Mutations in human mitochondrial isoleucine tRNA (hs mt tRNA\textsubscript{Ile}) are associated with cardiomyopathy and ophthalmoplegia. A recent study showed that ophthalmoplegia-related mutations gave rise to severe decreases in aminoacylation efficiencies and that the defective mutant tRNAs were effective inhibitors of aminoacylation of the wild-type substrate. The results suggested that the effectiveness of the mutations was due in large part to an inherently fragile mitochondrial tRNA structure. Here, we investigate mutant tRNAs associated with cardiomyopathy, and a series of rationally designed second-site substitutions introduced into both ophthalmoplegia- and cardiomyopathy-related mutant tRNAs. A source of structural fragility was uncovered. An inherently unstable T-stem appears susceptible to misalignments. This susceptibility sensitizes both domains of the L-shaped tRNA structure to base substitutions that are deleterious. Thus, the fragile T-stem makes the structure of this human mitochondrial tRNA particularly vulnerable to local and distant mutations.

The canonical tRNA cloverleaf folds into a two-domain L-shaped structure. One domain contains the amino acid attachment site and the 12-base pair acceptor-T\psiC mini helix. The other domain of 10 base pairs contains the dihydrouridine and anticodon-stem and -loop. The primary structures of hs mt tRNAs\textsuperscript{1} differ significantly from cytoplasmic tRNAs (1). Despite their analogous function in the decoding of genetic information, mt tRNAs are generally shorter than cytoplasmic tRNAs and feature higher numbers of AU pairs. In some cases, entire structural elements are deformed or even missing from the canonical cloverleaf, although a truncated L-shaped structure can still be made. The minimized structures of hs mt tRNAs appear to be susceptible to point mutations, as errors in the corresponding mt genes are associated with disease. Over 70 pathology-related mutations in mt tRNA genes are known (2). The impact of the base substitutions on the structure and function of hs mt tRNAs is difficult to predict \textit{a priori}, given the limited information available concerning the properties of these molecules.

Within the gene for hs mt tRNA\textsubscript{Ile}, eight different point mutations are correlated with pathologies (3–10). The genetic errors identified are generally associated with two diseases, cardiomyopathy (3–7) and ophthalmoplegia (8–10). The mutations can be classified according to the types of structural defects induced. The ophthalmoplegia mutations are associated with one specific type of structural defect, CA mispairs, whereas the cardiomyopathy mutations are more varied.

The ophthalmoplegia-related mutants of tRNA\textsubscript{Ile} are poor substrates for aminoacylation (11). Studies of the reactivity of these molecules with the hs mt isoleucyl-tRNA synthetase (Il\textsubscript{RS}) revealed kinetic defects that impeded the aminoacylation reaction. Because the binding affinities of the mutated tRNAs for the enzyme were not affected, the mutants were effective inhibitors of the charging of wild-type tRNA. The aminoacylation of these mutant tRNAs was restored with compensatory mutations that reintroduced base pairing. These results prompted the proposal that the structure of the hs mt tRNA\textsubscript{Ile} was inherently fragile and, thus, small perturbations introduced by the pathogenic mutations were magnified into large losses in function and the creation of effective inhibitors.

Here, using aminoacylation to probe structure-function properties of hs mt tRNA\textsubscript{Ile}, the effects of a subset of cardiomyopathy-associated mutations are investigated. An A59G mutation in the T\psiC-loop (hereafter called the T-loop) significantly decreased the aminoacylation efficiency of this molecule. In contrast, a C62U mutation (that introduced an AU pair in place of a CA pair within the T-stem), increased the aminoacylation efficiency. Because the C62U mutation both stabilized the T-stem and improved aminoacylation, we considered the possibility that the T-stem had an inherent fragility that affected the entire molecule. With this in mind, we set out to study systematically the effects of specific, rationally designed manipulations of the T-stem to elucidate structural properties that cause the hs mt tRNA\textsubscript{Ile} to be susceptible to pathogenic mutations. These investigations were aimed at not only exploring the local consequences of mutations in the T-stem and loop, but also at seeing whether these mutations affected distant parts of the tRNA.

**MATERIALS AND METHODS**

**Preparation of tRNA Transcripts—**Plasmids encoding tRNA trans- scripts were generated by ligating overlapping oligonucleotides into a pUC18 plasmid (12) between a BamHI and PsI cleavage site. The tRNA constructs were placed between a T7 RNA polymerase promoter sequence and a BstN1 cleavage site and adjoined at the 3' end with a hammerhead ribozyme derivative to increase the transcription yields obtained with this sequence (13). T7 RNA polymerase was purified from *Escherichia coli* as described (14). Plasmids encoding the tRNA constructs were linearized with BstN1 and incubated with T7 RNA polymerase for 2 h at 37 °C in the presence of 40 mM Tris, pH 8, 10 mM MgCl\textsubscript{2}, 5 mM dithiothreitol, 0.01%
Expression and Purification of Human Mitochondrial IleRS—The gene for human mitochondrial IleRS was cloned as described (15). The cloned gene was inserted into a yeast expression vector containing a 3′-untranslated region from the Saccharomyces cerevisiae actin gene (Invitrogen, Carlsbad, CA). After cell lysis performed in a French press, human mitochondrial IleRS-GST was batch purified by isolation on glutathione-agarose (Amersham Pharmacia Biotech) and subsequent elution with glutathione. The purification protocol employed produced highly active tRNA transcripts. Aminoacylation assays performed with high concentrations of tRNA (≥1 μM) were used to assess the activity of tRNAs that could be charged with isoleucine, and for both wild-type and mutant tRNAs, between 80 and 100% of the tRNA was quantitated by uv-visible absorbance as described below could be aminoacylated. These experiments indicated that the 3′-end of this tRNA was transcribed with high fidelity.

Aminoacylation Efficiencies of a Cardiomyopathy-related Mutant tRNA—Mutants of hs mt tRNAIle associated with cardiomyopathy (Fig. 1) that involve substitutions in the T-stem and loop were generated by in vitro transcription and tested as substrates for the cognate recombinant hs mt IleRS. (Previous studies showed that transcripts of hs mt tRNAIle were relatively robust substrates for the recombinant enzyme, Ref. 11.) A tRNA containing an A59G mutation that alters an unpaired base in the T-loop had a significantly decreased rate of aminoacylation (Fig. 2). A similar decrease in aminoacylation for this A59G mutant was observed in a previous study employing a C62U mutation (Fig. 3). This protocol yielded protein samples of activity comparable to that of the fusion protein.

Aminoacylation Assays—Aminoacylation assays were performed as described (18). Assays contained 2 μM tRNA and 25 nM enzyme, and [3H]isoleucine, ATP, and MgCl2. Samples were extracted with 25:24:1 phenol, pH 4.5, and precipitated with an equal volume of ethanol. After ethanol precipitation, tRNAs were desalted using a G-50 spin column (Amersham Pharmacia Biotech). There were no detectable differences in the efficiency of transcription or transcript length when mutations were introduced into hs mt tRNAIle.

RESULTS

Aminoacylation Efficiency of a Cardiomyopathy-related Mutant tRNA—Mutants of hs mt tRNAIle were introduced into the T-stem and loop (Fig. 1) that involve substitutions in the T-stem and loop. In vitro transcription and testing as substrates for the cognate recombinant hs mt IleRS. (Previous studies showed that transcripts of hs mt tRNAIle were relatively robust substrates for the recombinant enzyme, Ref. 11.) A tRNA containing an A59G mutation that alters an unpaired base in the T-loop had a significantly decreased rate of aminoacylation (Fig. 2). A similar decrease in aminoacylation for this A59G mutant was observed in a previous study employing a C62U mutation (Fig. 3). The introduction of an additional Watson-Crick base pair in the T-stem stabilized the cloverleaf structure and enhanced aminoacylation by locking out inactive conformations (Fig. 3). The enhanced charging seen with the C62U substitution suggests that, in this case, the associated cardiomyopathy is not because of a defect in aminoacylation.

Aminoacylation Assays—Aminoacylation assays were performed as described (18). Assays contained 2 μM tRNA and 25 nM IleRS. Data shown correspond to wild-type (•), A59G (△), or C62U (△) tRNAs. Assays contained 2 μM tRNA and 25 nM IleRS. The efficiency of transcription or transcript length when mutations were introduced into hs mt tRNAIle.

Aminoacylation Assays—Aminoacylation assays were performed as described (18). Assays contained 2 μM tRNA and 25 nM enzyme, and [3H]isoleucine, ATP, and MgCl2. Samples were extracted with 25:24:1 phenol, pH 4.5, and precipitated with an equal volume of ethanol. After ethanol precipitation, tRNAs were desalted using a G-50 spin column (Amersham Pharmacia Biotech). There were no detectable differences in the efficiency of transcription or transcript length when mutations were introduced into hs mt tRNAIle.
ture of tRNA\textsubscript{Ile} not being a major contact point for the synthetase in the bacterial system (22, 23).

Secondary structure calculations revealed that neither the U62:A52 nor the G62:C52 pairs altered the alignment of bases in the T-stem. The creation of a C62:G52 base pair is predicted to change the pairing such that two unpaired bases are introduced between the acceptor- and T-stems, and the size of the T-loop is reduced by one nucleotide (Fig. 3). Consistent with these predictions, the A52G mutant tRNA\textsubscript{Ile} (with a C62:G52 pair) was charged at low levels compared with the substrate containing this same base pair in the opposite orientation (Fig. 4).

Rescue of Pathology-related tRNA Mutants with a Stabilized T-stem—Having established the sensitivity to mutation and the fragility of the T-stem (particularly the capacity to form alternative structures), we wondered whether a stabilized stem could compensate for the deleterious effects of other pathology-related tRNA mutations. To address whether the impact of the A59G mutation on aminoacylation was related to the lability of the T-stem, this pathogenic mutation was incorporated into a tRNA\textsubscript{Ile} variant containing a C62U mutation. The substitution of C62 stabilizes the T-stem (by creating a U62:A52 pair) and, as described above, increases aminoacylation relative to the wild-type hs mt tRNA\textsubscript{Ile}.

Indeed, the C62U mutant was more resistant to the negative effect of the A59G mutation on charging (Fig. 5). Instead of the 80% decrease observed with the wild-type CA-containing T-stem, only a 10% decrease in charging was observed with an U62:A52 T-stem. The loss of charging caused by the A59G mutation in the wild-type construct therefore appears to reflect a greater tendency for the molecule to misfold. This tendency can be counteracted by strengthening the T-stem.

Consistent with the notion of misfolding contributing to the effects of the A59G mutation, the secondary structure calculated for this mutant tRNA\textsubscript{Ile} differs from that of the wild-type structure. Aminoacylation was monitored at pH 7.5, 37 °C in reactions containing 2 μM tRNA and 25 nM IleRS.

**FIG. 3.** Schematic of alternative secondary structures caused by the introduction of mutations into hs mt tRNA\textsubscript{Ile} as predicted by MFOLD. The introduction of the pathogenic A59G mutation (right) and the rationally designed A52G mutation (far left) produces structures with misaligned T-stems. A tRNA containing a C62U mutation (middle) retains the wild-type structure. For a construct containing the A59G mutation, the introduction of the C62U mutation restores the wild-type structure (far right). Mutated bases are italicized.
suggested as the source of attenuated reactivities displayed by these mutants (11). By reexamining these mutations in constructs where the T-stem is stabilized, the contribution of distal structural elements to the deleterious impact of the ophthalmoplegia mutations could be evaluated.

Aminoacylation efficiencies were compared for wild-type and mutant tRNAs by calculating the ratios of initial rates for tRNAs containing a C62:A52 (wild-type) versus an U62:A52 base pair. In all cases, the decreases in aminoacylation efficiency seen with the U12C, U27C, G40A, and A59G mutant tRNAs are less severe with a secondary C62U mutation. As previously discussed, even the charging of wild-type tRNA is improved because the substitution strengthens the proper alignment of the T-stem by replacing an A-C pair with a U-A (Fig. 3). That the aminoacylation efficiency with this mutant is actually increased through the stabilization of a canonical tRNA structure emphasizes that the nature of the connection between a pathology and a specific mutation may be different for each mutation. The higher activity of the more stable C62U mutant tRNA<sub>106</sub> raises the possibility that the inherent fragility of the wild-type hs mt tRNA structure may be important for function. For example, among other possibilities, a flexible, relatively loose structure may be required for optimal activity with the mt translation apparatus (23).

The inherent weakness of the structure hs mt tRNA<sub>106</sub> gives rise to a significant degree of domain-domain communication. Because of the capacity for “slippage” in the secondary structure caused by base pair misalignments (Fig. 3), the stability of one domain is reliant on the integrity the other. (Whereas interdomain communication is prevalent in this tRNA possessing a minimized structure, it has also been detected in other tRNAs, Ref. 26.) In particular, the slipped secondary structures can disrupt loose tertiary interactions and thereby make one domain particularly sensitive to perturbations in the other.

Although the cause-and-effect basis for the ophthalmoplegias and cardiomyopathies associated with mutations in hs mt tRNAs has not been conclusively established, these pathologies have nonetheless provided the motivation and rationale for investigating in more depth the structural features of hs mt tRNAs. In particular, were it not for the cardiomyopathy-associated mutations studied here, the fragility of the T-stem of hs mt tRNA<sub>106</sub> might not have been appreciated. This fragility is manifested by the ease with which single point mutations can lead to serious realignments of the T-stem loop (Fig. 3) that, in turn, have consequences that are global (Fig. 6). It is these global effects that may be most significant for pathology because, for example, they may influence not only aminoacylation, but also the ability of the tRNA to function in subsequent steps of protein synthesis or to be a substrate for processing or modification enzymes.

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Fragile T-stem in Disease-associated Human Mitochondrial tRNA Sensitizes Structure to Local and Distant Mutations
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