The 7472insC Mitochondrial DNA Mutation Impairs the Synthesis and Extent of Aminoacylation of tRNA_{Ser(UCN)} but Not Its Structure or Rate of Turnover*

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The 7472insC mitochondrial DNA mutation in the tRNA_{Ser(UCN)} gene is associated with sensorineural deafness combined, in some patients, with a wider neurological syndrome. In cultured cybrid cells it causes a 70% decrease in tRNA_{Ser(UCN)} abundance and mild respiratory impairment, previously suggested to be due to decreased tRNA stability. When mitochondrial transcription was blocked by ethidium bromide treatment, the half-life of the mutant tRNA was not significantly different from that of wild-type tRNA_{Ser(UCN)}. Over-expression of mitochondrial translational elongation factor EF-Tu also had no effect on the mutant phenotype. However, during recovery from prolonged ethidium bromide treatment, the synthesis of the mutant tRNA_{Ser(UCN)} was specifically impaired, without polarity effects on downstream tRNAs of the light strand transcription unit. We infer that the mutation acts posttranscriptionally to decrease tRNA_{Ser(UCN)} abundance by affecting its synthesis rather than its stability. The extent of aminoacylation of the mutant tRNA was also decreased by ~25%. In contrast, the mutation had no detectable effect on tRNA_{Ser(UCN)} base modification or structure other than the insertion of an extra guanosine templated by the mutation, which was structurally protected from nuclease digestion like the surrounding nucleotides. These findings indicate a common molecular process underlying sensorineural deafness caused by mitochondrial tRNA_{Ser(UCN)} mutations.

More than 50 pathological mutations in mitochondrial tRNA genes have now been reported and validated (Ref. 1, see also www.gen.emory.edu/mitomap.html), and the molecular effects of many have been at least partially elucidated via the creation of ρ₀ cybrids (fusions to cells lacking endogenous mitochondrial DNA, see Ref. 2) or other cell culture models. Heteroplasmic mutations, such as A3243G or other tRNA_{Leu(UUR)} mutations found in cases of MELAS/ (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes) and other syndromes or A8344G and T8356C found in cases of MERFF (myoclonic epilepsy with ragged-red fibers), tend to have severe quantitative and/or qualitative effects on mitochondrial protein synthesis and respiratory function (3–6). They are associated with abnormal base modification (7–9), decreased aminoacylation (4, 8, 10–12), and reduced steady-state levels of the corresponding tRNAs (5, 6, 8, 11). Mutations in tRNA_{Leu(UUR)} are associated frequently with defective RNA processing (6, 13–15) and also shortened half-life (8) of the affected tRNA. By contrast, mutations affecting tRNA_{Ser(UCN)}, which are frequently homoplasmic or implicated pathologically only at high levels of heteroplasmy, have rather modest effects on mitochondrial function and are generally associated with mild or tissue-restricted pathological states, principally sensorineural deafness.

The 7472insC mutation is one such mutation. Clinically, most subjects have an apparently non-syndromic hearing impairment (16), with a minority suffering a more widespread neurological disease including ataxia and myoclonic seizures (16–18), sometimes with a measurable deficit of cytochrome c oxidase (18). In 143B osteosarcoma-derived cybrid cells, homoplasy for the 7472insC mutation produces only a very modest biochemical phenotype, comprising a small decrease in complex I activity (17) and a growth deficit in galactose medium when the mutation is present in combination with a diminished copy number of mtDNA (19). Effects on mitochondrial protein synthesis are minimal, with only a slight quantitative decrease in mitochondrial translation products detectable by pulse labeling (19), an effect mildly exacerbated by doxycycline treatment (19). The only clear molecular effect of the mutation that can be seen in cybrid cells is a decrease of ~70% in the steady-state level of tRNA_{Ser(UCN)} (19), an effect shared with the A7445G deafness-associated mutation (20, 21). Unlike the case of the latter, which has been studied only in lymphoblastoid cells, 7472insC produces no systematic change to the level of the upstream (ND6) mRNA, leading to the suggestion that its effects are most likely on the half-life of tRNA_{Ser(UCN)} rather than on its processing (19). Electrophoretic analysis of [¹⁴C]serine-labeled tRNA also suggested only a minimal effect on aminoacylation.

To investigate in further detail the molecular effects of the

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The abbreviations used are: MELAS, mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes; MERFF, myoclonic epilepsy with ragged-red fibers; EtBr, ethidium bromide; np, nucleotide pair; RT, reverse transcription; LSTU, light strand transcription unit.

1 The paper is available on line at http://www.jbc.org.
7472insC mtDNA Mutation Impairs tRNA<sup>Ser(UCN)</sup> Synthesis

7472insC mutation, applied a variety of assays. These compared 143B osteosarcoma-derived cybrid cell lines homoplasmic for the mutation with those containing only wild-type mtDNA derived from the same patient. Mitochondrial tRNA half-lives and synthesis rates were measured via the use of ethidium bromide (EtBr) to block new transcription of mtDNA (8). Effects on aminoaoylation were studied using oxidation-circularization of tRNA (12) combined with minisequencing in the ratio of mutant to wild-type tRNA in the final product mixture. Effects on base modification were studied by primary sequence determination of tRNA<sup>Ser(UCN)</sup> (22, 23) and on secondary/tertiary structure by partial RNase digestion under non-denaturing conditions. Possible effects on RNA stability were also investigated by overexpression of mitochondrial EF-Tu. These assays revealed a pronounced decrease in the rate of synthesis but not the half-life of the mutant tRNA, combined with a small but clear decrease in the extent of aminoaoylation. Because of the absence of polarity effects on the synthesis of downstream tRNAs of the light strand transcription unit, the mutation is inferred to act posttranscriptionally. The synthesis of downstream tRNAs of the light strand transcription unit, the mutation is inferred to act posttranscriptionally, as was found. Considered alongside previous findings on the A7445G mutation (20, 21, 24), a consistent picture emerges of mtDNA localization of the mutation. 

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Culture**—143B osteosarcoma cell cybrids homoplasmic for the np7472insC mutation or for wild-type mtDNA from the same individual were as described previously (17, 19). Specific cell lines used in the experiments are indicated in figure legends. Except where indicated, line 43 was used as the source of control tRNA and line 47 was used as the source of mutant tRNA. Cells were routinely cultured in media supplemented with uridine and pyruvate as described previously (19) and passaged weekly.

**Oligonucleotides**—Custom-designed oligonucleotides were purchased from DNA Technology (Aarhus, Denmark) or Genset (Paris, France) and are as follows (all shown as 5'-3'). For Northern and dot-hybridization: Ser-11AAGEGAATGCA1GACCCCGCGGCTG (np 7451–7478 of human mtDNA, Ref. 25); U2-11GATTTAGGGCTTCTATCAAGGCCA (np 2965–3224) of human mtDNA; cser1-TGG-GAG (np 4341–4360); Tyr-ATTTACAGTCCAATGCTTCACTC (np 5679–5757); Lys-AGCCAACCCCATGGCCTCCATGACTTTTTC (np 7485–7514); Ser-Bio, with 3’ biotinylated oligonucleotide/Hi/H11032; for purification of tRNAser(UCN): Ser-Bio, with 3’ biotinylated oligonucleotide/Hi/H11032; for minisequencing: cser3-CTTGAAACCA-7478 of human mtDNA, Ref. 25); Leu21 (Ref. 6)-GTTTTAT-7479); for RT-PCR cloning of the human mitochondrial EF-Tu cDNA (from the 7467), and cser4-AAGGAAGGAATCGAAA-CGCGGATCCACCACAATGGCGGCCGCCACCCTGCT and 5S1 (Ref. 8)- as described previously (17, 19). Specific cell lines were as described previously (17, 19) and passaged weekly.

**Miscellaneous RNA and DNA Manipulations**—Total RNA was prepared from cells using the Trizol method as previously described (19). For sequencing, about 500 or 1000 ¢A590 units (depending on the purpose) of total RNA were extracted from 100–200 9-cm plates of semi-confluent cells (~10<sup>6</sup> cells) by Trizol extraction (Invitrogen). Total RNA was incubated at 37 °C for 2 h in 20 ml Tris-HCl, pH 9.0, to deacetyl tRNAs. After this treatment, the pH was adjusted to 7.5, and RNA was fractionated on 5% DEAE-Sepharose, which was eluted with random hexamers and Invitrogen M-MLV RTase. Except where indicated, PCR and RT-PCR reactions used 55 °C for the annealing step. Northern blotting to oligonucleotide probes used the same conditions as described previously (19). Each of the following modifications and specific RNases at 37 °C for 1.5 h in water to induce partial digestion. The same individual were as described previously (17, 19). Inspection of the results in experiments with ΔT4 was used for the second dimension. For enzymatic probing of differences in second-

**Aminoacylation Analysis by Oxidation-Circularization Assay**—The extent of aminoaoylation of tRNA<sup>Ser(UCN)</sup> was measured using the oxidation-circularization assay, essentially as described by Börner et al. (12). For this purpose, the following modifications. Total RNA was isolated by Trizol extraction from cells grown to 90% confluence and dissolved in ice in 0.1 M NaOAc, pH 5.0. In some experiments, small RNAs were isolated from 25 µg of total RNA, using chromatography on DEAE-Sepharose fast flow (Amersham Biosciences). Eluates were divided into four aliquots, precipitated, and used for two sets of oxidation-circularization reactions. RNA preparations from control and mutant cybrids were mixed in various arbitrary proportions, giving ratios of wild-type to mutant signal in the final assay of between 0.2 and 3. DNA was synthesized from circularized tRNA<sup>Ser(UCN)</sup> using primer cser1 and then amplified with primers cser1 and cser2 (92 °C, 2 min; 30 cycles of 94 °C, 30 s, 55 °C, 30 s, 72 °C, 30 s; final extension at 72 °C, 5 min). RT-PCR products were isolated by 10% PAGE, eluted, and purified (PCR purifica-

**rRNA Half-life and Synthesis Measurements**—For half-life measurements, cultures were grown to 70% confluence, giving 60–70% confluence, on 6-cm plates 14–16 h before the experiment. They were then incubated in fresh medium containing 250 ng/ml of EtBr for the times indicated in the figures. For synthesis measurements, cells were seeded at 50% confluence on 9-cm plates 14–16 h before the experiment and then incubated in medium containing 250 ng/ml of EtBr for two days after which they were passaged into fresh medium on 6-cm plates.
Medium was again replaced after 5–6 h of recovery and then daily until cells were harvested at the times indicated in figures. Cells were either seeded initially at different densities so that they reached approximately the same final densities at the time of harvesting or else were passaged to obtain single clones, which were placed under selection in 1.6 mg/ml Geneticin (Invitrogen). After 1 week the two sets of data thus obtained were measured by the Bradford method (28). For detection of EF-Tu mouse monoclonal MAB-68 (1:500 dilution) (Ref. 29, a kind gift of Frank Henkler) and goat or horse anti-mouse horseradish peroxidase (1:10,000 dilution) antibodies were used. Detection of PAK1 (p21-associated kinase) used rabbit polyclonal PAK4C-19 (Santa Cruz Biotechnology, 1:5000 dilution) and horse anti-rabbit horseradish peroxidase (1:10,000 dilution) antibodies. Fluorographs were analyzed by densitometry.

RESULTS
The 7472insC Mutation Has Only a Minimal Effect on tRNA<sup>Ser(UCN)</sup> Structure—To evaluate the structural effects of the 7472insC mutation, the primary sequence of tRNA<sup>Ser(UCN)</sup> from two different control cybrid cell lines was first determined (Fig. 1) via a combination of partial RNase digestion of end-labeled tRNAs (Donis-Keller method, see Ref. 22) and TLC of mononucleotide immobilized on streptavidin-coated beads (26). Modifications of four kinds were detected at five positions of the tRNA. Two pseudouridines were found, one located in the anticodon stem and only one nucleotide between the acceptor and D-stems, is the one proposed earlier for mammalian mitochondrial tRNA<sup>Ser(UCN)</sup> (21). The nucleotide analysis (phosphorimaging output) is shown for the five positions at which modified 5'-nucleotides were detected. For each position, two different solvent systems were used, A in the left-hand panel, B in the right-hand panel as described under “Experimental Procedures.” The identities of the 5'-modified nucleotides were deduced from standard chromatographic maps, as illustrated (see Ref. 23). All assignments were also consistent with partial digestions (Fig. 2) based on known RNase specificities and electrophoretic properties of RNA fragments containing modified nucleotides. Note the presence of additional nucleotides in the chromatographic maps, as illustrated (see Ref. 23). All assignments were also consistent with partial digestions (Fig. 2) based on known RNase specificities and electrophoretic properties of RNA fragments containing modified nucleotides.
position 51), adjacent to a ribothymidine (conventional position 54, actual position 50), giving the conventional T/C9023C motif. In the anticodon loop, two additional modifications similar to those found previously in bovine mitochondrial tRNA\textsubscript{Ser(UCN)} (31) were found: 3-methylcytosine at conventional position 32 (actual position 29) and N6-isopentenyladenosine (i\textsubscript{6A}) or 2-methylthio-N6-isopentenyladenosine (ms\textsubscript{2}i\textsubscript{6A}), which the solvent systems used for TLC do not resolve, at conventional position 37 (actual position 34). This modified base is probably ms\textsubscript{2}i\textsubscript{6A} because bovine mitochondrial tRNA\textsubscript{Ser(UCN)} has exclusively ms\textsubscript{2}i\textsubscript{6A} at this position.\textsuperscript{2} The wobble base U (conventional position 34, actual position 31) was found to be unmodified.

The equivalent tRNA was extracted from cells homoplasmic for the mutation and analyzed in parallel, via partial RNase and alkaline digestions (Fig. 2). No differences in the digestion patterns were seen, except for the presence of the additional G templated by the inserted nucleotide pair that defines the mutation, which lies within a homopolymeric tract spanning from the extra loop through the T-stem. All of the modifications identified by TLC produce characteristic changes in RNase sensitivity and/or mobility that allow their presence to be unambiguously scored by the partial RNase digestion method. In every case, the digestion pattern of the mutant tRNA indicated that the modified base detected by TLC in the wild-type tRNA was present also in the mutant tRNA.

Partial RNase digestion carried out on non-denatured, 5’-\textsuperscript{H}labeled tRNA\textsubscript{Ser(UCN)} from homoplasmic mutant and control cybrid cells also revealed only very subtle differences attributable to the mutation (Fig. 3). Like the other guanosines of the T-arm and extra loop, the inserted G was protected from digestion by RNase T1. The T-arm and extra loop were almost entirely inaccessible to all nucleases tested in both mutant and control tRNA\textsubscript{Ser(UCN)}; therefore it is not possible to ascertain whether the inserted G creates an additional base pair in the T-stem or is found in the extra loop or T-loop. However, the mutation appears to cause no major structural disturbance.

\textsuperscript{2} T. Suzuki, T. Suzuki, and K. Watanabe, unpublished data.

\textbf{Fig. 2. Sequence comparison of tRNA\textsuperscript{Ser(UCN)} from control and 7472insC mutant cybrid cell lines.} \textit{a}, a portion of the alkaline ladder and RNase T1 digest of the 5’-labeled tRNA from control cybrid cell line 43 (denoted \textit{c}) and mutant cybrid cell line 47 (denoted \textit{m}). The characteristic abnormal spacing associated with three of the modified nucleotides (conventional numbering) is seen clearly in the alkaline ladder for both control and mutant tRNA. Partial digestions of the same 5’-labeled tRNAs with each of five ribonucleases. The portion of the gel shown covers all of the modified nucleotides. Nuclease specificities were as expected from the literature, except that the available preparations of RNase U2 cut weakly also at pyrimidines. RNase CL was found to cut only weakly at some Cs adjacent to another C, and weakly also at some Us. RNase A was very variable in activity at different positions. RNase ONE, although largely nonspecific, was found to cut only weakly or not at all at most Gs. Control RNA from cybrid cell line 34 was also sequenced using RNase PhyM, which gave concordant results. The enzyme is no longer available from any source. The only reproducible difference between the mutant and control tRNAs is the inserted G templated by the 7472insC mutation itself. In both samples, the modified adenosine at conventional position 37 (actual position 34), the modified cytidine at conventional position 32 (actual position 29) and the ribothymidine at conventional position 54 (actual position 50) are not cut with any enzyme tested. The unmodified wobble-base U-34 (actual position 31) is cut as normally by RNases A and T2, and also very weakly by RNase CL, in both samples. The pseudouridines at conventional positions 28 (actual position 25) and 55 (actual position 51) are cut efficiently by RNase T2, but not by RNase A and only weakly or very weakly by any other enzyme. All nucleotides outside the region shown were cut as predicted, based on the known properties of the enzymes, in both control and mutant tRNAs.
a of the data on which contributes to the extra loop or to the T-loop without significantly disturbing the tertiary structures in which these loops participate. Tested, in both mutant and wild-type tRNAs, it is not possible to ascertain whether the extra G forms an additional base pair in the T-stem or by the inserted nucleotide pair of the mutant mtDNA, is arrowed. Because the whole region is protected from digestion by any of the nucleases tested, in both mutant and wild-type tRNAs, it is not possible to ascertain whether the extra G forms an additional base pair in the T-stem or contributes to the extra loop or to the T-loop without significantly disturbing the tertiary structures in which these loops participate. A sample of the data on which is based. Digestions of tRNA<sub>Ser(UCN)</sub> from control (denoted c) or mutant (denoted m) cybrid cells, with each of three informative RNases. For clarity, the top and bottom halves of the gel are shown separately. Note that the extra G in the mutant tRNA is not accessible to RNase T1, but that the very poorly accessible cytidines at conventional positions 56 (T-loop, actual position 52), 61 and 62 (T-stem, actual positions 57 and 58) are even less digestible by RNase CL in the mutant tRNA, although RNase A does not distinguish the two substrates in this region. The accessible guanosines of the anticodon (G-35 on the conventional scheme, actual position 52) and at the base of the acceptor stem (G-7, using conventional or actual numbering) are arrowed for reference.

aminoacylated and non-aminoacylated tRNA<sub>Ser(UCN)</sub> cannot be separated electrophoretically. The assay distinguishes aminoacylated from non-aminoacylated tRNA on the basis that periodate oxidation renders the latter incapable of being circularized by RNA ligase, whereas the former is protected by the amino acid and can be circularized after subsequent deacetylation. The amount of wild-type versus mutant RNA in the circularized product can then be determined by RT-PCR using a reverse transcription primer that anneals adjacent to the 5′ end of the tRNA and hence will only use circularized RNA as a template. We used fluorescent minisequencing to compare the amount of mutant and wild-type product derived from the total and aminoacylated RNA fractions. To exclude any systematic error from the choice of primer or fluorescent labels used for minisequencing, we analyzed each product twice, using unrelated primers (and different dNTP labels) for the two strands. The assay was originally developed for studies of heteroplasmic cells, whereas the 7472insC mutation in the test cells was homoplasmic. To circumvent this problem, we created various arbitrary mixtures of RNA from cybrid cells containing wild-type and mutant mtDNA and then used the assay to compare the ratio of mutant to wild-type product in total RNA and aminoacylated RNA for each such mixture. This approach is valid because the assay merely determines the ratio between the aminoacylation efficiencies of mutant versus wild-type tRNA.

As shown in Fig. 4, the proportion of mutant tRNA in the aminoacylated fraction relative to its proportion in unfractioated RNA, was uniformly around 75%, regardless of the mixing ratio used, at least across the 6-fold range employed. Minisequencing on the two strands gave essentially identical results (77.3 ± 3.8% using the coding-strand primer, or 73.0 ± 3.9% using the non-coding strand primer), as did several different RNA preparations or prior purification of the small RNA fraction by DEAE-Sephrose chromatography. The mutation therefore causes a 25% decrease in steady-state aminoacylation of tRNA<sub>Ser(UCN)</sub> in the 143B cell background. It should be noted, however, that the assay assumes that all of the aminoacylation that has occurred is by the correct amino acid (serine). Any mis-aminoacylation of the mutated tRNA would have been overlooked. Nevertheless, a substantial level of mis-aminoacylation would very likely have been detected electrophoretically in earlier studies because acylation of the 14 other mitochondrial tRNAs tested has produced a mobility shift detectable on acidic polyacrylamide gels (32).

The 7472insC Mutation Has No Effect on the Half-life of tRNA<sub>Ser(UCN)</sub>. Although the aminoacylation defect is modest, the observation is similar to that for the A3243G mutation on tRNA<sub>Leu(UUR)</sub>, which results in defective aminoacylation (8, 10–12) and is associated with a >70% decrease in the abundance of the tRNA in cybrid cells at or near homoplasm for the mutation (6, 11). In the latter case, the mutation also causes a large decrease in tRNA<sub>Leu(UUR)</sub> half-life (8). We therefore tested whether the 7472insC mutation similarly affects tRNA<sub>Ser(UCN)</sub> stability in cybrid cells. We used EtBr to block mitochondrial RNA synthesis as described previously (8, 33, 34).
Wild-type and mutant cybrid cells were initially treated with EtBr at various concentrations, and total RNA was extracted at various time points after the addition of the drug. Northern blots of these RNA samples, fractionated on urea-polyacrylamide gels, were probed for mitochondrial tRNASer(UCN) and tRNALeu(UUR) and for 5 S rRNA as a loading control. We selected the lowest concentration of EtBr (250 ng/ml) that appeared to give complete inhibition of mitochondrial RNA synthesis (i.e. above which the profiles of RNA degradation were indistinguishable) and then repeated the experiment a sufficient number of times using these conditions to minimize the effects of experimental variation. Within the margins of error, the halflives of both tRNASer(UCN) and the control tRNA, tRNA\textsuperscript{Leu(UUR)}, were the same in 7472\textsuperscript{insC} mutant and wild-type cybrid cells. The different steady-state levels of tRNA\textsuperscript{Ser(UCN)} in mutant and wild-type cells must therefore be due to differences in the rate of synthesis of the tRNA, not its stability.

During prolonged EtBr treatment, mitochondrial protein synthesis should be rapidly shut down due to the short halflives of mitochondrial mRNAs (35). To check whether this was accompanied by tRNA deacylation under conditions of ATP depletion, which might have influenced the relative stability of the mutant and wild-type tRNAs, we checked the aminoacylation status of mitochondrial tRNA\textsuperscript{Lys} and tRNA\textsuperscript{Leu(UUR)} electropheretically. The latter remained aminocytlated throughout the 24 h of EtBr treatment, although tRNA\textsuperscript{Lys} gradually became partially deacylated (Fig. 5b). Although aminocytlated and deacylated tRNA\textsuperscript{Ser(UCN)} cannot be distinguished electropheretically, the aminocytlation difference between wild-type and mutant tRNA allowed us to use the oxidation-circularization minisequencing assay to detect changes in aminocytlation during the course of the experiment. The relative extent of aminocytlation of the mutant tRNA was slightly lower after 24 h of EtBr treatment than in untreated cells (Fig. 5c), implying that a small amount of deacylation was occurring. However, this does not appear to have affected the half-life of the tRNA. On the other hand, the fact that the changes in aminocytlation are modest means that the minimal effects of the mutation on tRNA half-life are not an artifact attributable to deacylation.

As a further test of the effect of the mutation on tRNA\textsuperscript{Ser(UCN)} stability we made use of the observation that moderate overexpression of mitochondrial elongation factor EF-Tu in yeast, via manipulation of its gene dosage, can complement a variety of mutations affecting mitochondrial tRNAs (36, 37). EF-Tu binds aminocyt-tRNAs in a ternary complex with GTP, acting as a molecular chaperone to facilitate their delivery to the acceptor site of the ribosome. The complementation activity of yeast EF-Tu is presumably based on the stabilization of mutant aminocyt-tRNA structures that are otherwise unstable. We transfected an EF-Tu expression construct into cybrid cells containing either wild-type or 7472\textsuperscript{insC} mutant mtDNA, picked colonies, and verified the presence and expression of the transgene by PCR and RT-PCR. We then tested the expression level of EF-Tu by Western blotting and measured the representation of tRNA\textsuperscript{Ser(UCN)} by Northern blotting in stably transfected clones compared with those transfected with only the empty vector. Based on analysis of a panel of such transfectants (Fig. 6), modest (up to 3-fold) overexpression of mitochondrial EF-Tu in cybrid cells had no effect on the decreased abundance of tRNA\textsuperscript{Ser(UCN)} associated with the 7472\textsuperscript{insC} mutation, consistent with the above findings from EtBr-treated cells that the mutation did not grossly affect the structure or turnover of the tRNA.

The 7472\textsuperscript{insC} Mutation Impairs the Synthesis of tRNA\textsuperscript{Ser(UCN)} but Not That of Downstream tRNAs of the Light Strand Transcription Unit—The above results suggest that it is the synthesis of tRNA\textsuperscript{Ser(UCN)}, rather than its turnover, that is affected by the mutation, raising the additional question of whether the effect is transcriptional or posttranscriptional. To address these questions directly we followed the resynthesis of mitochondrial tRNAs in wild-type and mutant cells depleted of mitochondrial transcripts by prolonged EtBr treatment (Fig. 7). EtBr was removed after 48 h, and the cells allowed to recover for a further 7 days. After correcting for the loading control (5 S rRNA), the signal for each tRNA was normalized to its level in untreated control cells always included on the same gel blot. The data plotted in Fig. 7 represent the means from six experiments. No systematic effects were observed that depended on cell density or the time at which cells were passaged. The heavy-strand-encoded tRNA\textsuperscript{Ser(UCN)} was again included as a control to reveal any effects attributable to mtDNA copy number or other inherent properties of the mitochondrial transcription system in control versus mutant cell lines. To detect any polarity effects on the downstream tRNAs of the light strand transcription unit, we also probed for tRNA\textsuperscript{Thr} and tRNA\textsuperscript{Gln}. All tRNAs returned to their starting levels within 5–7 days of recovery, typically with a small “overshoot.”

![Image](http://www.jbc.org/)

**FIG. 4.** Extent of aminocytlation of tRNA\textsuperscript{Ser(UCN)} in control and mutant cybrid cells using the oxidation-circularization assay. The results plotted are from a total of 17 different experiments, using different, arbitrary mixtures of RNA from control and mutant cells and based on four independent RNA isolations from each source. In each case, the ratio of mutant to wild-type product was analyzed by RT-PCR and fluorescent mini-sequencing using primers for each strand (open circles, coding-strand primer; filled circles, non-coding strand primer). For each RNA mixture, parallel samples were analyzed that had and had not been deacylated in vitro prior to the oxidation step. Arbitrary mixtures are appropriate because the assay measures only a ratio, namely that of the proportion of mutant tRNA that is aminocytlated to the proportion of wild-type tRNA that is aminocytlated. It also assumes that the amount of unreacted (deacylated) tRNA is a small and constant proportion of the whole. The fact that the data points lie on a straight line confirms the validity of the approach. Despite the very different relative fluorescence signals for the diagnostic chain terminators on the two strands, the relative proportion of mutant to wild-type signal in aminocytlated RNA versus total RNA was virtually identical for the two strands in all samples analyzed. The line of slope 0.75 represents an unweighted mean ratio of all data points. Identical results were obtained when RNA samples were processed further to purify short RNAs, confirming that the differences between mutant and control RNAs are unlikely to result from differential lability during extraction. RT-PCR performed on the circularized products routinely generated a low proportion of apparently dimerized product, but this did not differ according to the ratio of input mutant and wild-type tRNAs, and in fact the minisequencing ratios obtained from excised, presumptive dimer bands were invariably identical to those obtained from monomer bands excised in the same experiment.
The rate of resynthesis of tRNA\(^\text{Ser(UCN)}\) in mutant cells, as judged by the recovery profiles shown in Fig. 7, was approximately one-third of that in wild-type cells. For the other tRNAs, control and mutant cells gave very similar recovery profiles. For all of the tRNAs analyzed, including the heavy strand-encoded control, tRNA\(^\text{Leu(UUR)}\), both the starting and final levels reached were slightly lower in the mutant than control cells, which indicates a small effect of mtDNA copy number. We conclude that the synthesis of the tRNA\(^\text{Ser(UCN)}\) is specifically impaired by the mutation, but the absence of polarity effects on downstream, light strand-encoded tRNAs indicates that the effect must be posttranscriptional.

To investigate this further, we probed Northern blots with oligonucleotides for tRNA\(^\text{Ser(UCN)}\) and tRNA\(^\text{Leu(UUR)}\) using RNA isolated from cells at different times during EtBr treatment. RNA levels expressed as a fraction of the signal obtained from a panel of replicates taken at zero time (the time at which EtBr was added) were converted to logarithms on the assumption of first-order decay kinetics. All hybridization signals were normalized against 5 S rRNA as loading control. The data plotted represent the mean ± S.D. of five separate experiments. Lines of best fit (least squares method) are shown, \(r^2\) for the four panels (clockwise, from top left) being \(-0.990\), \(-0.985\), \(-0.967\), and \(-0.999\), respectively. No significant differences in tRNA half-life were inferred between control and mutant cybrids or between the two tRNAs. b, analysis of the effects of EtBr treatment on aminoacylation of mitochondrial tRNAs by acidic gel electrophoresis and Northern hybridization. Samples deacylated at pH 9 denoted as ‘d’. Graphical values, shown alongside the gel image, are based on densitometric analysis. c, analysis of effects of EtBr treatment on relative aminoacylation of mutant versus wild-type tRNA\(^\text{Ser(UCN)}\), by oxidation-circularization-minisequencing assay, as in Fig. 4. Each data point is the mean of at least 4 experiments, using both sense- and antisense-strand oligonucleotides for minisequencing.

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To investigate this further, we probed Northern blots with oligonucleotides for tRNA\(^\text{Ser(UCN)}\) and the regions immediately upstream and downstream (antisense transcripts of COXI and COXII). To control for possible cross-reaction to other transcripts, we tested two different oligonucleotides for a given gene. We detected no high molecular weight precursor-like transcripts, even at the longest exposure times (data not shown). This applied to both mutant and wild-type cybrid cells.
The relevant RNA processing intermediates must therefore be heterogeneous and/or of very low abundance and do not accumulate in mutant cells to higher levels than in control cells.

**DISCUSSION**

**Primary Effects of the 7472insC Mutation on Mitochondrial Gene Expression**—Previous studies of 143B osteosarcoma cell cybrids homoplasmic for the 7472insC mutation (19) indicated that the mutation is associated with approximately a 70% decrease in the steady-state level of tRNA<sup>Ser(UCN)</sup> compared with control cybrids. The cells exhibit a modest, quantitative deficit of mitochondrial protein synthesis (19) and mild respiratory impairment (17, 19). These earlier findings left open the question of why mitochondrial gene expression was so severely affected in mutant cells to higher levels than in control cells.

In cells treated with EtBr, the half-life of the mutant tRNA was indistinguishable from wild-type. This finding suggests that EtBr treatment blocks mitochondrial RNA synthesis completely, but does not directly affect RNA stability. This is supported by the fact that across a wide range of concentrations (150 ng/ml to over 1 μg/ml), EtBr treatment gave essentially the same decay profiles for mitochondrial tRNAs. Another assumption is that the RNA used as loading control, 5 S rRNA, is unaffected by the treatment. Use of an alternate loading control in some experiments, a probe for a cytosolic tRNA (19, 38), gave similar results (data not shown). The half-life of tRNA<sup>Ser(UCN)</sup> as measured in these experiments (~18 h) is somewhat shorter than inferred previously in HeLa cell cybrids (8), probably reflecting cell type differences in gene expression that influence tRNA half-life.

In contrast, the 7472insC mutation profoundly and specifically affected the synthesis of tRNA<sup>Ser(UCN)</sup> during recovery from EtBr treatment. Quantitatively, the effect was similar to the decrease in steady-state level (in both cases ~70%). Because turnover was unaffected, simple first-order kinetic considerations support the view that this decreased synthesis is sufficient to account for the effects of the mutation on the tRNA<sup>Ser(UCN)</sup> level. In previous experiments, we found that ND6 mRNA, encoded upstream of tRNA<sup>Ser(UCN)</sup> in the light strand transcription unit (LSTU), was not systematically affected by the mutation (19). The absence of any effect on downstream tRNAs of the LSTU confirms that the effect of the mutation is posttranscriptional. In principle the defect could be in processing of the primary transcript or in a late step of maturation, such as CCA addition, possible 3′ end trimming, or the rate of base modification.

Although we cannot exclude an interference with processing at the termini of tRNA<sup>Ser(UCN)</sup>, the absence of detectable precursor accumulation in mutant cells suggests that the mutation most likely affects the efficiency of a later step in tRNA<sup>Ser(UCN)</sup> maturation. Because the mutant tRNA has the same termini as wild-type tRNA<sup>Ser(UCN)</sup>, any effect on RNA processing would have to be quantitative rather than qualitative, although it cannot be excluded that the mutation promotes mis-processing and that incorrectly processed transcripts are turned over very rapidly. Structural studies indicated only very subtle differences in the region of the extra loop/T-arm that may conceivably extend or bend the T-arm. The combined length of the acceptor and T-stems is a critical determinant for pre-tRNA processing by RNase P in *Xenopus* (39) and also by mammalian 3′-tRNA endonuclease (40). Nuclear and mitochondrial RNase P appear to be identical in human cells (41), and the same may be true of 3′-tRNA endonuclease (42). The A7445G mutation has recently been shown to
block processing of pre-tRNA$^{\text{Ser(UCN)}}$ by mitochondrial 3'-tRNA endonuclease in vitro (24). However, in the same assay, a substrate with the 7472insC mutation was not processed at a rate that was substantially below wild-type. An interference with pre-tRNA processing remains a plausible mechanism, but effects on later maturation steps seem more likely. Testing this will require the development and validation of appropriate in vitro assays.

The finding of a reduced extent of aminoacylation raised the possibility that inefficient charging of the mutant tRNA might contribute to its decreased abundance. However, partial deacylation during prolonged EtBr treatment was not accompanied by any detectable destabilization of the mutant tRNA. We conclude that the poor aminoacylation of the mutant tRNA is unlikely to have any bearing on its decreased steady-state level: the two are independent effects of the mutation.

The length and orientation of the extra arm, but not its specific nucleotides, are known to be important for the interaction of tRNA$^{\text{Ser}}$ with seryl-tRNA synthetase in both Escherichia coli (43) and humans (44). However, a G insertion equivalent to the 7472insC mutation did not block in vitro aminoacylation of a bovine tRNA$^{\text{Ser(UCN)}}$ substrate by bovine mitochondrial seryl-tRNA synthetase recombinantly expressed in E. coli (45). The reaction showed only a small change in kinetic parameters, consistent with the modest decrease in aminoacylation efficiency in vivo reported here.

The combined effects of the 7472insC mutation on tRNA synthesis and aminoacylation reduce the steady-state level of aminoacylated tRNA$^{\text{Ser(UCN)}}$ by 75–80%. This is probably sufficient to account for the modest protein synthesis deficiency and respiratory phenotype and may also explain why the mutation only causes a severe disease when homoplasmic or at high percentages of mutant mtDNA. The fact that the impairment in protein synthesis is modest suggests that tRNA$^{\text{Ser(UCN)}}$ may be present in excess in normal cells, which may be of critical importance in understanding the tissue specificity of the phenotype. The drop in abundance may not be critical unless some other component of the mitochondrial protein synthesis apparatus is limiting.

Other mitochondrial tRNA disease mutations, such as
A3243G, profoundly affect base modification (7, 8), and lack of wobble-base modification seems to correlate with a more severe effect on protein synthesis (8, 46). A3243G also has a more drastic effect on aminoacylation both in vivo (12) and in some cybrid models (10, 11), as well as affecting tRNA stability (8). However, in cells 80–90% heteroplasmic for A3243G the combined effects on the level of functional, aminoacylated tRNA
\textsuperscript{ser}(UUR) may be similar to the effects of homoplasy for 742insC on tRNA\textsuperscript{ser}(UCN). This level of heteroplasmic for A3243G also has only modest effects on mitochondrial functions in cybrid cells (3, 6), but is indisputably pathological.

A Similar Pathogenic Mechanism for Deafness-associated Mutations Affecting Mitochondrial tRNA\textsuperscript{Ser}?—The molecular effects of two deafness-associated mitochondrial mutations, A7445G and 742insC, are strikingly similar (19–21, 24, and this paper), suggesting a similar underlying pathogenic mechanism, based on impaired synthesis of (aminoacylated) tRNA\textsuperscript{Ser}(UCN).

Three other pathological mutations have been found in the same gene, mapping to consecutive residues on the 5′ side of the aminoacyl stem (18, 47–49). T7512C has been reported in association with a disease similar to the extended phenotype of 742insC (18), elsewhere described as “MERFF/MELAS overlap syndrome” (49). T7511C and T7510C are found in families with maternally inherited hearing impairment (47, 48). These mutations each disrupt base-pairing, converting an A-U to a G-U base pair. The C12258A mutation in the acceptor stem of tRNASer(UCN) within the LSTU, separated from other functional domains, leads to a similar end result.

Genotype-Phenotype Correlations in Mitochondrial tRNA Disease—Mutations affecting tRNA\textsuperscript{Ser}(UCN) mainly cause sensorineural deafness, whereas mutations in other mitochondrial tRNA genes are usually associated with wider, multi-system disorders. This might reflect the rather unusual situation of tRNA\textsuperscript{Ser}(UCN) within the LSTU, separated from other functional transcripts on either side by several kilobases. Virtually all other mitochondrial tRNAs are immediately adjacent to another same-strand transcript on one or both sides, the only other prominent exceptions being tRNA\textsuperscript{lno} and tRNA\textsuperscript{vo} (see Fig. 7). Because of this punctuation model of mitochondrial RNA synthesis (54), virtually all mutations that affect tRNA processing will have multiple effects on adjacent transcript(s).

Although both A7445G and 742insC manifest primarily as hearing impairment, both are associated, at least in some individuals, with a disorder affecting other tissues. In A7445G families many individuals suffer also from palmitoeran kera
toderma (55, 56). In 742insC families some individuals have a wider neurological syndrome including ataxia and myoclonus (16–18), sometimes associated with a clear cytochrome c oxidase deficiency (18). The wider but distinct features of these disorders may be attributable to specific effects of each mutation on processes other than on the synthesis of tRNA\textsuperscript{Ser}(UCN).

In the case of 742insC the aminoacylation defect may produce tissue-specific features in individuals where the expression of, for example, mitochondrial seryl-tRNA synthetase (serine-tRNA ligase, SARS) may be limiting. The recent identification of single nucleotide polymorphisms in the SARS gene (57) may provide a useful tool to assess whether the clinical phenotype of 742insC disease shows linkage with the locus, provided enough families can be collected. In turn, the specific features of A7445G disease may be attributable to effects of the mutation on the expression of the upstream gene of the LSTU, ND6 (21), which are not shared with 742insC (19).

In yeast, a variety of mitochondrial tRNA gene defects, including at least one example of an apparent RNA processing defect, are suppressible by modest increases in gene dosage of TUFM, encoding mitochondrial EF-Tu (36, 37). The suppression mechanism is unknown, although the steady-state level of the affected tRNA is invariably increased. In the case of 742insC no such suppression was found, consistent with the minimal effects of the mutation on tRNA structure and turnover; although it is possible to hypothesize an undetected effect on the translational properties of the tRNA without an increase in its steady-state level. However, it would be virtually impossible to detect this reliably, using currently available techniques. Alternatively, there may be species differences in the interaction of EF-Tu with tRNA or mitoribosomes, or EF-Tu may already be present in excess, although a recent estimate (58) suggested otherwise. In either case, our finding suggests that overexpression of TUFM may not be a useful general strategy for correcting the effects of mitochondrial tRNA mutations in humans.

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The 7472insC Mitochondrial DNA Mutation Impairs the Synthesis and Extent of Aminoacylation of tRNA Ser(UCN) but Not Its Structure or Rate of Turnover
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