicated time points at 37°C and were interrupted by the addition of a denaturing gel loading buffer. Samples were subjected to high-resolution Northern hybridization for the detection of the mitochondrial tRNA^{Asn}. The graph represents the decrease in densitometric units which corresponds to a decrease in the amount of full-length tRNA^{Asn}. The insert shows the X-ray film from which the data were derived.

previously identified in a patient with a mitochondrial myopathy. The presence of the mutation was associated with a severe defect in oxidative phosphorylation function and a marked reduction in mitochondrial protein synthesis. The asparagine contents of mtDNA-encoded polypeptides are similar, ranging from 2.28% (ND6) to 7.35% (A8), with most polypeptides having between 3 and 5.5% asparagine. These small differences did not influence the translation impairment, as all polypeptides were reduced in similar fashion in mutant clones. These defects correlated with an equally marked reduction in the steady-state levels of the tRNA^{Asn}. Mutant clones had approximately 20 to 30% of the levels of tRNA^{Asn} observed in wild-type clones. We searched for conformational changes which could be responsible for decrease stability of tRNA^{Asn} by adapting an electrophoretic system commonly used to detect single nucleotide changes in small DNA fragments (SSCP [29]). In contrast to the SSCP analysis, the RNA sample was not denatured before loading into the gel, and we assume that the tRNA^{Asn} molecules were folded in their thermodynamically preferred conformation. The mutated tRNA^{Asn} showed an abnormal migration suggesting that it had an altered conformation because of the mutation. The smeary appearance of the band also suggested a relatively unstable structure. Computer analysis indicated that the native secondary structure was still the most energetically favorable conformation for the mutated tRNA^{Asn} (although the wild-type C:G base pair in the anticodon stem was substituted for a U:G pair [Fig. 1]). However, in vivo conformational changes may not follow these simple base-pairing rules because of tertiary interactions and chemical modifications (27).

Conformational changes could have a major impact on tRNA function by affecting aminoacylation, interaction with translation factors, or stability. We attempted to determine the in vivo levels of aminoacylated tRNA^{Asn}, but unfortunately we were not able to completely separate the aminoacylated form from the free tRNA by using the standard procedure developed by Varshney et al. (30). This procedure has been used to analyze in vivo aminoacylation levels of several human mitochondrial tRNAs (10). Although the mitochondrial tRNA^{Asn} was not included in these series, some human mitochondrial tRNAs could not be analyzed because of lack of separation between the charged and uncharged forms. After testing a large number of experimental conditions, we were able to detect a slight change in migration between acylated and nonacylated tRNA^{Asn} from wild-type samples. Mutated samples, however, did not show any alteration in migration upon removal of the amino acid, suggesting that these molecules were poorly acylated. Impaired aminoacylation may be associated with the marked decrease in tRNA steady-state levels, and together, these factors could play a major role in the observed impairment in mitochondrial protein synthesis.

The destabilization of the tRNA^{Asn} secondary or tertiary structure and a possible impairment in aminoacylation could lead to the low steady-state levels of the tRNA^{Asn} transcripts by increasing the degradation kinetics of the tRNA^{Asn} by mitochondrial RNases. We attempted to test this hypothesis in vitro by incubating total RNA preparations with mitochondrial extracts and by measuring the kinetics of degradation of the tRNA^{Asn} by high-resolution Northern hybridization. These experiments showed that the wild-type tRNA^{Asn} was actually degraded slightly faster than the mutated, but they seemed to reach similar residual levels which were relatively resistant to degradation by mitochondrial RNases. Overnight incubations, however, did reduce the amounts of full-length tRNAs (mutated and wild type) to undetectable levels, indicating that the

RNases present in the extract were not inactivated by the long incubation time.

Although these findings appear contradictory, there are a couple of scenarios which could explain the results. It is possible that the in vitro degradation of tRNAs does not mimic the in vivo metabolic pathways. The RNA preparations, for example, were not obtained and kept in acid conditions and were most likely nonacylated. In addition, it is possible that there is a small subpopulation of $tRNA^{Asn}$ which is relatively resistant to degradation, whereas the majority of the $tRNA^{Asn}$ (possibly the functional pool) is relatively sensitive to RNases. This hypothetical RNase-sensitive functional pool may be absent in the mutant clones because it was already degraded in vivo. It is important to keep in mind that the mutant clones have only 20 to 30% of the $tRNA^{Asn}$ levels observed in the wild-type clones, and because of the >90% impairment in respiration and protein synthesis, this residual pool may not be fully functional. What would make a subpopulation of $tRNA^{Asn}$ resistant to mitochondrial RNases? Although a great deal of work performed on *Escherichia coli* has elucidated different pathways for tRNA processing and degradation (20), not much is known about these pathways in mammalian mitochondria (21). The presence of an amino acid covalently bound to the 3′ end of the tRNA could have such an effect, and even though the RNA preparations used in these experiments were not isolated in acid conditions, a certain level of aminoacylation could still be preserved in the mitochondrial $tRNA^{Asn}$. To test for this possibility, we treated the RNA sample with an alkaline solution known to completely remove amino acid residues from tRNAs (7) before incubating it with a mitochondrial enzyme extract. The kinetics of degradation of these treated samples were the same as for untreated samples, ruling out aminoacylation as the factor protecting the residual tRNA pool against degradation. These findings are in agreement with the aminoacylation studies which suggested that the mutated $tRNA^{Asn}$ is poorly acylated. Although this possible poor aminoacylation would be compatible with an increased degradation in vivo because of the unprotected tRNA 3′ end, it does not explain why the residual mutated $tRNA^{Asn}$ is relatively resistant to RNases.

Despite the in vitro findings, we still favor increased in vivo degradation of the mutated $tRNA^{Asn}$ as the molecular basis for the translational defect and consequently the disease. The arguments supporting this concept are (i) the extremely low steady-state levels of the $tRNA^{Asn}$, (ii) the nonaccumulation of partially processed or unprocessed transcripts containing $tRNA^{Asn}$ sequences, and (iii) the normal levels of $tRNA^{Ala}$, located immediately downstream of the $tRNA^{Asn}$. These observations strongly suggest that the decrease in $tRNA^{Asn}$ steady-state levels is not a consequence of impaired transcription or processing. Although the aminoacylation studies were not conclusive, they did suggest that the mutated $tRNA^{Asn}$ is poorly acylated, which could be the cause for the increased degradation. In any case, a poor aminoacylation would reduce even further the levels of functional $tRNA^{Asn}$ available for translation.

Reduction of mitochondrial tRNA steady-state levels as a mechanism of disease. The steady-state levels of the different mitochondrial tRNA species are remarkably similar in HeLa cells (15). These observations suggested that the control of tRNA levels is not at the level of transcription, as $tRNA^{Val}$ and $tRNA^{Phe}$ are transcribed 20 times more frequently than other H-strand transcripts, and the L-strand transcripts (which includes $tRNA^{Asn}$) are synthesized 10 to 16 times more frequently than the complete H-strand transcripts. These findings also underscore the importance of maintaining normal levels of the different tRNAs for proper mitochondrial function. King and Attardi suggested that the parity in mitochondrial tRNA levels could be caused by stabilizing factors, such as tRNA aminoacyl synthetases (15). If this is correct, it is possible that the decrease in steady-state levels of the mutated $tRNA^{Asn}$ is actually a consequence of its reduced affinity for the cognate synthetase, possibly caused by the observed conformational change.

The role of reduced steady-state level of mature, functional tRNAs may be a more common mechanism of disease than previously anticipated. Enriquez et al. (8) suggested that a 50 to 60% reduction in the levels of functional $tRNA^{Lys}$ in transmitochondrial cybrids harboring the A8344G mutation was the basis for the translational defect in their mutant cybrids. This decrease took into consideration a 16 to 33% reduction in steady-state levels and a 37 to 49% reduction in aminoacylation. Masucci et al. (22) found a 41% decrease in the levels of $tRNA^{Lys}$ harboring the A8344G mutation but no decrease in RNA^{Lys} harboring a T8356C mutation. Decrease in the steady-state levels of the mitochondrial $tRNA^{Leu(UUR)}$ have also been associated with different mutations. Chomyn et al. found a 20% decrease in $tRNA^{Leu(UUR)}$ in transmitochondrial cybrids harboring the A3243G mutation (5). However, King et al. (14) did not detect alterations in the levels of $tRNA^{Leu(UUR)}$ in their cybrids harboring the same mutation. Hao and Moraes (12) found that a mutation in the $tRNA^{Leu(UUR)}$ gene at position 3256 led to a 30% reduction in steady-state levels of the $tRNA^{Leu(UUR)}$. Bindoff et al. (2) described a loss of approximately 50% of mature $tRNA^{Leu(UUR)}$ in fibroblasts harboring a mtDNA mutation at position 3302. In the two latter studies, it was suggested that impairment in processing of RNA19 was responsible for the decrease in the steady-state levels of $tRNA^{Leu(UUR)}$ and ND1. The role of a decrease in the levels of mature, functional $tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$ in the pathogenesis of mitochondrial disorders is still obscure, but it may be more relevant than previously suspected.

Correlations between phenotype and molecular pathogenesis. Although levels of heteroplasmy and tissue distribution play an important role in the development of clinical syndromes, different mtDNA mutations tend to cause specific symptoms (e.g., stroke-like episodes associated with the A3243G mutation and myoclonic seizures associated with the A8344G mutation [19, 31]). The G5703A mutation was associated with a pure myopathy most noticeable in the extraocular muscles. The levels of the mutation in the patient's tissues were 69% (muscle), 4% (erythrocytes), and 6% (fibroblasts) (25). This pattern of tissue distribution and clinical features is very similar to what is observed in most patients with mtDNA deletions (23), and our results suggest that the similarities are also present at the molecular level. The mitochondrial protein synthesis defect associated with the G5703A mutation is almost as severe as the one observed with mtDNA deletions, what makes this base change a near-null mutation. We propose that null or near-null mtDNA mutations are more frequently associated with muscle pathologies (with ophthalmoparesis), whereas mutations with a less severe consequence on general mitochondrial protein synthesis (e.g., A3243G and A8344G) are more frequently associated with central nervous system symptoms. Biochemical assays on patients tissues suggested that the activities of the various oxidative phosphorylation complexes are differently affected by the various mutations. There is a predominant complex I deficiency in mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) patients with the A3243G mutation (17) and a more generalized deficiency of complexes containing mtDNA-coded subunits in patients with mtDNA deletions or the G5703A mutation (23, 25). It is interesting that another mtDNA mu-

tation, affecting the tRNA^{Ile} gene (T4285C), localizes to the same position in the tRNA cloverleaf structure as the G5703A mutation (position 27, at the top of the anticodon stem). This mutation was identified in a patient with a myopathy and ragged-red fibers (RRF). The mutation was present at high levels in muscle (90%) but was undetectable in blood (28). This case resembles our patient in several clinical and molecular aspects. Although the correlations described above are not absolute, there is an obvious trend. The basis for the differential clinical and biochemical involvement has not yet been comprehensively connected to molecular abnormalities. The continuing molecular analyses of different mtDNA mutations should help elucidate some of these puzzling correlations.

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