Award Accounts

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Role of Modified Nucleosides in the Translation Function of tRNAs from Extreme Thermophilic Bacteria and Animal Mitochondria

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This report characterizes the structure and function of four modified nucleosides first identified by myself or members of my research group in thermophilic bacteria and animal mitochondria over the past 30 years. I identified 2-thioribothymidine (s^2T) (or 5-methyl-2-thiouridine (m^5s^2U)) in 1974 at position 54 in the T loop of tRNAs from an extreme thermophile, *Thermus thermophilus*, as well as related thermophiles, and a good deal of evidence has shown that it is required for thermostabilization of tRNAs, functioning at high temperatures. The functional roles and the biosynthetic pathway of s^2T are outlined here. My research group has identified three novel modified nucleosides, 5-formylcytidine (f^5C), *N*-(uridine-5-ylmethyl)taurine (τm^5U), and *N*-(2-thiouridine-5-ylmethyl)taurine (τm^5s^2U), at the first anticodon position of mitochondrial (mt) tRNAs from higher animals (f^5C is present in $tRNA^{Met}$, τm^5U in $tRNA^{Leu}$(UUR) and $tRNA^{Trp}$, and τm^5s^2U in $tRNA^{Gln}$, $tRNA^{Lys}$, and $tRNA^{Glu}$). Their chemical structures were determined and their functional roles in translation were examined. Additionally, f^5C at the first anticodon position of mt $tRNA^{Met}$ plays a crucial role in decoding the AUA codon (decoded as isoleucine in the universal genetic code system) as methionine, which will form the second topic of this article. Defective modification of τm^5U and τm^5s^2U at the first anticodon position in human mt $tRNA^{Leu}$(UUR) and $tRNA^{Lys}$, respectively, is the result of point mutations in these tRNAs in mitochondria. It is strongly suggested that these defects are the direct cause of two mitochondrial diseases, MELAS (mitochondrial encephalomyopathies, encephalopathy, lactic acidosis, and stroke-like episodes) and MERRF (myoclonus epilepsy associated with ragged-red fibers). Their molecular mechanisms are also discussed here. These studies would serve to highlight again the importance of modified tRNA nucleosides in the structure and function of tRNAs.

Introduction

More than 100 species of modified nucleosides in tRNAs from various sources have been reported, but their roles in tRNA function are still largely unidentified.[1–3] I have been interested in the functional roles of tRNA modified nucleosides since 1972, when I received my Ph.D. from the University of Tokyo and started research focusing on tRNAs from an extreme thermophilic bacterium, *Thermus thermophilus*, which was isolated from a Japanese thermal spa, Mine-Onsen, in 1971 by Tairo Oshima, an assistant Professor in the University of Tokyo at that time.[4] Ten years later, I also began to study tRNAs from animal mitochondria, which possess some intriguing structural as well as functional characteristics including coding problems, and have been deeply involved in this field since that time.

Since tRNAs from *Thermus thermophilus* have acquired thermostability[4,5] and several kinds of genetic code changes occur in animal mitochondria,[6,7] I hypothesized that such phenomena might have been caused by modified nucleosides embedded in the tRNAs. I first succeeded in identifying 2-thioribothymidine (s^2T) (or 5-methyl-2-thiouridine (m^5s^2U)) at position 54 in the T loop in thermophile tRNAs.[8] The s^2T (or m^5s^2U) nucleoside had previously been found in rat tRNA at the first anticodon position (position 34),[9] but this was the first time that s^2T was found at position 54 in the T loop in thermophile tRNAs, where it replaces the usual ribothymidine (rT) and suggested that s^2T played a crucial role in thermostabilization of tRNA.[10] However, the mechanism by which 2-thiolation of rT54 is achieved, and whether 2-thiolation is dependent on the growth temperature of the bacterium, had not been clarified. Here, I describe the initial characterization of the biosynthetic pathway of s^2T and the mechanism of tRNA recognition by the presumed s^2T-modifying enzyme.

In the process of pursuing thermo-unstable tRNAs in different organisms, as an antithesis of thermostable tRNAs, I encountered an exciting finding by F. Sanger's group in 1981. These researchers found that animal mitochondrial genomes included an unusual-shaped tRNA gene consisting of 2 leaves of the tRNA cloverleaf structure instead of the usual 3,[7] and

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that some of the animal mitochondria genetic code deviated from the universal genetic code.[6,7] On the basis of this information, I and my research group at the University of Tokyo began to develop methods to purify the tiny amounts of mt tRNAs in cells in order to clarify their structure and function and creating a method to detect modified nucleosides in the tRNAs.

Almost 10 years later, Tsutomu Suzuki and the students in my group finally created a new purification device referred to as chaplet-column chromatography.[11] The device is based on the idea of H. Tsurui et al.[12] which we developed further,[13,14] and coupled to a new LC/MS detection system,[11] to provide adequate detection. Thus, rapid and effective purification of mt tRNAs from whole animal cells (bovine liver) and the analysis of the very small amounts of modified nucleosides contained therein (≈10 pg) were made possible for the first time.[11,13]

We were able to find three novel modified nucleosides at the first anticodon position in individual mt tRNA species. We determined that one, 5-formylcytidine (f^5C), which contains a formyl group and is located at the first anticodon position in $tRNA^{Met}$,[15,16] could recognize A in addition to G at the third codon position. Thus, an AUA codon can be read as methionine (Met) instead of the usual isoleucine (Ile)[17] (C. Takemoto et al., unpublished data). The other two modified nucleosides, *N*-(uridine-5-ylmethyl)taurine (τm^5U) at the first anticodon position in $tRNA^{Leu(UUR)}$ and $tRNA^{Trp}$, and *N*-(2-thiouridine-5-ylmethyl)taurine (τm^5s^2U) at the same position in $tRNA^{Glu}$, $tRNA^{Gln}$, and $tRNA^{Lys}$,[18,19] were found to be indispensable for correct decoding of the respective codons.[20–24] Additionally, we found that these modified nucleosides were lacking in cells from animals with some mitochondrial diseases. This lack results in loss of certain mitochondrial proteins containing the relevant codons, and is presumed to be the cause of mitochondrial diseases such as MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) and MERRF (myoclonus epilepsy associated with ragged-red fibers).[24]

Following the identification of the modified nucleosides contained in tRNAs of a thermophilic bacterium and animal mitochondria (Fig. 1), their functions in tRNAs have been elucidated and some of them are directly connected to some human diseases, as described above. The results reported in this article are summarized in Fig. 2.

Thus, the study of modified nucleosides in tRNAs is intriguing not only from the basic science perspective, but also from the applied perspective, including prevention and cure of some diseases, as well as artificial protein production. Since modified nucleosides have recently been found in ribosomal RNAs, mRNAs, and small RNAs such as snRNAs, snoRNAs, and miRNAs,[25–27] functional study of these newly identified modified nucleosides will also be very important for understanding RNA function within the cell, as well as for their application to various RNA technologies.

1. A Modified Nucleoside, 2-Thioribothymidine (s^2T) Located in the TΨC Loop of tRNA from an Extreme Thermophilic Bacterium, *Thermus thermophilus*

1.1 Current Results Concerning the Thermo-Stabilization Function of s^2T.

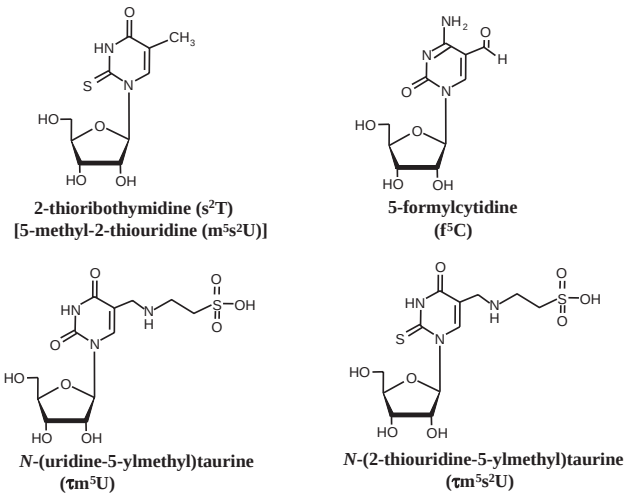

Fig. 1. Chemical structures of 4 modified nucleosides. 2-Thioribothymidine (s^2T) (or 5-methyl-2-thiouridine (m^5s^2U)) is found in tRNAs of *Thermus thermophilus* (upper left). 5-Formylcytidine (f^5C) (upper right), *N*-(uridine-5-ylmethyl)taurine (τm^5U) (lower left), and *N*-(2-thiouridine-5-ylmethyl)taurine (τm^5s^2U) (lower right) are found in tRNAs from human and bovine mitochondria.

The melting temperatures of tRNAs from the extreme thermophile, *Thermus thermophilus* sp. are 5–10 °C higher than those of the corresponding tRNA species from the mesophilic bacterium *Escherichia coli* (Table 1),[10] and this difference cannot be explained solely by the higher G–C pair content in the thermophile tRNAs.[28] Analysis of modified nucleosides in tRNAs from *T. thermophilus* revealed a thermophile-specific sulfur-containing modified nucleoside s^2T (see Fig. 1),[8,28] a 2-thiolated derivative of ribothymidine (T), located at position 54 in the T loop of almost all tRNAs.[29] Because s^2T54 is also present in tRNAs from hyperthermophilic Archaea such as *Pyrococcus furiosus*, which contains about 0.77 mol % of s^2T when cultured at 100 °C,[30] 2-thiolation of T54 is postulated to be a common modification responsible for the thermostabilization of tRNAs in both thermophilic eubacteria and Archaea.

The 2-thiolation of T54 increases concomitantly with elevation of the cultivation temperature without any changes in other modified nucleosides, such as 1-methyladenosine at position 58 (m^1A58) or 2′-*O*-methylguanosine at position 18 (Gm18). Almost 60% of the tRNAs in *T. thermophilus* HB8 cells grown at higher than 80 °C contained s^2T54 instead of T54, whereas, at 50 °C, less than 30% of the tRNAs had s^2T, while the sum of s^2T54 and T54 was approximately 1 mole.[10] In addition, the tRNA melting temperature increased in direct proportion to the increase in s^2T content, as shown in Fig. 3.[10] These findings indicate that 2-thiolation of T54 is responsible for the thermostability of *T. thermophilus* tRNA under diverse cultivation temperatures, thereby ensuring the thermal adaptation of protein synthesis.

The temperature of the inflection point in the specific CD signal[31] and in the characteristic chemical shift in the NMR spectra of s^2T in *T. thermophilus* $tRNA$[32] was correlated to the melting temperature monitored by UV absorbance, suggesting a close correlation between the local conformation of

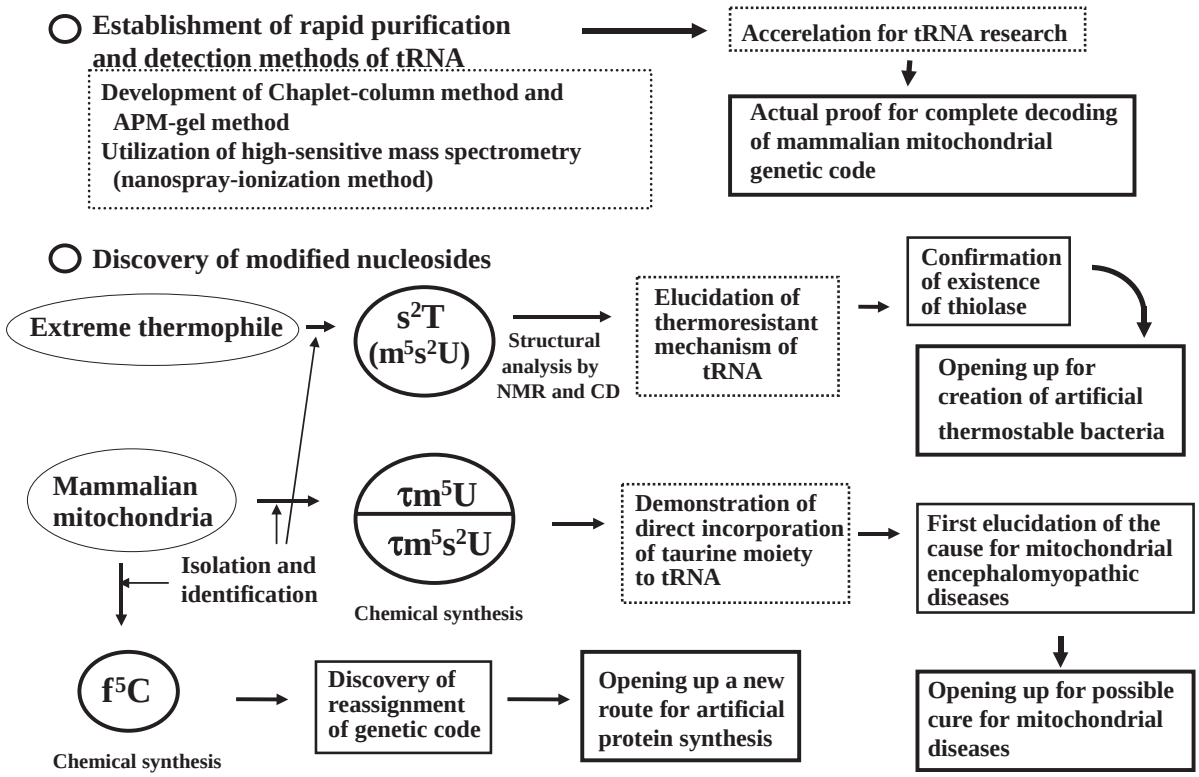

Fig. 2. Summary of research results.

Table 1. Comparison of Melting Temperatures (T_m) of tRNAs Isolated from *Thermus thermophilus* and *Escherichia coli*[35]

	T_m/°C (in the presence of 10 mM Mg^{2+})		ΔT_m
tRNA	*T. thermophilus*	*E. coli*	
$tRNA^{Met}_{f1}$	88.0	83.0	5.0
$tRNA^{Met}_{f2}$	89.0		6.0
$tRNA^{Met}_{m}$	86.7	77.0	9.7
$tRNA^{Phe}$	84.0	76.0	8.0
$tRNA^{Tyr}$	85.5	78.0	7.5
$tRNA^{Ile}$	85.8	78.3	7.5
Unfractionated tRNA	85.5	75.0	10.5

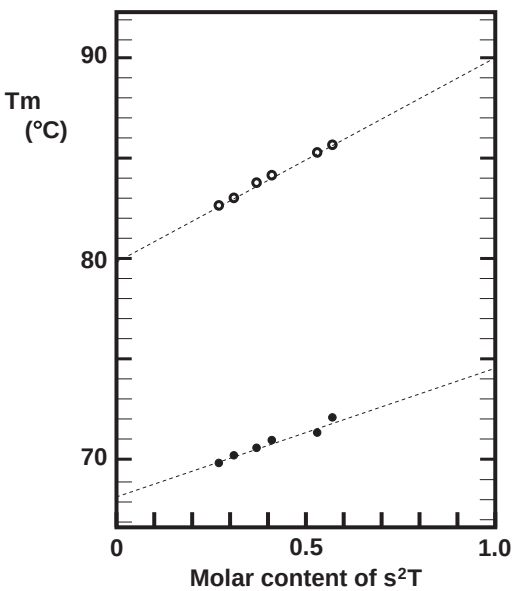

Fig. 3. The relationship between melting temperature (T_m) of the thermophile tRNA and mole fraction of s^2T,[10] in the presence (open circle) and absence (closed circle) of Mg^{2+}.

s^2T54 and the structural stability of tRNA. The mechanism of the structural stabilization of tRNA conferred by s^2T has been elucidated by proton NMR analysis:[33] the ribose puckering of s^2T preferentially conforms to the C3′-*endo-gg-anti* conformation, as do all residues in the A-form RNA, because of the steric effects of the interactions between the bulky 2-thiocarbonyl group and the 2′-hydroxy group. This inherent rigidity of s^2T54 gives structural stability to the elbow region formed by the D loop–T loop interaction, resulting in an increased thermostability of tRNA tertiary structure.[34]

1.2 The Importance of s^2T in tRNA Function. The importance of s^2T in the tRNA function has been demonstrated by experiments in vitro as well as in vivo.

In in vitro experiments, aminoacylation reactions, binding of aminoacyl-tRNA to EF-Tu, and poly(U)-dependent poly(phenylalanine) synthesis were examined by comparing the activities of $tRNA^{Phe}$ containing s^2T54 ($tRNA^{Phe}(s^2T54)$) and $tRNA^{Phe}$ containing T54 ($tRNA^{Phe}(T54)$). In the aminoacyla-

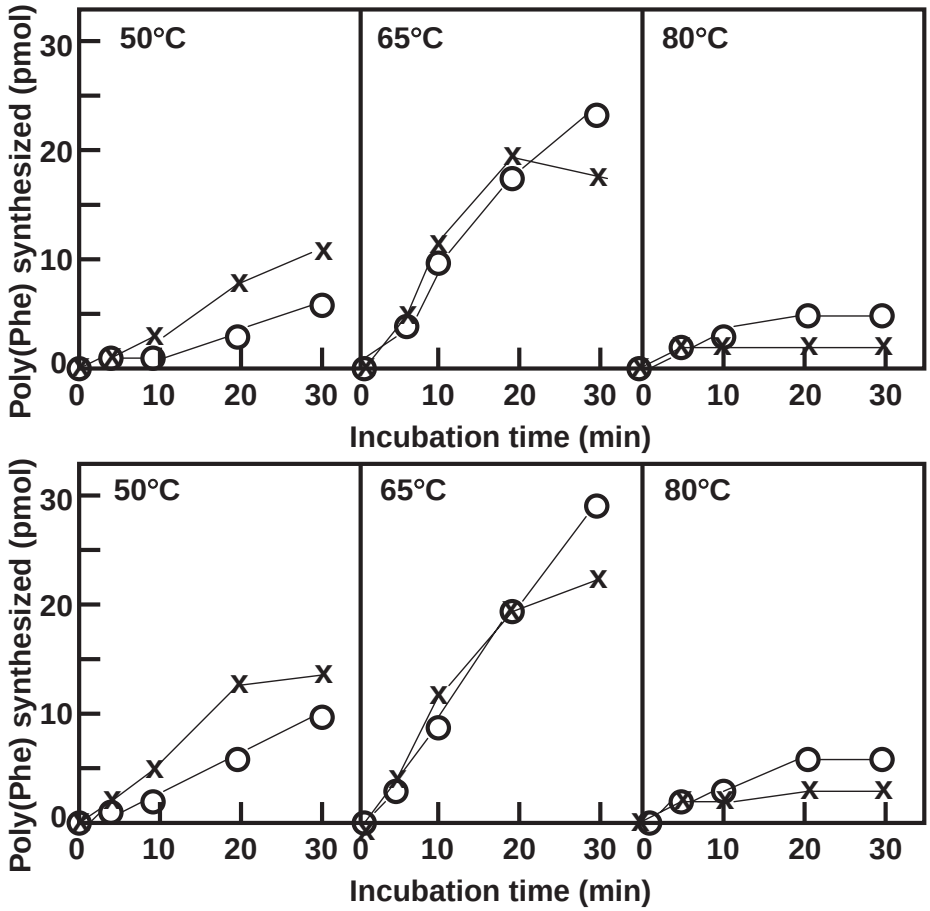

Fig. 4. Temperature dependence of poly(U)-dependent poly(Phe) synthesis using *T. thermophilus* $tRNA^{Phe}$ (○) and *E. coli* $tRNA^{Phe}$ (×).[35] Upper panel: Reactions using ribosomes and S100 isolated from cells grown at 50 °C. Lower panel: Reactions using ribosomes and S100 isolated from cells grown at 80 °C.

tion reaction, no difference in the initial velocity of aminoacylation was detected at temperatures up to 75 °C, but at 80 °C, the aminoacylation velocity of $tRNA^{Phe}$(T54) decreased more rapidly than that of $tRNA^{Phe}$(s^2T54). No differences in the activity of enzymes obtained from cells cultured at 50 °C and at 80 °C were observed.[35]

As shown in Fig. 4, in the overall protein synthesis reaction, the poly(U)-dependent poly(phenylalanine) synthesis activity of $tRNA^{Phe}$(T54) was about 2 times higher than that of $tRNA^{Phe}$(s^2T54) at 50 °C, the activities of the tRNAs were approximately equal at 65 °C, and the activity of $tRNA^{Phe}$(s^2T54) was twice that of $tRNA^{Phe}$(T54) at 80 °C. No differences in the activities of enzymes and ribosomes obtained from cells cultured at 50 °C and at 80 °C were observed. These results show that at lower temperatures $tRNA^{Phe}$(s^2T54) is less effective than $tRNA^{Phe}$(T54), but the effectiveness is reversed at higher temperatures.

In the in vivo experiments, in order to quantify T- and s^2T-containing tRNAs in ribosomes involved in protein synthesis, active polysomes were isolated by sucrose density gradient centrifugation from the thermophile cells cultured at 3 different temperatures: 55, 65, and 77 °C. tRNAs were separated from the polysomes and the T- and s^2T-contents of the tRNAs were determined by HPLC.[36] The relative content of s^2T-containing tRNA in the polysomes from cells cultured at 77 °C was much higher than that in bulk tRNAs from whole cells cultured at the same temperature (75% versus 50%), but the situation was reversed in cells cultured at 55 °C (23% versus 32%). At 60 °C the bulk vs. polysome associated contents were approximately equal (36%), as shown in Fig. 5. These results clearly show that the protein synthesizing systems of the thermophile have some selection mechanism to utilize either T- or s^2T-containing tRNAs preferentially depending on the environmental temperature.

Thus, it is evident that s^2T54 in the thermophile tRNA is a key modification for tRNA stabilization and function at elevated temperatures.

1.3 Simple Identification of s^2T and the Biosynthesis of s^2T in tRNAs of *Thermus thermophilus*. Sixteen years ago, G. Igloi, my long-time colleague in Germany, reported an affinity electrophoresis system in which a polyacrylamide gel was co-polymerized with (acryloylamino)phenylmercury(II) chloride (APM).[37] In this system, the electrophoretic mobility of thiolated tRNAs was retarded compared with that of non-thiolated tRNAs due to the specific interaction between the thiocarbonyl group and APM in the gel. This system successfully detected tRNAs with the modified nucleosides 4-thiouridine (s^4U) and 5-methylaminomethyl-2-thiouridine (mnm^5s^2U).

We therefore used this approach to detect s^2T at position 54

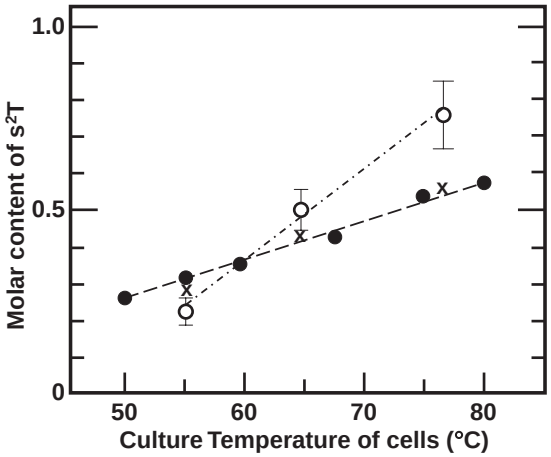

Fig. 5. Correlation between s^2T content of tRNAs and culture temperature of cells from which the tRNAs were isolated.[36] tRNAs were isolated from intact cells (closed circle), spheroplasts (cross), and polysomes (open circle). The vertical lines on the open circles show the experimental error range from multiple analyses.

in a specific tRNAs of *Thermus thermophilus*, and finally found that the thiolated nucleosides in a particular tRNA species could be detected by combining this APM gel electrophoresis technique with Northern hybridization using a radioactive probe specific for the relevant tRNA.[38] Using this technique, my group, lead by N. Shigi, sought to determine the recognition sequence in tRNAs, if any, for s^2T-synthesizing enzymes in thermophile cells.[38]

An artificial $tRNA^{Asp}$ gene, in which the base-pairs in the anticodon, acceptor stem, and T stem regions were altered, was introduced into thermophile cells using the pEx-Asp* vector[38,39] (Fig. 6). The artificial $tRNA^{Asp}$ expressed in the thermophile cells was modified with s^2T. Using a series of artificial $tRNA^{Asp}$ genes, in which the T loop sequence was mutated, the region recognized by the s^2T-modifying enzyme(s) was determined to be $U_{54}U_{55}C_{56}N_{57}A_{58}N_{59}N'_{60}$, where N is any four nucleosides and N′ is any three nucleosides except A.[38] Therefore, the base residues at positions 55, 56, and 58, in addition to U54, form a strict target site for s^2T-modification. We hypothesize that 2-thiolation activity at position 54 requires the canonical T loop structure formed by the conserved U_{54}–A_{58} bases together with the conserved residue C_{56}.

In order to characterize the 2-thiouridylation reaction in detail, we assessed the s^2T-modifying enzyme activity in vitro.[40] The activity could be detected in crude extracts of *T. thermophilus* cells using *E. coli* tRNA and ATP. Using this assay, we found a strong temperature dependence of the 2-thiolation reaction in vitro, and expression of the 2-thiolation enzymes was strongly temperature dependent in vivo. Additionally, 1-methyladenosine (m^1A) at position 58 was required for efficient 2-thiolation of T_{54} both in vivo and in vitro.

Currently, we have identified two genes that are essential for the synthesis of s^2T in *T. thermophilus*, *ttuA* and *ttuB* (tRNA-two-thiouridine) using a proteomic approach.[41] The gene product of TtuA (TTC0106) appears to be an ATPase, possessing a P-loop motif (Ser–Gly–Gly–Xaa–Asp–(Ser/Thr)–) found in tRNA-binding ATPases, which activates target nucleosides

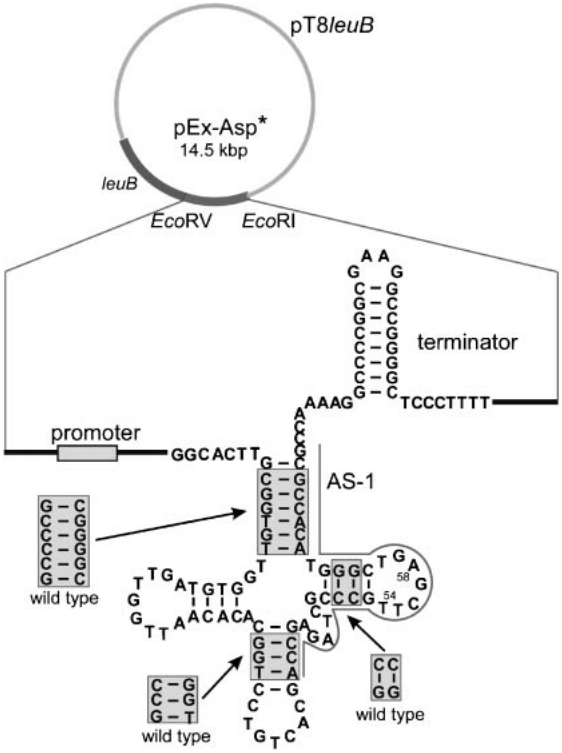

Fig. 6. Construction of the $tRNA^{Asp*}$ expression vector, pEx-Asp*.[38] The tRNA expression operon was ligated into the EcoRI and EcoRV sites of the *E. coli–T. thermophilus* shuttle vector pT8*LeuB*.[38,39] The sequences of the aminoacyl stem, T loop, and anticodon stem were altered from those of the native $tRNA^{Asp}$ gene to allow the expressed tRNA to be discriminated from the native tRNA in *T. thermophilus* cells. AS-1 indicates the positions of the DNA probe used to detect mature $tRNA^{Asp*}$.

by forming an adenylate intermediate. TtuB (TTC0105) is a putative thio-carrier protein that exhibits significant sequence homology to ThiS of the thiamine synthesis pathway. We were able to reconstitute the in vitro 2-thiolation reaction with both these proteins and a small amount of cell extract. Both TtuA and TtuB are required for in vitro s^2T formation in the presence of cysteine and ATP. The addition of cysteine desulfurases such as IscS (TTC0087) or SufS (TTC1373) enhanced the sulfur-transfer reaction in vitro. Untagged TtuA was co-purified with His6-tagged TtuB using nickel affinity column chromatography, suggesting that TtuA and TtuB form a heterocomplex, which may function as the s^2T synthetase in vivo.

In the thiamine biosynthesis pathway, a sulfur atom derived from cysteine is eventually incorporated into the thiazole moiety.[42] The sulfur-transfer pathway, from cycteine to thiazole, uses a C-terminal thiocarboxylate of ThiS (ThiS-COSH) as an intermediate sulfur donor. In this pathway, the C-terminus of ThiS is first activated to an adenylate form (ThiS-COAMP) by the ATPase ThiF, then ThiS-COSH is formed by cysteine and IscS, a cysteine desulfurase.

A putative s^2T biosynthesis pathway is presented in Fig. 7, and the potential roles played by TtuA, TtuB, IscS, and SulS are summarized below. Given that TtuB shares significant sequence homology with ThiS, it is also possible that C-terminal

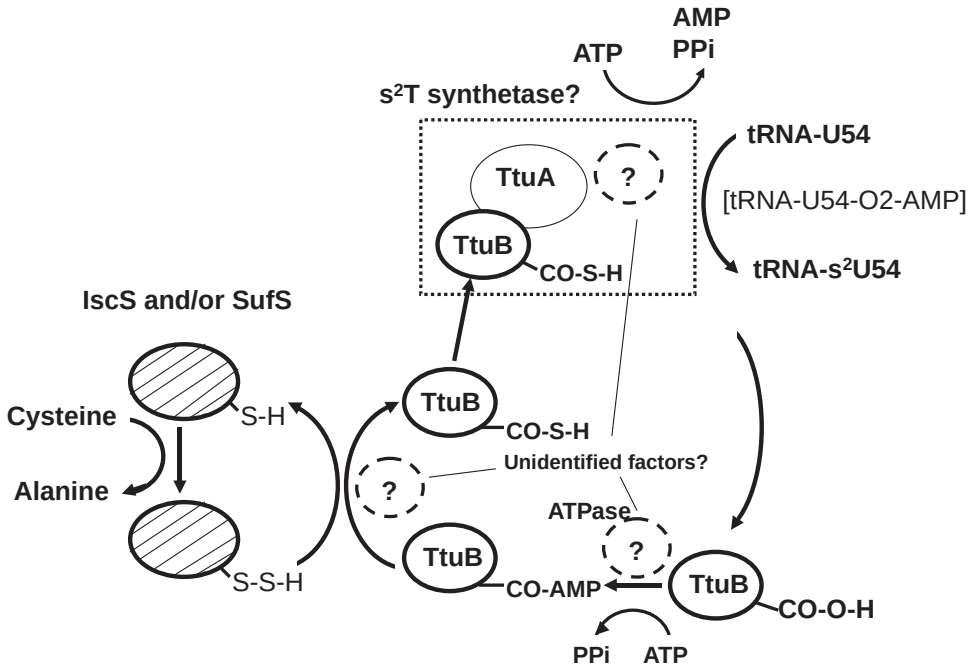

Fig. 7. Putative sulfur-transfer scheme in s^2T biosynthesis.[41] See text for details.

thiocarboxylation of TtuB (TtuB-COSH) by IscS or SufS using cysteine as a sulfur donor, may generate the immediate sulfur donor for s^2T formation. In this scenario, the sulfur atom of TtuB-COSH is incorporated into s^2T, either by direct transfer to the tRNA or via TtuA (possibly to a conserved cysteine residue). If this scenario is correct, then we expect a C-terminal acyl-adenylated form of TtuB (TtuB-COAMP) to be generated by an ATPase (corresponding to the activity of ThiF in ThiS-COAMP synthesis). The results of our in vitro s^2T reaction demonstrate that ATP was an absolute requirement for s^2T synthesis, suggesting the involvement of a TtuB-activating ATPase present in the *T. thermophilus* cell extract, in addition to the tRNA-activating ATPase (TtuA). A TtuB-activating ATPase is a major candidate for one of the unidentified but strictly required components of the in vitro s^2T reaction. IscS is a primary sulfur donor for all of the thio-modifications of tRNA in *E. coli*.[43,44] However, in *T. thermophilus*, mutants of *iscS* and *sufS* remain capable of synthesizing s^2T when the two genes are disrupted independently.[40] These data suggest that both cysteine desulfurases can serve as sulfur donors in *T. thermophilus*. The involvement of a TtuB-like sulfur carrier protein is a novel feature of the s^2T biosynthesis pathway.

Mutant strains of *ttuA* and *ttuB* demonstrated a temperature-sensitive phenotype, when compared with the wild-type strain. This is the first result directly suggesting that s^2T in tRNA is most likely linked to bacterial growth at high temperatures.

2. Novel Modified Nucleosides, 5-Formylcytidine (f^5C), *N*-(Uridine-5-ylmethyl)taurine (τm^5U), and *N*-(2-Thiouridine-5-ylmethyl)taurine (τm^5s^2U), Are Found in Animal Mitochondrial tRNAs

2.1 f^5C in the Methionine $tRNA^{Met}_i$ from Bovine Mitochondria Causes Codon Change. In the process of pursuing thermo-unstable tRNAs from different organisms as an antithesis of thermophile tRNAs, I encountered an exciting finding by Sanger's group in which they showed that animal mitochondrial genomes are very A–T rich and include an unusual-shaped tRNA gene consisting of 2 leaves of the tRNA cloverleaf structure instead of the usual 3. In addition, some genetic codes in animal mitochondria have been shown to deviate from the universal genetic code.[6,7] I was strongly impressed by these findings and convinced that the mitochondrial tRNAs described would be thermo-unstable and that they might include some novel modified nucleosides which are functional in decoding non-universal genetic codes.

At this time, our group began to develop methods to purify mt tRNAs from bovine cells and, finally, J. Moriya and co-workers succeeded in purifying 1 mg of initiator tRNA ($tRNA^{Met}_i$) from 100 kg of bovine liver and found a novel modified nucleoside at the first anticodon position of $tRNA^{Met}_i$ (Fig. 8). The nucleoside was determined to be 5-formylcytidine (f^5C; Figs. 1 and 8) in collaboration with J. A. McCloskey's lab at the University of Utah, U. S. A.[15]

In order to examine the functional role of this modified nucleoside in decoding, C. Takemoto and co-workers in my lab constructed an in vitro translation system from bovine mitochondria in collaboration with L. L. Spremulli's lab at the University of North Carolina, U. S. A. Using synthetic mRNAs in which 11 repeats of AUG or AUA triplets [$(AUG)_{11}$ or $(AUA)_{11}$] were connected to poly(U), Takemoto et al. proved that bovine mt $tRNA^{Met}_i$ possessing f^5C at the first anticodon position possessed a poly(Met)-synthesizing activity dependent on $(AUG)_{11}$ or $(AUA)_{11}$ (Table 2, and manuscript in preparation). This was the first experimental verification that in animal mitochondria the AUA codon was decoded as not isoleucine (Ile) but as methionine (Met). Further, $tRNA^{Met}_i$ possessing f^5C at the first anticodon position is required to decode the AUA codon as Met, because the artificial bovine mt $tRNA^{Met}_i$ in which f^5C was replaced by an unmodified C, bovine cytosolic $tRNA^{Met}_i$, and *E. coli* $tRNA^{Met}_m$ possess-

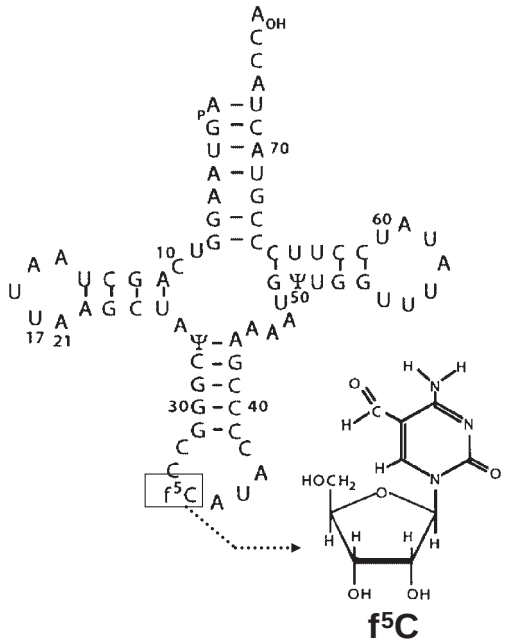

Fig. 8. Nucleotide sequence of $tRNA^{Met}$ from bovine mitochondria[15] and the chemical structure of f^5C at the first anticodon position.

Table 2. Decoding Capacities of Initiator tRNAs with Various Anticodons toward AUG, AUA, and AUC Codons

tRNA	Anticodon	Decoding capacity toward		
		AUG	AUA	AUC
Bovine mt $tRNA^{Met}_i$	f^5CAU	+	+	−
Artificial bovine mt $tRNA^{Met}_i$ (f^5C is replaced by C)	CAU	+	−	−
Bovine cyt. $tRNA^{Met}_i$	CAU	+	−	−
E.coli $tRNA^{Met}_m$	ac^4CAU	+	−	−

ing an unmodified C or a 4-acetylcytidine (ac^4C) at the anticodon first position, each decoded the AUG codon, but not AUA codon, as Met (Table 2, and manuscript in preparation). Thus, the AUA codon is translated by native bovine mt $tRNA^{Met}_i$ not as Ile, but as Met, in a bovine mitochondrial translation system.

^1H NMR analysis showed that f^5C has a normal amino tautomeric form at position 4 of the base moiety, indicating the presence of an intramolecular hydrogen bond between the carbonyl of the 5-formyl group and the 4-amino proton (Fig. 9).[16] This conformation analysis revealed that f^5C is one of the most conformationally rigid nucleosides yet analyzed, taking the C3′-*endo*-anti form, and may contribute to regulation of codon recognition by $tRNA^{Met}_i$ (Fig. 9).

2.2 A New Purification Method for Isolating Very Small Amounts of Mitochondrial tRNAs—Chaplet Column Chromatography. It is extremely difficult to isolate mt tRNAs by conventional column chromatography, because of the limited amounts of mt tRNAs that exist in the cell. To overcome this difficulty, Tsutomu Suzuki and co-workers in my lab developed a new tRNA isolation technique[11,13,14] employing a solid-phase DNA probe method, an affinity chromatography approach, the principle of which was originally devised by H. Tsurui et al.[12] In this method, to entrap the desired tRNA, a biotinylated DNA probe complementary to the target mt tRNA is immobilized on streptavidin Sepharose (Fig. 10a) and a crude tRNA fraction that includes about 0.1% of each mt tRNA is circulated through the column by a pump at 65 °C. After washing out the non-specific tRNAs, the trapped tRNA is eluted from the column with low salt buffer at 65 °C (Fig. 10b). Continuous circulation proved to be the most important factor in maximizing the individual tRNA yields. To isolate individual mt tRNAs, 22 columns, each specific for a different tRNA, were connected in tandem, and it was possible to simultaneously isolate all 22 individual mt tRNA species from the same crude tRNA preparation using this approach (Fig. 10c).[11] Tsutomu Suzuki named this system "chaplet" column chromatography. The crude tRNA fraction from bovine liver or human placenta was then circulated through this chaplet column, and 0.5–1 mg of individual tRNAs could be isolated from 1 kg of bovine liver by this single chromatography step. The purity of each tRNA was close to 100%. This method is applicable to the isolation of not only tRNAs but also small RNAs such as micro RNAs and sno RNAs.

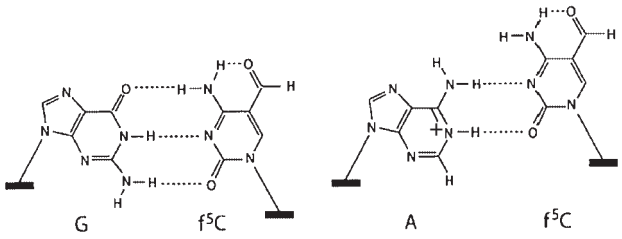

Fig. 9. Chemical structure of f^5C and base-pairing models of f^5C–G (left) and f^5C–A.[16]

2.3 Identification of τm^5U and τm^5s^2U at Position 34 in Five Species of tRNA from Bovine Mitochondria and the Decoding Capacities of These tRNAs. In 2001, a graduate student, Takeo Suzuki and co-workers in my lab identified new modified uridines with molecular masses of 381 and 397 Da at the first anticodon positions of $tRNA^{Leu(UUR)}$ ($tRNA^{Leu}$ with an anticodon corresponding to the UUR codon) and $tRNA^{Lys}$, respectively. Both tRNAs were isolated by the chaplet column chromatography method and each tRNA fraction was treated with phosphodiesterase and phosphatase to hydrolyze the tRNA into nucleoside components, which were then subjected to LC/MS analysis to identify the modified nucleosides.

Using high-resolution mass spectrometry utilizing a Fourier-transforming ion cyclotron mass spectrometry (FT-ICR MS) method, Takeo Suzuki determined the molecular formulas of these compounds. In combination with split pattern analysis by collision-activated estrangement and structural information through analysis by high-resolution NMR, these new modified nucleosides were deduced to be *N*-(uridine-5-ylmethyl)taurine (τm^5U) and *N*-(2-thiouridine-5-ylmethyl)tau-

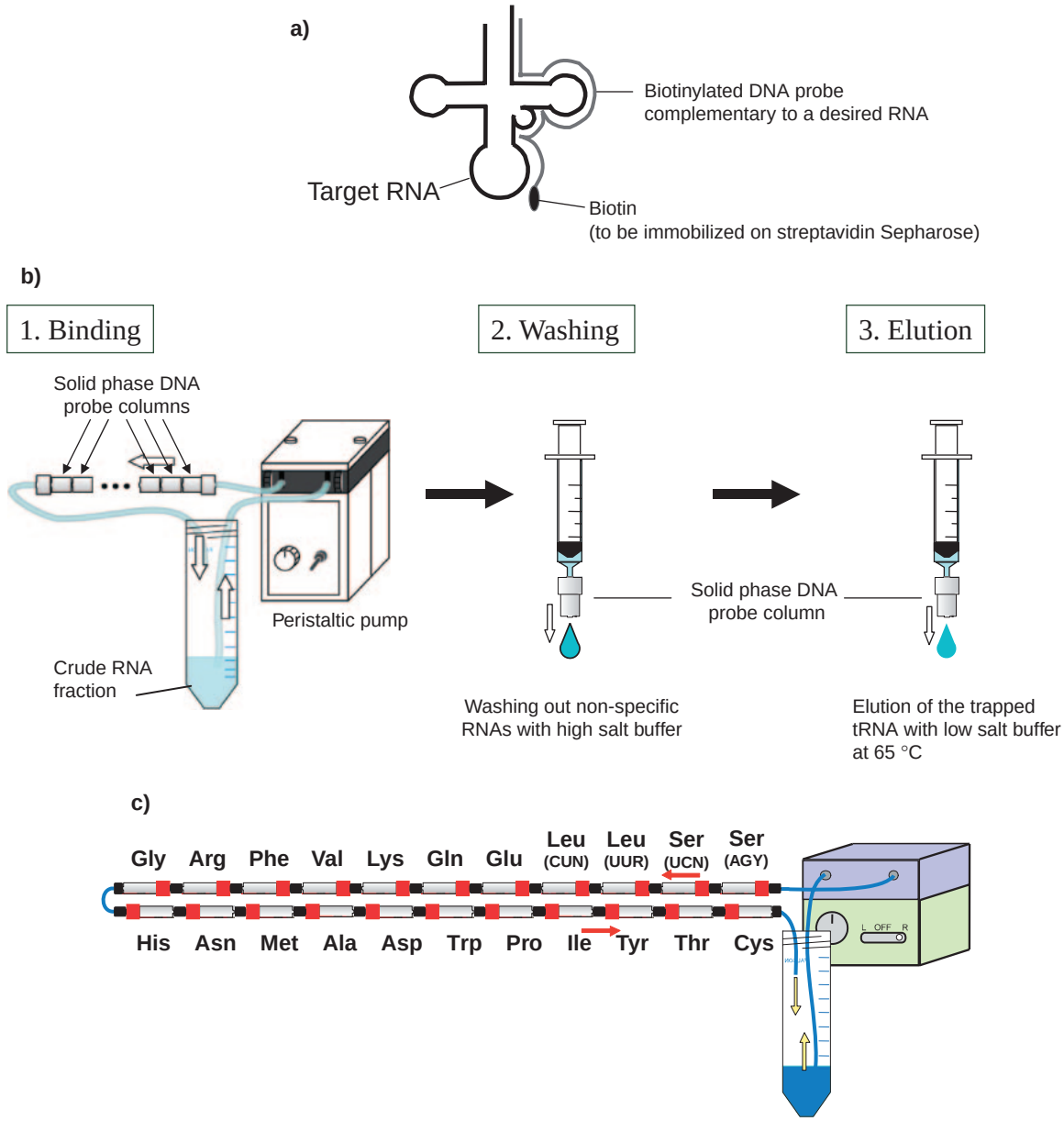

Fig. 10. Chaplet column chromatography. a) 3′-Biotinylated DNA probe complementary to target RNA was immobilized on Streptavidin Sepharose. b) Several DNA columns described above were connected in tandem (the "chaplet" column). A crude tRNA fraction was circulated through this chaplet column by a pump at a temperature of 65 °C to entrap the desired tRNA (1). After washing out non-specific RNA (2), the trapped tRNA was eluted from the column with low salt buffer at 65 °C (3). c) The "chaplet" column can be applied to parallel isolation of multiple species of RNA molecules at the same time. The DNA columns for each mt tRNA are tandemly connected and the crude tRNA fraction from bovine liver or human placenta is circulated through this chaplet column to entrap each mt tRNA. We could successfully isolate multiple species of mt tRNA at the same time.

rine ($\tau m^5 s^2 U$) from $tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$, respectively. The *N*-(2-sulfoethyl)aminomethyl groups were attached at position 5 of U in both cases, and a thio group was attached at position 2 of U in the case of $\tau m^5 s^2 U$ (see Fig. 1). The chemical structures were verified by comparison with authentic chemically synthesized samples (in collaboration with K. Saigo and T. Wada of Graduate School of Frontier Sciences, Univ. Tokyo).[14]

These two modified uridines are the first examples of modified nucleosides which include a sulfo group in their side chains. Since these taurine-containing modified nucleosides exist not only in human and bovine mitochondria but also in fish and ascidian mitochondria, they are thought to be commonly used in mt tRNAs of at least protochordates and vertebrates.

These modified nucleosides have been found in additional tRNAs, $\tau m^5 U$ exists in $tRNA^{Trp}$, and $\tau m^5 s^2 U$ exists in $tRNA^{Glu}$ and $tRNA^{Gln}$. In mt translation systems any tRNA possessing an unmodified uridine at the first anticodon position can decode all 4 codons which are assigned to a single amino acid. In contrast, a tRNA possessing a modified uridine at this position is restricted to decoding only the two NNR (R = A

Table 3. Genetic Code Table Including Codon–Anticodon Pairing Relations in Mammalian Mitochondrial Decoding System

Codon	Amino acid (anticodon)	Codon	Amino acid (anticodon)	Codon	Amino acid (anticodon)	Codon	Amino acid (anticodon)
UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys
UUC	(GAA)	UCC	(UGA)	UAC	(QUA)	UGC	(GCA)
UUA	**Leu**	UCA		UAA	Stop	**UGA**	**Trp**
UUG	**(τm^5UAA)**	UCG		UAG		**UGG**	**(τm^5UCA)**
CUU	Leu	CCU	Pro	CAU	His	CGU	Arg
CUC	(UAG)	CCC	(UGG)	CAC	(QUG)	CGC	(UCG)
CUA		CCA		**CAA**	**Gln**	CGA	
CUG		CCG		**CAG**	**(τm^5s^2UUG)**	CGG	
AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser
AUC	(GAU)	ACC	(UGU)	AAC	(QUU)	AGC	(GCU)
AUA	Met	ACA		**AAA**	**Lys**	AGA	Stop
AUG	(f^5CAU)	ACG		**AAG**	**(τm^5s^2UUU)**	AGG	
GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly
GUC	(UAC)	GCC	(UGC)	GAC	(QUC)	GGC	(GCA)
GUA		GCA		**GAA**	**Glu**	GGA	
GUG		GCG		**GAG**	**(τm^5s^2UUC)**	GGG	

and G) codons.[45] Thus, mt translation systems are capable of operating with a small number of tRNAs, such as the 22 species shown in Table 3.

2.4 Biosynthesis of Taurine-Containing Uridines. Taurine (2-aminoethanesulfonic acid) is a β-amino acid existing in all organisms other than bacteria and plants and is the most abundant amino acid in the human body (about 0.1% by weight). The physiological role of taurine is the maintenance of homeostasis for internal circulation, and its biochemical roles include assisting in excretion of cholesterol, regulating calcium ion content in the heart, and functioning as a neurotransmitter in the brain. Taurine has not been found in high-molecular mass compounds such as protein and RNA, and has been thought to exist as a monomer or a component of low-molecular mass chemicals.

In order to characterize the biosynthetic pathway of taurine-modified uridines in tRNA, HeLa cells were cultured in medium containing [^{18}O]- or [^{15}N]-labeled taurine, from which mt tRNAs were isolated. When the molecular masses of τm^5U and τm^5s^2U in the mt tRNAs were measured, both had increased by 90% in accord with [^{18}O]- or [^{15}N]-labeled taurine added into the medium. Thus, HeLa cells actively incorporate external taurine through the taurine transporter in the plasma membrane, and the taurine is transported into mitochondria, where it is used in modification of mt tRNAs (Fig. 11).

2.5 Relationship to Mitochondrial Diseases—Modification Defects of Taurine in $tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$ Found in Mitochondrial Brain and Muscle Diseases. In 1994–1995, we initiated a collaboration with Prof. S. Ohta's group at Nippon Medical School, Tokyo, on the hypothesis that our mt translation research over the previous ten years might be useful in elucidating the molecular basis of the mitochondrial diseases on which Ohta's lab had concentrated. The sequence of the human mt genome, which was completed by Sanger's group in 1981,[7] contains only 13 protein, 2 ribosomal RNA, and 22 tRNA genes which are arranged with almost no

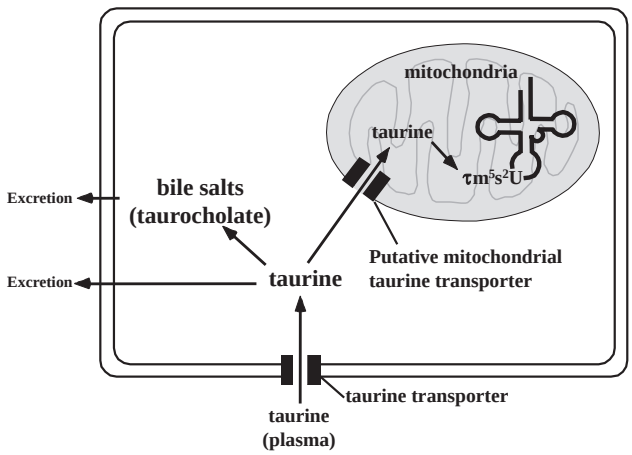

Fig. 11. Catabolic flow of intracellular taurine, in which new pathway proposing transport into mitochondria is shown.

gaps and total ≈16500 base pairs (Fig. 12). MELAS and MERRF, which are representative of mitochondrial diseases, originate from point mutations in mt tRNA genes, as discovered by several research groups including Ohta's group (Fig. 12).[46–49] An A to G transition at position 3243 of the $tRNA^{Leu(UUR)}$ gene occurs in approximately 80% of MELAS patients[46,47] and a T to C transition at position 3271 of the same tRNA gene occurs in about 10% of patients.[48] In most MERRF patients, an A to G transition at position 8344 of $tRNA^{Lys}$ occurs.[49] These are representative mutations occurring in mt tRNA genes (Fig. 13).

In 2000, T. Yasukawa, a graduate student in my lab, succeeded in isolating the mutant $tRNA^{Leu(UUR)}$ from a large scale culture of MELAS-derived cybrid cells (artificially fused cells containing the wild-type nucleus and mutant mitochondria). Yasukawa's detailed analysis of the tRNA showed that τm^5U was present at the first anticodon position in the wild-type

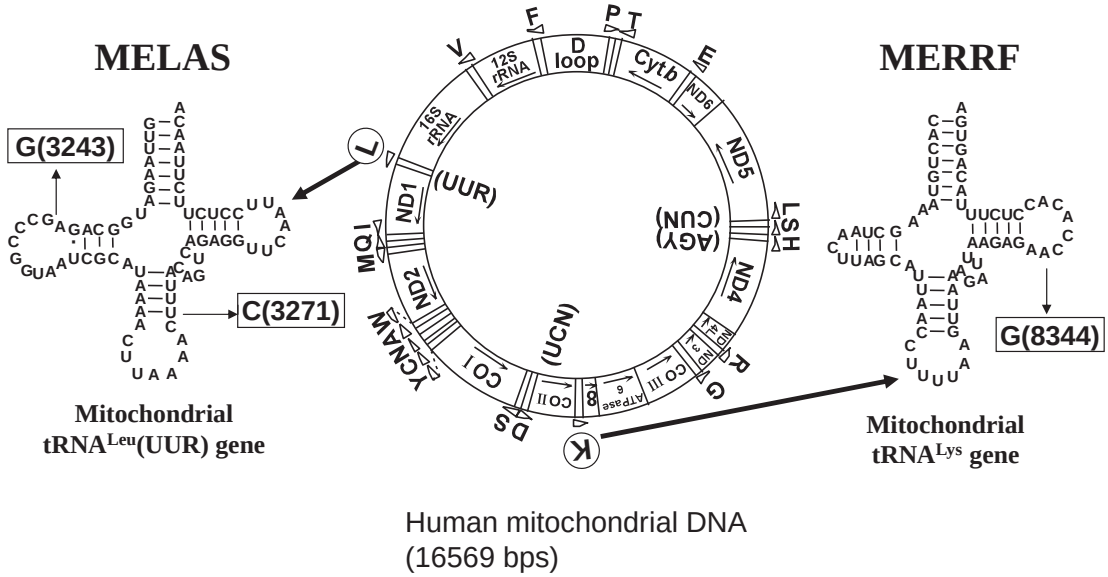

Fig. 12. Relative positions of genes encoding proteins, tRNAs, and ribosomal RNAs in the human mitochondrial genome (center), and secondary structures of mitochondrial mutant tRNA genes from MELAS3243 and 3271 (left), and MERRF8344 (right).

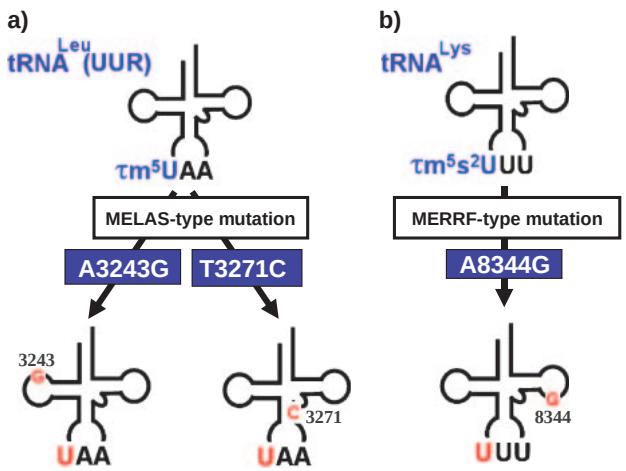

Fig. 13. Wobble modification defect in mutant tRNAs from mitochondrial diseases. The wobble modification defect is found in three mutant tRNAs from MELAS3243 and 3271, and MERRF8344. These point mutations work as negative determinants for taurine-containing wobble uridines in mt tRNAs.

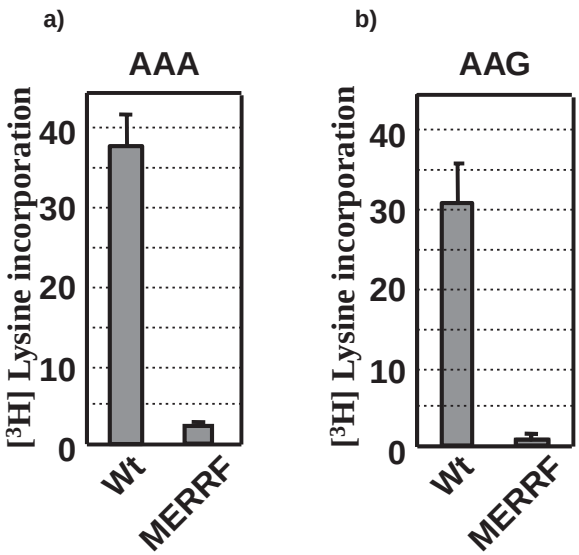

Fig. 14. In vitro translational elongation activities of the wild-type $tRNA^{Lys(sU^{*}UU)}$ (Wt) and mutant $tRNA^{Lys(UUU)}$ with the A8344G mutation (MERRF) toward codons AAA (a) and AAG (b).

$tRNA^{Leu(UUR)}$, but in the A3243G and T3271C mutant $tRNA^{Leu(UUR)}$, the position was occupied by an unmodified U.[18] Since all the sequences other than the first anticodon position were identical between the wild-type and mutant $tRNA^{Leu(UUR)}$, these data imply that the taurine-modification defect is the result of a defect in recognition of the mutant tRNA by the τm^5U-modification enzyme. Analogous results were obtained in the case of MERRF mutants.[19] Thus, we conclude that the point mutation resulted in the taurine-modification defect in the respective tRNAs in these 2 types of mitochondrial diseases. These taurine-modification defects were also found in native tRNAs isolated from patient tissues.[50]

2.6 Translational Abnormalities Caused by the Modification Defect in the Mutant tRNAs. In order to clarify the relationship between the taurine modification defect in mitochondrial tRNAs and the translational activity of the mitochondria, we examined the translation activity of the A8344G-mutant $tRNA^{Lys}$ in the bovine mitochondria in vitro translation system which was developed in my lab. This mutant tRNA lacks the ability to decode either AAA or AAG codons, which results in a total loss of protein synthesis activity (Figs. 14 and 15b).[20]

In the MELAS mutation, a wild-type-like tRNA which lacks only the taurine modification blocks the ability to translate leucine containing proteins. Y. Kirino, a Master's course student in my lab, conducted a large scale purification of the wild-type $tRNA^{Leu(UUR)}$ from approximately 27 kg of human placenta

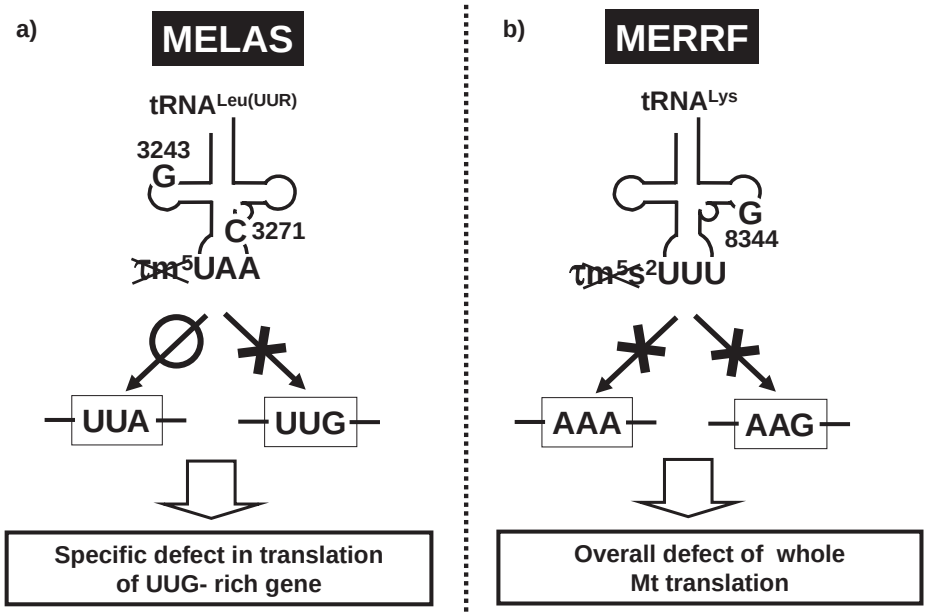

Fig. 15. Distinct patterns of codon recognition found in mutant tRNAs lacking wobble modification. The pathogenic point mutation (A3243G or U3271C) in mutant $tRNA^{Leu(UUR)}$ from MELAS patients causes a deficiency in τm^5U-modification, which results in a UUG codon-specific translational defect. The MERRF 8344 mutation also causes a deficiency in τm^5s^2U-modification that results in a translational defect for both cognate codons, AAA and AAG.

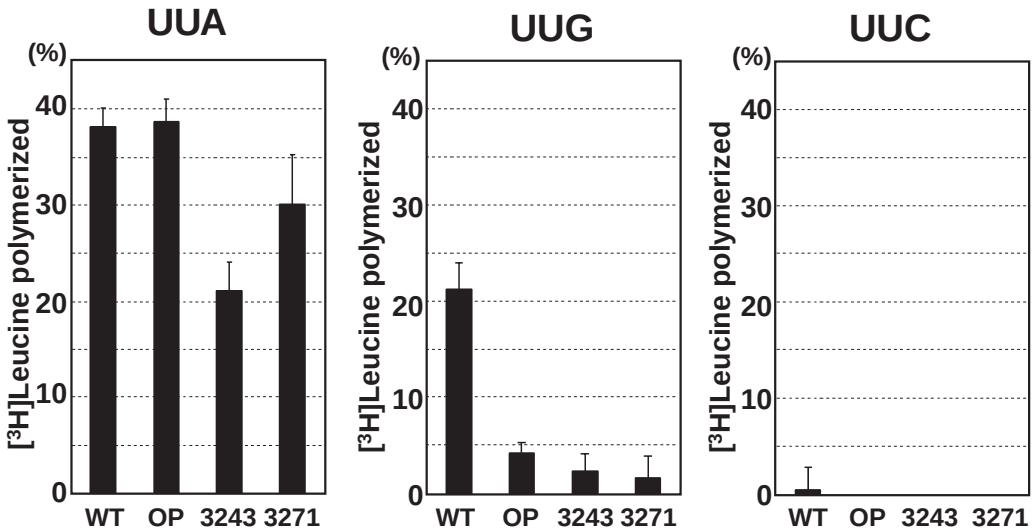

Fig. 16. Translational activity of the operated $tRNA^{Leu(UUR)}$ without the wobble modification and of the MELAS mutant $tRNA^{Leu(UUR)}$. In vitro mitochondrial translation of test mRNAs containing the UUA (left), UUG (center), or UUC (negative control) (right) codons was performed with wild-type $tRNA^{Leu(UUR)}$ (WT), operated $tRNA^{Leu(UUR)}$ with an unmodified wobble uridine (OP), and two MELAS mutants, $tRNA^{Leu(UUR)}$ bearing the A3243G (3243) or the U3271C (3271) mutations, with an unmodified wobble uridine. The radioactivity of the [^{3}H]leucyl-tRNA input to the reaction mixture was defined arbitrarily as 100. The means ± SD of three independent experiments are shown.

and constructed a wild-type-like $tRNA^{Leu(UUR)}$, lacking only the taurine modification, by molecular surgery to produce a site-selective excision and linkage of RNA fragments. When the translation activity of this tRNA containing an unmodified U at the first anticodon position, but no other mutations, was measured using the mitochondrial in vitro translation system, the tRNA was able to decode the UUA codon, but not the UUG codon for Leu (Figs. 15a and 16).[23] Further, the loss of this translation activity came from the loss of tRNA binding at the ribosomal A site.[23]

Synthesis of ND6, a subunit of the respiratory chain complex I, decreases rapidly as the mutation rate at position 3243 increases.[51,52] ND6 has an extraordinarily high content of UUG codons among the 13 protein genes of the human mitochondrial genome (Fig. 17),[11] and therefore, the decrease in UUG codon-specific translation by the mutant tRNA would

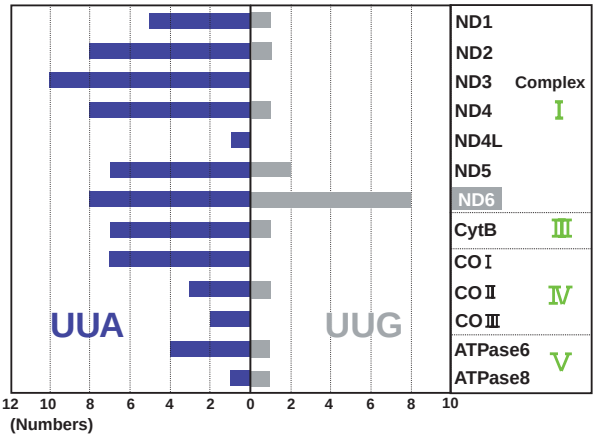

Fig. 17. Usage of UUR codons in human mitochondrial DNA. Leu codon (UUA/G) usage for 13 protein genes encoded by mitochondrial DNA. The number of UUA/G codons are shown for each gene.

specifically depress ND6 synthesis. These observations have resolved a long-standing question as to why the activity of respiration chain complex I decreases drastically only in MELAS, but not in MERRF, patients.

2.7 Relationship between Taurine-Deficiency Disease and Mitochondrial Brain and Heart Disease. Although human adults are capable of synthesizing taurine de novo, newborn babies have an undeveloped de novo taurine synthesizing system. Thus, taurine intake is a prerequisite for normal growth of new-borns. In some animals such as cats and foxes biosynthesis of taurine is completely lacking, so taurine is an essential amino acid.[53] Taurine deficient cats suffer from myocardial diseases.[54] Since myocardial diseases have been linked to mitochondrial dysfunctions, taurine-deficient cats may suffer from myocardial diseases resulting from the same mechanism as found in MELAS and MERRF. Some fish, for example, yellowtail and flatfish, contain abundant levels of taurine, but cannot synthesize taurine themselves.[55] Flatfish cultured with casein-containing but taurine-deficient feed did not grow well and were more sluggish than control fish. Since taurine is also involved in contraction of the heart muscle, the influence of taurine on the activity of the body must be complex. However, it is strongly suggested that defects in taurine modification of mt tRNAs correlates with functional defects in mitochondria, and clarification of the relationship between taurine deficiency diseases and decreases in taurine modification in mitochondrial tRNAs will be an important area of investigation in mitochondrial research.

The taurine-deficiency found in MELAS and MERRF is the first example of diseases which originate from disorders in RNA modification. The concept of diseases which result from RNA dysfunctions has not been common, because most genetic diseases are thought to originate from protein dysfunctions. However, complete analysis of the human genome has revealed that approximately half of the human genome is occupied by non-coding RNA genes, RNAs which do not code for proteins.[56,57] Thus, qualitative disorders in RNA may be the underlying cause of some diseases. As examples of such diseases increase, RNA research will become more and more important.

3. Summary and Future Perspectives

One already-known and three new modified nucleosides were found in tRNAs from an extreme thermophilic bacterium and animal mitochondria, respectively, and the chemical structures of the new nucleosides were determined. The known nucleoside, 2-thioribothymidine (s^2T) which had previously been found in the first anticodon position of rat liver tRNAs[9] was identified at position 54 in the T loop in tRNAs from a thermophilic bacterium, *Thermus thermophilus*, and was found to be indispensable for thermostability of the tRNA.[8,10] The three new nucleosides are 5-formylcytidine (f^5C), *N*-(uridine-5-ylmethyl)taurine (τm^5U), and *N*-(2-thiouridine-5-ylmethyl)taurine (τm^5s^2U), each found at the first anticodon position (position 34) of $tRNA^{Met}$, $tRNA^{Leu}$(UUR) and $tRNA^{Trp}$, and $tRNA^{Gln}$, $tRNA^{Lys}$ and $tRNA^{Glu}$, respectively, from animal mitochondria.[14,15] These nucleosides play a crucial role in recognizing cognate codons.

3.1 s^2T in *Thermus thermophilus* tRNA. A new method was devised for simple identification of s^2T-bearing tRNAs. Samples of tRNA were separated on APM gels [(acryloylamino)phenylmercury(II) chloride][37] and Northern hybridization was adapted to detect the s^2T-bearing tRNA bands on the gel.

To search for s^2T-modifying enzyme(s), an artificial $tRNA^{Asp}$ gene, in which the sequences in the anticodon and acceptor stem regions were changed, was introduced into thermophile cells using an *T. thermophilus–E. coli* shuttle vector.[38,39] The artificial $tRNA^{Asp}$ expressed in the thermophile cells was modified with s^2T. Using artificial $tRNA^{Asp}$ genes in which the T loop sequences were mutated, the region recognized by the s^2T-modifying enzyme(s) was determined to be $U_{54}U_{55}C_{56}N_{57}A_{58}N_{59}N'_{60}$, where N is any of the four nucleosides and N′ is any nucleoside except A. The s^2T-modifying enzyme activity was detected in crude extracts of *T. thermophilus* cells in an in vitro assay using *E. coli* tRNA and ATP.[38]

We have identified 2 genes, *ttuA* and *ttuB*, essential for s^2T synthesis in *T. thermophilus.* TtuA appears to be an ATPase possessing a P-loop motif found in tRNA-binding ATPases, and TtuB is a putative thio-carrier protein which may be the sulfur donor for s^2T formation, either by direct transfer to the tRNA or via transfer to TtuA (Fig. 7).

What can we expect when the entirety of thiolases have been elucidated? All the tRNAs in the cell can be made thermostable, and further a whole mesophilic cell might be made thermoresistant to some extent, if the thiolase genes are introduced into the cell. Artificial thermostable bacteria might be created through this procedure (Fig. 2). If mesophilic bacteria with various properties (synthesis of antibiotics, resistance towards various pharmaceutical agents including antibiotics, etc.) would be made thermostable, a variety of applications would be possible.

3.2 Modified Nucleosides in Mammalian Mitochondrial tRNAs. A new separation method, named chaplet-column chromatography was devised for isolation of individual mitochondrial tRNAs in amounts necessary for studying their structures and functions (Fig. 10). Using this method, 0.5–1 mg of individual tRNAs could be isolated from 1 kg of bovine liver in a single chromatographic step. This method is applicable

to the isolation not only of tRNAs but also of small RNAs such as micro RNAs and sno RNAs.

Using liquid column chromatography, high performance mass spectrometry (LC/MS) analysis, and NMR measurement, the chemical structures of three novel modified nucleosides were determined to be 5-formylcytidine (f^5C), *N*-(uridine-5-ylmethyl)taurine (τm^5U), and *N*-(2-thiouridine-5-ylmethyl)taurine (τm^5s^2U) (Fig. 1), in which the formyl group and *N*-(2-sulfoethyl)aminomethyl group were attached at position 5 of C (for f^5C) and U (for τm^5U), respectively, and at positions 5 and 2 of U for the *N*-(2-sulfoethyl)aminomethyl and thio groups, respectively (for τm^5s^2U).

In order to examine the functional roles of these modified nucleosides, we developed an in vitro mitochondrial translation system from bovine mitochondria in collaboration with L. L. Spremulli's lab. at the University of North Carolina. Using this system, we showed that these modified nucleosides at the first anticodon position play crucial roles in decoding.

The f^5C of $tRNA^{Met}$ is capable of decoding not only AUG but also AUA, revealing that the AUA codon is translated as not Ile, but as Met, in mammalian mitochondrial translation systems. In the future, we will isolate the modification enzyme(s) for f^5C. Using the enzyme(s), introduction of f^5C into the $tRNA^{Met}$ of any cell may be possible, which may enable the AUA codon to be changed to Met, if the original gene for AUA codon-specific $tRNA^{Ile}$ is deleted. Thus, reassignment of the genetic code may be possible, leading to new methods for artificial protein synthesis.

The taurine-containing U's at the first anticodon position of five species of tRNA (Leu(UUR), Trp, Gln, Lys, and Glu) are important for correct decoding of the cognate codons, UUR, GUR, CAR, AAR, and GAR (R = A and G). Of the known mitochondrial diseases, MELAS and MERRF are representative diseases known to be caused by a single base replacement in mitochondrial $tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$, respectively.

We isolated these two tRNAs from the placentas of both healthy individuals and patients suffering from these diseases, using the "chaplet" column chromatography, and found that τm^5U is absent in $tRNA^{Leu(UUR)}$ in MELAS patients and τm^5s^2U is absent in $tRNA^{Lys}$ in MERRF patients, although all the other modified nucleosides are present in the patients' mitochondrial tRNAs. Using the in vitro bovine mitochondrial translation system, we clearly demonstrated that the τm^5U-lacking $tRNA^{Leu(UUR)}$ can decode UUA, similar to the native tRNA, but cannot decode UUG, and that the τm^5s^2U-lacking $tRNA^{Lys}$ lost the ability to decode either AAA or AAG.

The most important task at present is to isolate the enzymes which generate the τm^5U and τm^5s^2U modifications. Next, these enzymes should be altered by genetic engineering or other methods, so that the altered τm^5U-forming enzyme recognize the mutant $tRNA^{Leu(UUR)}$ in which A3243 and U3271 are replaced by G and C, respectively (in the case of MELAS), and the altered τm^5s^2U-forming enzyme recognizes the mutant $tRNA^{Lys}$ in which A8344 is replaced by G (in the case of MERRF). Subsequently, the $tRNA^{Leu(UUR)}$ and $tRNA^{Lys}$ could be modified with τm^5U and τm^5s^2U, respectively at the first anticodon position, and these modified tRNAs would be able to decode the UUG and AAR (R = A and G) codons, respectively.

If these altered τm^5U-forming and τm^5s^2U-forming enzymes could be introduced into cells of MELAS and MERRF patients, the mitochondrial translation system could recover the ability to decode UUG and AAR codons so that the cells could grow normally and MELAS and MERRF diseases could be cured. This may be still a dream at present, but the dream will be realized when the above-mentioned scenario is completed.

Most of the results described here have already been published in a number of papers cited in the Text, which were obtained by many colleagues and graduate students in my previous laboratories at the University of Tokyo and the Tokyo Institute of Technology for many years, and also by domestic as well as foreign collaborators, to whom my great appreciation is given, although I cannot list all the name of these contributors here (please refer to the papers cited in the References). Most of the results in this paper originated from the deep endeavors of Dr. Tsutomu Suzuki and his co-workers (Univ. Tokyo). Additionally, the data concerning the f^5C and s^2T synthetases were contributed by Dr. C. Takemoto (Riken Institute) and Dr. N. Shigi (Biological Information Research Center, AIST), respectively. I thank Drs. J. A. McCloskey (University of Utah), L. L. Spremulli (North Carolina University), and S. Ohta (Nippon Medical School) for their kind collaboration.

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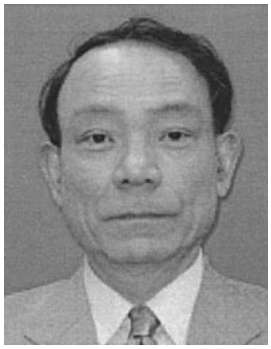

Kimitsuna Watanabe was born in 1941 in Osaka. He graduated from the University of Tokyo in 1967 and received his Ph.D. in 1972 under the guidance of Professor Kazutomo Imahori. He got a researcher position at Mitsubishi-Kasei Institute of Life Sciences in 1972. From 1975 to 1977 he stayed as a post-doctral fellow at Max-Planck Institut für Experimentelle Medizin, West Germany. In 1980, he became an associate Professor in the University of Tokyo, Fuculty of Agriculture, and in 1984 he moved to Fuculty of Engineering as an associate Professor. In 1988 he became a Professor at Tokyo Institute of Technology, Graduate School of Integrated Science and Technology, and afterwords at Graduate School of Bioscience and Biotechnoogy. In 1991, he became a Professor at the University of Tokyo, Graduate School of Engineering and in 2001 he moved to Graduate School of Frontier Sciences at Kashiwa Campus. From 2004, he has been a director of Biological Information Research Center, National Institute of Advanced and Industrial Science and Technology (AIST). His research field is RNA and translation, especially focused on structure and function of tRNA and origin and evolution of genetic code.