A pathogenic point mutation reduces stability of mitochondrial mutant tRNA\textsubscript{Ile}

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Received June 2, 2000; Revised and Accepted August 10, 2000 DDBJ/EMBL/GenBank accession no. AB043958

ABSTRACT

Point mutations in mitochondrial tRNA genes are responsible for individual subgroups of mitochondrial encephalomyopathies. We have recently reported that point mutations in the tRNA\textsubscript{Leu}(UUR) and tRNA\textsubscript{Lys} genes cause a defect in the normal modification at the first nucleotide of the anticodon. As part of a systematic analysis of pathogenic mutant mitochondrial tRNAs, we purified tRNA\textsubscript{Ile} with a point mutation at nucleotide 4269 to determine its nucleotide sequence, including modified nucleotides. We found that, instead of causing a defect in the post-transcriptional modification, a pathogenic point mutation in the mitochondrial tRNA\textsubscript{Ile} reduced the stability of the mutant tRNA molecule, resulting in a low steady-state level of aminoacyl-tRNA. The reduced stability was confirmed by examining the life-span of the mutant tRNA\textsubscript{Ile} both in vitro and in vivo, as well as by monitoring its melting profile. Our finding indicates that the mutant tRNA\textsubscript{Ile} itself is intrinsically unstable.

INTRODUCTION

Point mutations as well as large scale deletions in mitochondrial DNA (mtDNA) are associated with a wide spectrum of human diseases arising from mitochondrial dysfunction (1). While there are an increasing number of reports on point mutations in mitochondrial tRNA genes, pathogenic tRNA molecules themselves remain poorly characterized. Hence, it is unknown how tRNA species that have mutations actually specify the clinical features they give rise to, for example, the clinically distinct mitochondrial encephalomyopathy subgroups MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) and MERRF (myoclonus epilepsy associated with ragged-red fibers), which are caused by point mutations in the tRNA\textsubscript{Leu}(UUR) and tRNA\textsubscript{Lys} genes, respectively (2).

Point mutations in the tRNA\textsubscript{Ile} gene are associated with cardiomyopathy (1). One of these is an A→G transition at nt 4269 [nucleotide position numbering conforms to that used by Anderson et al. (3)] in the mitochondrial tRNA\textsubscript{Ile} gene, which was found in a patient with fatal cardiomyopathy (CM) (4). The patient, who died from progressive intractable cardiac failure, predominantly harbored this mutated mtDNA in cardiac muscle as well as in skeletal muscle and blood cells (4). It has been confirmed by intercellular transfer of the mutant mtDNA to human \textsuperscript{ρ0} HeLa cells lacking mtDNA that accumulation of the mutant is sufficient in itself to bring about mitochondrial dysfunction, i.e. almost complete loss of overall mtDNA-encoded polypeptide synthesis and a significant reduction in respiratory chain enzyme activities, without nuclear gene involvement (5). The significant decrease observed in respiratory activity can be explained by a loss of mitochondrial translation activity, as subunits of the enzymes are encoded by the mitochondrial genome (5).

In order to undertake a systematic analysis of mutant tRNAs, we have developed a method of purifying them from large-scale cultures of cybrid cells. All three of the purified pathogenic mutant tRNAs so far examined have been found to lack a post-transcriptional modification at the first letter of the anticodon (6,7). This finding raises questions as to whether such a post-transcriptional modification deficiency is universal in pathogenic mutant tRNAs and whether the manner in which these mutants affect cells takes the form of a loss of function or the acquisition of a cytotoxic function. An anticodon modification defect may lead to the misincorporation of amino acids into proteins or to suppression of translation resulting in the generation of premature proteins in mitochondria.

In this study, we purified the wild-type tRNA\textsubscript{Ile} and a mutant that has been confirmed to be pathogenic (5) and observed the post-transcriptional modification. We also examined the stability of the mutant tRNA\textsubscript{Ile} both in vivo and in vitro and monitored the dependence of the tRNA hyperchromicity on temperature.

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MATERIALS AND METHODS

Cybrid cell lines

Using the method of intercellular transfer of patient mtDNA to pβ HeLa cells (EB8), two cybrid cell lines were previously constructed by fusing EB8 cells with enucleated fibroblasts from a CM patient with the heteroplasmic A4269G mutation. The CM114-5 cell line exclusively contains mtDNA with the 4269 mutation and the CM1-9 cell line contains only normal mtDNA for use as a control (5). For the study, cells were cultured in normal medium [DMEM/F-12 (1:1) (Gibco BRL), 10% fetal calf serum].

Purification of tRNAIle from cybrid cells by solid-phase probing

Total RNA (~500 A260 U) was extracted from ~10⁶ semiconfluent cultured cybrid cells by Isogen (Nippon Gene, Toyama, Japan). Total RNA was incubated at 37°C for 3 h in 50 mM Tris–HCl (pH 9.5) to discharge amino acids from the tRNAs. The decacylated total RNA preparation was adjusted to pH 7.5 and fractionated on a DEAE–Sepharose Fast Flow column (1 × 45 cm; Amersham Pharmacia Biotech) with a linear gradient of NaCl and MgCl₂ between 250 and 500 mM and between 8 and 16 mM, respectively, in a buffer containing 20 mM Tris–HCl (pH 7.5). Fractions enriched with tRNA Ile were monitored by dot hybridization with an oligonucleotide probe specific for mitochondrial tRNAIle, 5′-TAGAAATAAGGGGGTTTAA-GCTCTATTAT-3′ (not including the mutation position). tRNAIle was purified by solid-phase probing column chromatography using a 3′-biotinylated oligonucleotide with a sequence identical to that used for the dot hybridization, which was immobilized on Streptavidin agarose (Gibco BRL) as previously described (8). The tRNAIle was finally purified by gel electrophoresis.

Determination of wild-type and mutant tRNAIle nucleotide sequences including modifications

Purified tRNAIle was sequenced by a combination of the methods of Donis-Keller (9) and Kuchino et al. (10). For Donis-Keller’s method (9), the homogeneous tRNA was labeled at the 5′-terminus with [γ-32P]ATP (110 TBq/mmol; Amersham Pharmacia Biotech) and T4 polynucleotide kinase (Toyobo, Osaka). The nucleotide-specific RNases used for restricted digestion of tRNA were RNase T₁ (Amersham Pharmacia Biotech), U₃ (Seikagaku Kogyo, Tokyo), PhyM (Amersham Pharmacia Biotech) and CL₃ (Boehringer Mannheim). For the method of Kuchino et al. (10), each nucleotide in the tRNA was analyzed by two-dimensional thin-layer chromatography using two different solvent systems. Solvent system A consisted of isobutyric acid/concentrated ammonia/H₂O (66:13:3 by volume) in the first dimension and 2-propanol/HCl/H₂O (70:15:15 by volume) in the second. In solvent system B, the first dimension was the same as for solvent A, but 0.1 M sodium phosphate (pH 6.8)/ammonium sulfate/1-propanol (100 ml:60 mg:2 ml) was used for the second dimension.

Examination of tRNA aminoacylation levels in cybrids

Total RNA from semiconfluent cultured cybrid cells was prepared under acidic conditions in a cold room and then dissolved into 0.1 M NaOAc (pH 5.0) according to the literature (11) in order to prevent deacylation. A portion of the RNA sample was incubated at 37°C for 3 h in a buffer containing 50 mM Tris–HCl (pH 9.5) for complete deacylation of tRNA. The same amount of total RNA containing aminoacyl-tRNAs or forcibly deacylated tRNAs was mixed with an acid loading solution containing 0.1 M NaOAc (pH 5.0) and 8 M urea and electrophoresed at 4°C through a 6.5% polyacrylamide gel containing 0.1 M NaOAc (pH 5.0) buffer and 8 M urea in order to separate aminoacyl-tRNAIle and uncharged tRNAIle. Then RNA was blotted onto a nylon membrane, Hybrid N (Amersham Pharmacia Biotech), and fixed by ultraviolet light irradiation. Northern hybridization was performed by using a 32P-5′-end-labeled oligonucleotide probe complementary to tRNAIle as described above. Aminoacyl- and non-acylated-tRNAIle were quantified by exposing the membrane to an imaging plate, followed by analysis with a BAS 1000 bioimaging analyzer (Fuji Photo Film).

Analysis of tRNA life-spans in cybrids

Semiconfluent cultured cybrid cells were trypsinized and divided accurately into equal volumes in fresh dishes. After the cells had adhered to the dishes, the medium was replaced with one containing ethidium bromide (250 ng/ml), which is a potential inhibitor of mitochondrial transcription (12). Culture of the cells with ethidium bromide was continued for the indicated periods, after which total RNA was isolated by Isogen. A sample (5 µg) of total RNA was electrophoresed into denaturing polyacrylamide gel and northern hybridization was carried out using the same probe specific for mitochondrial tRNAIle, as described above, or a 32P-5′-end-labeled probe specific for mitochondrial tRNAlys: 5′-TCACTGTAAAGAGGGTGTTGG-3′. The tRNA amounts were normalized by the amount of nuclear-encoded 5S ribosomal RNA (a probe specific for 5S rRNA, 5′-GGGTTGTATGCGCCGTAGAC-3′ complementary to the 3′-region, was used). RNAs were quantified by exposing the membrane to an imaging plate on which the radioactivities of the bands were measured with a BAS 1000 bioimaging analyzer.

Degradation of tRNAIle in mitochondrial extract

The wild-type and mutant tRNAIle were purified from the respective cybrid cells (CM1-9 and CM114-5) and labeled at the 5′-termini as described above. Mitochondrial enzymatic extract, which was a kind gift from Dr C. Takemoto of RIKEN, was prepared from bovine liver mitochondria as described by Schwartzbach et al. (13). The 32P-5′-labeled tRNAIle was mixed with 1.5 A₂₆₀ of purified carrier Escherichia coli tRNA mixture and incubated at 37°C with the mitochondrial enzyme extract in 50 µl of a reaction mixture containing 50 mM Tris–HCl (pH 7.5) and 50 mM MgCl₂. At appropriate times (5, 10, 15 and 20 min), 8 µl aliquots were taken and immediately subjected to phenol extraction. The extracted RNA fractions were then electrophoresed in a denaturing polyacrylamide gel and the gel was exposed to an imaging plate. Remaining intact tRNAIle was quantified with a BAS 1000 bioimaging analyzer.

Preparation of synthetic tRNAIle

A synthetic DNA polynucleotide corresponding to the phage T7 promoter directly connected to the downstream tRNAIle gene with or without the mutation and terminating at the
discriminator nucleotide of the tRNA was constructed and cloned into pUC 18, followed by transformation into E. coli strain JM109 (Toyobo). Since T7 RNA polymerase has a high preference for the synthesis of transcripts starting with G, we replaced the A1–U72 base pair with G1–C72 of tRNA\textsubscript{Ile} in the template DNA construction. The transcriptional template harboring the T7 promoter and tRNA\textsubscript{Ile} gene was prepared from the cloned plasmid by PCR amplification and \textit{in vitro} transcription was performed according to the literature (14) with slight modification. The resulting solution was subjected to phenol/chloroform extraction, applied onto an anion-exchange tip (QIAGEN Plasmid Kit) and fractionated. The transcript was further purified by denaturing polyacrylamide gel and the sequences of the wild-type and mutant transcript tRNAs were confirmed by Donis-Keller’s method (9) and 5′-end nucleotide analysis.

**Measurement of tRNA\textsubscript{Ile} melting profiles**

Melting profiles from 25 to 95°C were measured automatically at a speed of 0.5°C/min with a Gilford Response II spectrophotometer as described by Watanabe \textit{et al.} (15) using 0.5 A\textsubscript{260} U/mL of transcribed tRNA samples in a buffer consisting of 50 mM sodium cacodylate (pH 7.0), 10 mM MgCl\textsubscript{2} and 200 mM NaCl.

**RESULTS**

**Purification of a mutant tRNA\textsubscript{Ile} and determination of its sequence**

Mitochondrial tRNA\textsubscript{Ile} was purified in amounts sufficient for structural analysis from mass cultures of cybrid cells with or without the pathogenic mutation at nucleotide position 4269. Since human mitochondrial tRNA\textsubscript{Ile} has not been sequenced directly, we first determined the wild-type tRNA\textsubscript{Ile}, including nucleotide modifications (DDBJ/EMBL/GenBank accession no. AB043958), by a combination of the methods of Donis-Keller (9) and Kuchino \textit{et al.} (10). Five post-transcriptional modifications were found: 1-methylguanosine (m\textsubscript{1}G) at position 9 [tRNA position numbering conforms to that used by Sprinzl \textit{et al.} (16)], 2,2-dimethylguanosine (m\textsubscript{2}G) at 26, two pseudouridines (Ψ) at 27 and 28 and N\textsubscript{6}-threoninocarbonyl-adenosine (t\textsubscript{6}A) at 37 (Fig. 1a). As shown in Figure 1b, sequencing ladders obtained by Donis-Keller’s method (9) revealed that the tRNA\textsubscript{Ile} from CM114-5 cybrid cells harboring the 4269 mutant mtDNA had the A→G transition at the bottom of the acceptor stem (position 7), confirming the accurate transcription of the mutant tRNA gene in the mitochondria of the mutant cybrid cells. In addition, the sequence ladder demonstrated that the tRNA preparation with the 4269 mutation [tRNA\textsubscript{Ile}(A4269G)] was essentially homogeneous. Four of the modified nucleotides found in the tRNA\textsubscript{Ile} were resistant against the corresponding RNases (m\textsubscript{1}G and m\textsubscript{2}G against RNase T1 and each Ψ against RNase PhyM). In the alkaline-treated lines, the two pseudouridines produced rather faint bands and t\textsubscript{6}A gave an upward shift of the band (Fig. 1b). The sequence ladder patterns, including the positions of the modified bases, were exactly the same for the wild-type and mutant tRNAs\textsubscript{Ile}, which indicates that all the sequences of the wild-type and mutant tRNAs, including the modified bases apart from the mutation point (position 7), were identical. From this, it can be deduced that no modification defect occurred and that the mutation does not trigger the appearance of any additional modification which would not also be present in the wild-type. This is different from the cases of MELAS mutant tRNA\textsubscript{Leu}(UUR) and MERRF mutant tRNA\textsubscript{Lys}, which were found to be deficient in the modification at the anticodon (6,7).

**Decreased aminoacylation level of tRNA\textsubscript{Ile} with the 4269 mutation**

The aminoacylation properties of \textit{in vitro} tRNA\textsubscript{Ile} transcripts with or without the A4269G point mutation have previously been compared (17). However, it is also important to examine the extent to which the mutant tRNA\textsubscript{Ile} is aminoacylated in cells. To do this, aminoacyl-tRNA and uncharged tRNA in the mutant CM114-5 and in the control CM1-9 cybrid cells were subjected to northern hybridization. The results are shown in Figure 2. The proportion of aminoacyl-tRNA\textsubscript{Ile} relative to the total tRNA\textsubscript{Ile} was significantly, though not markedly, lower in the mutant (64%) compared with that in the control (84%) as quantified by the BAS 1000 biotming analyzer.
Drastically decreased life-span of tRNA\textsubscript{Ile} with the 4269 mutation

To investigate the effect of the A4269G mutation in the tRNA\textsubscript{Ile} molecule on its life-span, we cultured wild-type and mutant cybrid cells (CM1-9 and CM114-5) in a medium containing ethidium bromide to specifically inhibit mitochondrial transcription (12) and monitored the degradation rate of mitochondrial tRNAs in the respective cells by northern hybridization. As a control for normalization, we used nuclear-encoded 5S rRNA. Figure 3a shows that the mutant tRNA\textsubscript{Ile} in the CM114-5 cells degraded dramatically faster than the wild-type counterpart in the CM1-9 cells: the half-lives of the mutant and wild-type tRNAs \textsubscript{Ile} treated with ethidium bromide were estimated to be 3.6 and 33 h, respectively (Fig. 3b). As an internal mitochondrial control, we also observed the stability of tRNALys, a tRNA with a normal sequence in both CM1-9 and CM114-5 cells. Figure 3c and d show that tRNALys was as stable in the mutant cybrid clone as in the wild-type. From these results, it can be presumed that the drastically reduced stability of the mutant tRNA \textsubscript{Ile} was intrinsic and not due to a secondary effect such as an unhealthy environment in mitochondria of the mutant cybrid cells.

The extreme and specific instability of tRNA\textsubscript{Ile} with the 4269 mutation suggested the susceptibility of the mutant to degradation by mitochondrial RNases in vivo, although the candidate nuclease(s) has not been identified. We thus examined its life-span in vitro by incubating the tRNA with mitochondrial enzymatic extract. Since we used homogeneous tRNA\textsubscript{Ile}s purified from the respective cybrid cells and then labeled isotopically, we were able to precisely adjust the amounts of the wild-type and mutant tRNAs so that they were the same, based on their radioactivity. We measured the amounts of the residual intact tRNA\textsubscript{Ile} with or without the mutation at various times (Fig. 4a) and again found that the mutant tRNA degraded faster than the wild-type (Fig. 4b). However, the instability of the mutant was less prominent than in the experiment using cybrid cells, which could be due to environmental differences between the in vitro assay system and cell mitochondria. From these results, it appears that the greatly decreased life-span of the tRNA with
the A4269G mutation observed in vivo might ensue from a susceptibility to mitochondrial nucleolytic attack.

**Comparison of wild-type and mutant tRNA melting profiles**

When nucleic acids are denatured by heating or denaturing reagents, their ultraviolet absorption increases, an effect known as hyperchromicity. Thus, monitoring the dependence of the hyperchromicity on temperature is an effective method of directly determining the stability of tRNAs. Since we had already established that the point mutation did not cause any deficiency in the nucleotide modification, instead of isolating tRNAs from cells, we considered it reasonable to use samples prepared by in vitro transcription with or without the mutation (although the possibility that the same modified nucleotide might influence the melting profiles of the wild-type and mutant tRNA^Ile in a different way cannot be completely excluded). As shown in Figure 5, the melting profiles of the two tRNAs differed substantially; the melting temperature of the mutant was 55°C, while that of the wild-type was 57.5°C. Judging from the melting profile, the mutant began to melt at around 37°C, indicating that it was partially denatured even at the physiological temperature. This could explain why the mutant tRNA is apparently easily attacked by mitochondrial nucleases.

**DISCUSSION**

Although in the past decade a variety of point mutations in mitochondrial tRNA genes have been reported to have pathogenic roles underlying a range of mitochondrial diseases, the intrinsic properties of the mutant tRNA molecules themselves remain poorly understood, probably because of technical problems involved in the purification of mutant tRNA. We have been able to overcome the difficulty by mass-culturing cybrid cells with a homoplasmic mutation and using a solid-phase hybridization method (8) to purify small amounts of mitochondrial tRNAs. As part of a systematic analysis of the pathogenic mutant tRNAs that we are undertaking, we here characterized the tRNA^Ile molecule with the 4269 mutation derived from a patient with cardiomyopathy. Our findings showed that (i) tRNA^Ile has no modified nucleotide in its anticodon, although both tRNA^{Leu(UUR)} and tRNA^{Lys} do and (ii) post-transcriptional modification was normal in the mutant tRNA^Ile.

We recently found that mutant tRNA^{Leu(UUR)}s with a point mutation at 3243 or 3271 (associated with MELAS) and tRNA^{Lys} with a point mutation at 8344 (associated with MERRF) are deficient in a post-transcriptional modification at the anticodon wobble position (6,7). In the case of the MELAS 3243 and 3271 mutations, there have been several reports implying the production of qualitatively abnormal proteins in mitochondria when the translation rates are maintained at near the normal level or decrease only slightly (18–20). In the case of the MERRF 8344 mutation, mutant cybrid cells have been shown to produce abortive polypeptides and to exhibit reduced mitochondrial protein synthesis (21). Since the wobble modification is presumably essential for the two kinds of tRNA to decode their cognate codons, the common modification defect is considered to be responsible, particularly through the translational process, for the pathogeneses of both MELAS and MERRF. However, from the findings in the present study that none of the tRNA^{Leu} anticodon bases was modified and the mutant had the normal post-transcriptional modifications, it can be inferred that a post-transcriptional modification deficiency is not a universal phenomenon in mitochondrial diseases.

Instead of a modification deficiency, the life-span of the mutant tRNA^Ile was found to be markedly decreased in vivo as well as in vitro and as a result it had a steady-state level ~50% lower than the wild-type tRNA (5). In addition, aminoacylation in the cybrid cells was significantly, though not markedly, reduced. The aminoacylation ratios, 64% for the mutant and 84% for the wild-type, do not contradict previously reported findings using partially purified mitochondrial isoleucyl-tRNA synthetase that neither the charging level nor the kinetic parameters of isoleucylation of the A4269G mutant tRNA^Ile decreased significantly in comparison with a wild-type transcript (17). Also, in the case of E.coli tRNA^Ile, the identity elements do not include the residue corresponding to the mutation point (22). Taken together with the findings of these previous studies, the reduced aminoacylation in the mutant tRNA^Ile observed in vivo in the present work does not arise directly from the mutation point but is probably associated with the marked instability of the mutant tRNA in cells.

Mitochondrial protein synthesis has been shown to be almost completely abolished in cybrid cells with a homoplasmic 4269 point mutation in the tRNA^Ile gene (5). Similarly, mitochondrial translation was abolished when a deletion mutant mtDNA exceeded 60% of total mtDNA (23). Therefore, when normal mitochondrial tRNAs make up <40% of the total, mitochondrial translation may not function due to a lack of aminoacyl-tRNA. In the case of the mutant tRNA^Ile examined in this study, a decrease in the amount of isoleucyl-tRNA may be essential to affect the translation, since the amount of isoleucyl-tRNA in the mutant was only ~30% of that in the wild-type [this percentage is deduced from (i) the fact that the steady-state level of the mutant tRNA^Ile has been shown to be <50% of that of the wild-type (5) and (ii) aminoacylation ratios of 64% for the mutant and 84% for the wild-type as observed in this study)].

We confirmed the instability of the mutant tRNA^Ile by the more direct method of comparing the melting profiles. Interestingly, the mutant began to be partially denatured even at around 37°C and its melting temperature was about 2.5°C.
lower than that of the wild-type. This could explain why the mutant tRNA was apparently easily digested, presumably by nucleolytic attack, leading to a reduction in its steady-state level. In the melting experiment, we used in vitro transcripts instead of a tRNA preparation purified from cells. The intrinsic sensitivity of the transcripts to the mitochondrial extract showed that the mutant transcript was also more susceptible to the extract than the wild-type, which is in accordance with the results for the native tRNAs shown in Figure 4. The wild-type and mutant transcripts were somewhat more sensitive than their respective native counterparts, suggesting the protective contribution of post-transcriptional modification (data not shown). Hence, the transcripts are considered suitable for demonstrating the intrinsic instability of the mutant tRNA resulting from the single point mutation using the melting profile method. We therefore conclude that the mutation at only a single point has a dramatic effect in making the mitochondrial tRNA molecule intrinsically unstable and apparently pathogenic.

ACKNOWLEDGEMENTS

We would like to express our thanks to Dr C. Takemoto of RIKEN and Prof. J.-I. Hayashi of Tsukuba University for providing the mitochondrial extract and for supporting the cybrid cell lines, respectively, and to Ms K. Nishimaki of the Institute of Gerontology, Nippon Medical School for her technical assistance. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture (Japan).

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