Conserved Bases in the TΨC Loop of tRNA Are Determinants for Thermophile-specific 2-Thiouridylation at Position 54*

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2-Thioribothymidine (s^2T) is a post-transcriptionally modified nucleoside of U54 specifically found in thermophilic bacterial tRNAs. The 2-thiocarbonyl group of s^2T54 is known to be responsible for the thermostability of tRNA. The s^2T content in tRNA varies depending on the cultivation temperature, a feature that confers thermal adaptation of protein synthesis in Thermus thermophilus. Little is known about the biosynthesis of s^2T, including the sulfur donor, modification enzyme, and the tRNA structural requirements. To characterize 2-thiolation at position 54 in tRNA, we constructed an in vivo expression system using tRNAAsp with an altered sequence and a host-vector for T. thermophilus. We were able to detect in vivo activity of s^2T54 thiolase using phenyl mercuric gel electrophoresis followed by Northern hybridization. 2-Thiolation at position 54 was identified in the precursor form of the tRNA, indicating that 2-thiolation precedes tRNA processing. To ascertain the elements that determine 2-thiolation in tRNA, systematic site-directed mutagenesis was carried out using the tRNAAsp gene. Conserved residues C56 and A58 were identified as major determinants of 2-thiolation, whereas tertiary interaction between the T and D loops and non-conserved nucleosides in the T loop were revealed not to be important for the reaction.

A characteristic structural feature of tRNA is post-transcriptional modification. The roles of modified nucleosides in tRNA function are important and wide-ranging. They are known to include codon recognition, reading-frame maintenance, stabilization of the tertiary structure, and serving as identity determinants for amino acid specificity (1).

The melting temperatures of tRNAs from the extreme thermophile Thermus thermophilus sp. are 3–10 °C higher than those of corresponding tRNA species from the mesophilic bacterium Escherichia coli, a feature that cannot be explained solely by the higher G-C pair content in Thermus tRNAs (2). Analyses of modified nucleosides in tRNAs from T. thermophilus revealed a thermophile-specific sulfur-containing modified nucleoside that was identified as 2-thioribothymidine (s^2T) (2, 3), a 2-thiolated derivative of 5-methyluridine (ribothymidine (T)) located at position 54 in the T loop of almost all tRNAs (4). Because s^2T54 is also present in tRNA from hyperthermophilic Archaea such as Pyrococcus furiosus, which contains about 0.77 mol % of s^2T when cultured at 100 °C (5), 2-thiolation of T54 is postulated to be a common modification responsible for the thermostabilization mechanism of tRNA in both thermophilic eubacteria and Archaea. The 2-thiolation of T54 increases along with elevation of the cultivation temperature without any changes in other modifications such as 1-methyladenosine at position 58 (m^1A58) or 2'-O-methylguanosine at position 18 (Gm18) (6); more than half of the tRNAs in T. thermophilus HB8 cells grown at more than 80 °C were found to contain s^2T54 instead of T54, whereas at 50 °C only a small proportion had s^2T. In addition, the tRNA melting temperature increased concomitantly with increases in the s^2T content (6). 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 temperatures of the inflection point in the specific CD signal (7) and in the characteristic chemical shift in the NMR spectra of s^2T in T. thermophilus tRNA (8) show a good match with the melting temperature monitored by UV absorbance, suggesting a close correlation between the local conformation of s^2T54 and the structural stability of tRNA. The mechanism of tRNA structure stabilization conferred by s^2T has been elucidated by proton NMR analysis (9); the ribose puckering of s^2T preferentially takes the C3'-endo-gg-anti conformation as do all residues in A-form RNA because of the steric effect of the bulky 2-thiocarbonyl group toward the 2'-hydroxyl group. This inherent rigidity of s^2T54 gives stability to the elbow region formed by D loop-T loop interaction, resulting in the thermostability of the overall tRNA tertiary structure (10).

Although s^2T54 is clearly a key modification for tRNA stability and function at elevated temperatures, information on its biosynthesis is very limited. Features that remain to be elucidated include the sulfur donor, modification enzymes or related genes, and the tRNA structural elements necessary for 2-thiolation. A genetic approach is, thus, indispensable for the characterization of this modification. Using the leuB gene as a selective marker, some of the present authors recently developed a host-vector system for T. thermophilus (11) that facilitates studies on the thermostability of proteins or RNAs from the bacterium. Here, we describe the detection of 2-thiolation activity in vivo in a reporter tRNA expressed using this T. thermophilus host-vector system. A systematic mutation analysis enabled us to characterize 2-thiolation in the maturation process of the tRNA and to identify the structural requirements for 2-thiolation at position 54.

Received for publication, July 22, 2002, and in revised form, August 7, 2002
Published, JBC Papers in Press, August 12, 2002, DOI 10.1074/jbc.M207323200

39128 This paper is available online at http://www.jbc.org
Plasmid Construction—A T. thermophilus HBB trRNAAsp* gene was amplified by PCR: the trRNAAsp* was designed so that trNAAsp could be discriminated from the intronic trNAAsp* and other tRNAs in the cells by Northern hybridization (Fig. 1). The promoter/terminator sequences from the T. thermophilus HBB trNAAsp* gene (GenBank accession number: X07394) were attached to the designed trNAAsp* so it would be expressed in T. thermophilus cells. A synthetic DNA fragment comprising the trNAAsp* operon (about 300 bp; Fig. 1A) was constructed from 6 DNA fragments in the following manner. The 5′-half of the operon was synthesized by the Klenow reaction with the primer set 5-F and 5-R, each of which has an overlapping 16-bp complementary sequence at its 3′ terminus. The 3′-half was synthesized in the same way with the primers 3I-F and 3I-R. These two half-fragments were ligated using the primers 5-F and 3I-R, respectively corresponding to the 5′- and 3′-termini of the operon. The ligated DNA fragment was then introduced into the pCR-XL-TOPO vector (Invitrogen). To confirm the 5′- and 3′-termini of the operon, the sequence of the operon was determined by the primer extension method (17).

Mass Spectrometry—An LCQ ion trap mass spectrometer (Thermo Finnigan) equipped with an electrospray ionization source and a MAGIC 2002 liquid chromatography system (Michrom BioResources) was used to analyze RNA fragments digested with RNase T1. Purified trNAAsp* (0.4 μg) was digested with RNase T1 (2.5 units) in 25 mM ammonium acetate (pH 5.5) at 37 °C for 1 h, and the digest was subjected to mass spectrometric analysis. Oligonucleotides produced by RNase T1 digestion were detected by LC/MS in the negative ion mode as described by Qiu and McCloskey (18) with the following slight modifications. An ODS reversed-phase column (Inertial ODS3, 1.0 × 200 mm; GL Sciences) was used. The solvent system, consisting of 0.4 mM N-hexane-2-propanol (pH 7.0, adjusted to 0.1 M ammonium acetate) in H2O (A) and 50% methanol (B) was used as follows: 15–95% B in 0–15 min, 95% B for 15–25 min, 95–15% B in 25–26 min. Negative ions were scanned over an m/z range from 620 to 2000.

Results

Expression of T. thermophilus trNAAsp* Gene with an Altered Sequence in E. coli and T. thermophilus Cells—To detect activity of the sT (54)-thiole in T. thermophilus cells and to investigate its recognition elements in trNAAsp* (Fig. 1), we first expressed an artificial trNAAsp gene encoded on a plasmid. We selected T. thermophilus HBB trNAAsp* as our model trNAAsp species because the presence of s2T at position 54 has been a barrier in the expression of trNAAsp* in E. coli (14). To express trNAAsp* in E. coli, we first cloned the 5′- and 3′-flanking regions from the full-length tRNA sequence (as above), and the fragment was inserted into p蝎blev to construct the respective tRNA expression vectors.

Northern Hybridization—Total RNAs were extracted from cultured cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method (15). The transformants were selected on minimal medium plus uracil (50 μg/ml) supplied to the minimal medium. The total RNAs extracted from T. thermophilus TTY1 (11) and T. thermophilus HB8 tRNAAsp* were electrophoresed separately in lanes on a 15% denaturing polyacrylamide gel along with the undigested control and alkaline-hydrolyzed tRNAs. Modified nucleotides were identified by the post-labeling method (17).

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Detection of 2-Thio Modification in trNAAsp* and Its Precursor—The 2-thiolation at position 54 in trNAAsp* and its precursor were detected by gel retardation in an electrophoresis system containing 10% polyacrylamide gel (10 × 12 × 0.1 cm) containing 7 M urea polymerized in the presence of 25 or 100 μM [N-acetyl-l-lysino]phenylmercuric chloride (APM), which was synthesized as described by Igoi (19). Total RNA (1.6–3.2 μg) was resolved on the APM gel. After electrophoresis, the gel was soaked in 0.2 M β-mercaptoethanol for 1 h to reduce and/or break the sulfur linkages formed between the thiolated RNA and the APM in the gel. The RNA was transferred onto a nylon membrane (Hybond-N*, Amersham Biosciences) by blotting using TBE Buffer, which was hybridized with the 5′-32P-labeled DNA probes for trNAAsp* or its precursor. The retarded band resulting from the presence of thioacylated nucleotides in RNA was visualized by a Fuji BAS1000 bioimaging analyzer.

Results

Expression of T. thermophilus trNAAsp* Gene with an Altered Sequence in E. coli and T. thermophilus Cells—To detect activity of the sT (54)-thiole in T. thermophilus cells and to investigate its recognition elements in trNAAsp* (Fig. 1), we first expressed an artificial trNAAsp gene encoded on a plasmid. We selected T. thermophilus HBB trNAAsp* as our model trNAAsp species because the presence of sT at position 54 has been verified in this trNAAsp (20). We introduced mutations at 11 base positions of the trNAAsp* (Fig. 1) so that the resultant trNAAsp (named trNAAsp*) could be discriminated from the native trNAAsp* by Northern hybridization using the DNA probes for trNAAsp*. The trNAAsp* was integrated into the trNAAsp* operon of T. thermophilus.
of T. thermophilus cells resulted in strong accumulation of the tRNA precursor CACT (Thermus ever, the heterologous expression of consensus sequence (TTGACA (Thermus can lead to the tRNA expression operon of this gene (TTGACG (Thermus 

function even in E. coli, which can be explained by the likely low copy number of pEx-Asp* in T. thermophilus, because this expression vector has a replication origin derived from pT8TS, whose copy number is about eight (26).

Expressed tRNAAsp* with the s2T (54) Modification—Our next task was to determine whether tRNAAsp* expressed in T. thermophilus cells was processed normally and modified in the same manner as the native tRNA and, in particular, whether or not it contained s2T. To isolate tRNAAsp*, total RNA from T. thermophilus cells harboring pEx-Asp* was fractionated by anion exchange chromatography so as to enrich the fractions that included tRNAAsp*. In this step, we were able to successfully concentrate the tRNAAsp* fractions and exclude those containing the precursor. tRNAAsp* was then purified to homogeneity by solid-phase DNA probing (Fig. 3A). End-labeling with 32P and sequencing by Donis-Keller’s enzymatic digestion method (Fig. 3B) showed that the purified tRNAAsp* had the expected sequence, including both the 5’ and 3’ ends. Abnormalities in the bands suggested the presence of some modified residues (the expected modifications are parenthesized in Fig. 3B). The primary sequence of tRNAAsp* was further determined by post-labeling and LC/MS analysis, which enabled us to identify 7 post-transcriptional modifications at 8 positions: 4-thiouridine (s2U) at position 8, pseudouridine (Ψ) at positions 13 and 55, 2’-O-methylguanosine (m1G) at position 18, dihydrouridine (D) at positions 20 and 20a, 1-methyladenosine (m2A) at position 58, ribothymidine (T) at position 54, and 2-thioribothymidine (s2T) at position 54 (Fig. 3C). The post-labeling method clearly identified both T and s2T at position 54 (data not shown), which is consistent with a previous report that s2T is derived from a partial modification of T induced by the cultivation temperature (6). The presence of s2T at position 54 was further examined by LC/MS with RNAse T1-digested fragments of tRNAAsp* (Fig. 4). Although the frag-

strain HB8 by replacing the tRNAAsp* gene, as depicted in Fig. 1, A and B). The reason for employing this strategy is that the tRNAAsp* operon is the only class I tRNA cluster, was synthesized by PCR using six DNA oligos and ligated into the vector, pT8 (11), resulting in an expression vector for tRNAAsp* (pEx-Asp*).

Total RNA from cells of E. coli JM109 harboring pEx-Asp* was separated by 10% denaturing PAGE followed by Northern hybridization with the probe AS-2. Two distinct RNA bands associated with tRNAAsp* could be observed (Fig. 2A). The longer band (asterisk), which appeared in close proximity to 5 S rRNA, was considered to be a precursor of tRNAAsp*. The shorter band, located in the E. coli class I tRNA cluster, was presumed to be the mature form of tRNAAsp*. The result demonstrated that the tRNA operon from T. thermophilus could function even in E. coli cells, which can be explained by the fact that the promoter sequence of this gene (TTGACG (−35)/TATACT (−10)) (Fig. 1A) is similar to the E. coli tRNA promoter consensus sequence (TTGACA (−35)/TATAAT (−10)) (21). However, the heterologous expression of Thermus tRNA in E. coli cells resulted in strong accumulation of the tRNA precursor (−70% of the total expression), indicating that the slight disparity with the consensus sequence and the high copy number of the shuttle vector derived from pUC118 in E. coli cells (22, 23) may have led to the tRNA precursor being produced too abundantly to be processed sufficiently by tRNA maturases such as RNase P (24) or 3’ ribonucleases (25).

When the expression of tRNAAsp* in T. thermophilus was examined by Northern hybridization with the probe AS-1 (Fig. 2B), tRNAAsp* originating from the vector was detected mainly in the mature form, although as much as 30% of the total product was observed as the putative precursor (asterisk). Judging from the intensity of the Northern blotting, the expression of total RNA in T. thermophilus cells was lower than that in E. coli cells. This can be accounted for by the likely low copy number of pEx-Asp* in T. thermophilus, because this expression vector has a replication origin derived from pT8TS, whose copy number is about eight (26).

FIG. 2. Detection of expressed tRNAAsp* by PAGE followed by Northern hybridization. A, total RNA (0.8 μg) from cells of MC1061/pT8leuB and JM109/pEx-Asp* was separated by 10% denaturing gel electrophoresis, and the gel was stained by ethidium bromide (lanes 1 and 2, respectively). Northern hybridization patterns of the same gel with the probe AS-2 are shown in lanes 3 and 4, respectively. tRNAAsp* and its precursors are indicated by the arrow and asterisk, respectively. B, electrophoresis on 10% denaturing PAGE of total RNA (1.6 μg) from TTY1/pT8leuB (lane 1), TTY1/pEx-Asp* (lane 2), and their Northern blots with the probe AS-1 (lanes 3 and 4, respectively). The arrow and asterisk show tRNAAsp* and its precursors, respectively.
Our experimental results demonstrated that tRNA^{Asp*} was expressed in T. thermophilus cells in the mature form and that s^2T was detected even though the LC/MS was highly sensitive, suggesting that tRNA^{Asp*} preferentially possesses s^2T at position 54.

Our experimental results demonstrated that tRNA^{Asp*} was expressed in T. thermophilus cells in the mature form and that it carried 8 modifications including s^2T at position 54. We thus successfully detected s^2T synthesis activity in vivo.

Detection of 2-Thiouridylation at Position 54 in tRNA^{Asp*} by APM Gel Electrophoresis—Because the procedures we employed to detect s^2T54 are unsuitable for routine assays of multiple samples, we searched for a simpler method that does not require purification of the expressed tRNA. Igloi (19) reported an affinity electrophoresis system in which a polyacrylamide gel is co-polymerized with APM. In this system, which was reported to be successful in detecting tRNA with the s^U or 5-methylaminomethyl-2-thiouridine modification (19), the electrophoretic mobility of thiolated tRNAs is retarded compared with that of non-thiolated tRNAs due to the specific interaction between the thiocarbonyl group and the mercuric compound. By combining this technique with Northern hybridization using a probe specific for the relevant tRNA, the thiolated nucleosides in a particular tRNA species can be detected (27). We therefore used this approach to identify the 2-thiouridylation at position 54 in tRNA^{Asp*} expressed in T. thermophilus cells.

When APM gel electrophoresis was performed, specific retardation of tRNA^{Asp*} was observed (data not shown). However, because tRNA^{Asp*} has 2 thiouridines, s^U8 and s^2T54, this retardation would have been mediated by both of them. To differentiate gel retardation due to 2-thiolation at position 54 from that caused by s^U8, we first eliminated s^U8 from the expressed tRNA^{Asp*} by introducing a U to A point mutation at position 8 in the expression vector pEx-Asp*. As shown in Fig. 5A (lanes 1 and 4), the mutant tRNA^{Asp*} (USA) without the s^U8 modification was expressed in T. thermophilus cells.

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**Fig. 3.** Characterization of expressed tRNA^{Asp*}. A, purification of tRNA^{Asp*}. Total RNA of TTY1/pEx-Asp* (0.4 μg; lane 1) and the purified tRNA^{Asp*} (0.08 μg; lane 2, shown by the arrow) were separated by 10% denaturing gel electrophoresis and stained by ethidium bromide. B, determination of expressed tRNA^{Asp*} sequence by Donis-Keller’s method (16). The results of 3′-labeled (left) and 5′-labeled samples (right) are shown. Control, L, G, A, U, C, and C > U indicate no treatment and digestion by alkali, RNase T1, RNase U1, RNase PhyM, and RNase CL4, respectively. The nucleotide sequence is shown at the right side of each figure. The expected modifications are in parentheses. C, cloverleaf structure of the in vivo expressed tRNA^{Asp*} determined in this study. The s^2T at position 54 may be partially replaced by ribothymidine (T). The other modified nucleotides are 4-thiouridine (s^4U), pseudouridine (ψ), methylguanosine (Gm) at position 18, dihydrouridines (D) at positions 20 and 21, and 1-methyladenosine (m^1A) at position 58.

**Fig. 4.** Mass spectrometric analysis of oligonucleotides derived from tRNA^{Asp*}. A, mass chromatograms of oligonucleotides obtained by RNase T1 digestion of tRNA^{Asp*}. The upper panel shows base peaks in the mass chromatogram of m/z = 620–2000, within which each oligonucleotide was detected. Peak numbers represent RNA fragments derived from tRNA^{Asp*}: 1, m^1AGp; 2, CGp; 3, UGp; 4, AGp; 5, ψAGp; 6, UGp; 7, UUGmGp; 8, ACCGp; 9, AUCGp; 10, UCCUGp; 11, UCACGp; 12, UACCCGp; 13, Us^U8Gp; 14, DDAACACCGp. The middle and lower panels show mass chromatograms of m/z = 1309 and m/z = 654 for [M-H]^− and [M-2H]^2− ions of s^2T4CGp, respectively. B, mass spectrum in the range 19.64–20.50 min. [M-H]^− and [M-2H]^2− ions of s^2T4CGp were detected. Single and double asterisks, respectively, indicate the [M-H]^− ion and [M-2H]^2− ion peaks derived from UUGmGp, which gave the same retention time.
The electrophoretic mobilities were normalized by the position of 5S tRNA. The band corresponding to the expressed tRNAAsp*(U8A) was retarded in accordance with the concentration of APM. Next, which contains no thiolated nucleotides, the tRNA cluster was isolated and confirmed the feasibility of using this simple assay system in vivo. 5-methylaminomethyl-2-thiouridine, by the APM method (19), was increased (lane 6) from 3′-end heterogeneity of transcription termination (28) and/or 3′-trimming (25). All the precursors were shifted in the APM gel (Fig. 6, lane 4), from which we conclude that 2-thiolation occurs in the precursor form with both 5′-leader and 3′-trailer sequences. Hence, thermophile-specific 2-thiolation most probably precedes tRNA processing.

Evidence That 2-Thiolation Precedes tRNA Processing—In the APM gel electrophoresis, specific retardation of the tRNAAsp*(U8A) precursor was observed (asterisks in lanes 4–6 of Fig. 5A) and verified by the fact that the tRNAAsp*(U8A/U54A) precursor showed no such retardation (Fig. 5B, lanes 10–12). These observations suggested that 2-thiolation at position 54 occurs in the precursor form and precedes tRNA processing. To investigate this hypothesis experimentally, we constructed the vector tRNAAsp*(U8A/G19C). As shown in Fig. 6, lanes 3 and 4), precursors of tRNAAsp*(U8A/G19C) (asterisks) were shifted in the APM gel in the same manner as the mature tRNA (indicated by arrows), which is a clear indication that the 2-thiolation occurred in precursor tRNAs.

To differentiate the type of precursor tRNA that is 2-thiolated, we performed Northern blot analysis. Two oligo DNA probes, AS-5′ and AS-3′, were designed to detect tRNA precursors with 5′-leader and 3′-trailer sequences, respectively (Fig. 1B). The fact that the mature tRNA was not detected in lanes 1 and 2 of Fig. 6 shows that these probes were able to specifically detect precursor tRNAs. Because the longer precursors (asterisks in Fig. 6) were hybridized by both probes, this type of precursor was considered to have the 5′-leader and 3′-trailer sequences. The shorter precursors (*) were only detected by AS-3′, suggesting that they have the 3′-trailer sequence but no 5′-leader. Primer extension analysis revealed that the 5′ end of the precursors was at position 7 from the 5′ end of mature tRNA (Fig. 6B). Judging from the presence of heterologous Northern bands (Fig. 6A), the longer and shorter precursors contained several species of different sizes. Because the primer extension analysis indicated that these precursors have a uniform 5′ end, it is presumed that their unequal lengths arise from 3′ end heterogeneity of transcription termination (28) and/or 3′-trimming (25). All the precursors were shifted in the APM gel (Fig. 6, lane 4), from which we conclude that 2-thiolation occurs in the precursor form with both 5′-leader and 3′-trailer sequences.
tRNA structure is not required for 2-thiolation at position 54. To identify which sites are required, we first examined the effect of D loop-T loop interaction. We did so because earlier work revealed that s2T plays a critical role in stabilizing the interaction between the D and T loops by extending the A-form double-stranded helix into the T loop (10), thereby giving stability to the tRNA structure as a whole. To disrupt the D-T loop interaction, we constructed three mutants bearing point mutations at G18 (G18A, G18U, and G18C) and one with a mutation at G19 (G19C), because both G18 and G19 are involved in the tertiary interaction between the two loops (29, 30). When these mutated tRNAs were subjected to APM gel electrophoresis, they were all retarded in the APM gel (Fig. 7A), from which we concluded that disruption of D-T loop interaction has no effect on the 2-thiolation at position 54. Therefore, the T loop itself appeared to be the most likely recognition site for s2T-thiolase.

To verify this, we investigated 18 point mutations at all the T loop positions of tRNA8(U8A) except for U54 (i.e. at positions 55–60), in which each base was systematically substituted by each of the other three bases (Table I; Figs. 7B and 8A). The mutants bearing U55A, U55G, C56G, C56U, A58C, A58G, and A58U gave no mature tRNA detectable by Northern hybridization but only strong accumulations of longer precursors (data not shown). The most likely reason is that these mutations had a marked influence on the tRNA processing activity similar to the effect of the U54A mutation noted earlier (Fig. 5). In these cases, we evaluated the 2-thiolation activity of the shifts from the precursors, because we demonstrated above that 2-thiolation occurs in the precursor form when both the 5'-leader and 3'-trailer sequences are present (Fig. 6). Seven of the mutations, G57A, G57C, G57U, G59A, G59C, G59U, and U60G, had no influence on the 2-thiolation activity (Table I and Fig. 7B).

A slight reduction in 2-thiolation activity was observed in the case of mutant U55C (Fig. 7B), whereas U55A and U55G showed no activity (Table I). Although conserved U55 (normally modified to s2T in the cell) is involved in the tertiary base-pairing of G18 and U55 (29, 30), the slight reduction in the thiolation of the U55C mutant is considered to result from sequence-specific recognition by s2T-thiolase and not from disruption of D-T loop interaction, because there was no effect on the 2-thiolation of three G18 mutants (Fig. 7A).

| Mutationa | Expression of mature tRNAb | 2-Thiolationc |
|-----------|---------------------------|--------------|
| D loop    |                          |              |
| G18A      | +                        | +            |
| G18C      | +                        | +            |
| G18U      | +                        | +            |
| G19C      | +                        | +            |
| T loop    |                          |              |
| U55A      | -                        | -            |
| U55C      | +                        | -            |
| U55G      | -                        | -            |
| C56A      | +                        | +            |
| C56G      | -                        | -            |
| C56U      | -                        | -            |
| G57A      | +                        | +            |
| G57C      | +                        | +            |
| G57U      | +                        | +            |
| A58C      | -                        | -            |
| A58G      | -                        | -            |
| A58U      | -                        | -            |
| G59A      | +                        | +            |
| G59C      | +                        | +            |
| G59U      | +                        | +            |
| U60A      | +                        | +            |
| U60C      | +                        | +            |
| U60G      | +                        | +            |

a Mutants of tRNA8(U8A) are shown. b + represents expression of mature tRNA; – represents no expression of mature tRNA (longer precursors only were expressed).

In the cases of mutants expressing mature tRNA, the extent of 2-thiolation modification was determined as the ratio of the band strength at the shift position in the presence of APM to that of the whole amount of total expressed RNA and classified as fully modified (+, 1–0.65), partially modified (≥, 0.65–0.25), or not modified (<, 0.25–0). In the cases of mutants not expressing mature tRNA, the band-shift tendency of the precursor is indicated as + or – (although only – is presented in the table).

All the position 57 mutants, G57A, G57C, and G57U, were well thiolated (Fig. 7B), which strongly suggests that s2T-thiolase does not recognize the conserved purine at position 57 (Fig. 8B).

Mutation at G59 to any other nucleoside (G59A, G59C, and G59U) did not affect the 2-thiolation activity (Fig. 7B), which is consistent with the fact that any of the four nucleosides can occur at position 59 in the T. thermophilus tRNA consensus sequence (Fig. 8B).

In the case of position 60, the U60G and U60C mutants were both 2-thiolated with efficiencies similar to and slightly lower than that of the wild type, respectively. In contrast, mutant U60A was not 2-thiolated (Fig. 7B). Because a pyrimidine is located at position 60 in T. thermophilus tRNAs (Fig. 8B), it is reasonable that the U60C mutation resulted in the formation of a T stem with 6 base pairs, destroying the canonical 7-membered T loop structure, which may not be recognized by s2T-thiolase.

A strong reduction in 2-thiolation activity was observed in the cases of mutations at positions C56 and A58 (which is normally modified to m3A58 (1-methyladenosine at position 58) in cells) (Fig. 7B, Table I). These bases are highly conserved residues in Thermus tRNAs (Fig. 8B), and A58 is directly involved in tertiary base pairing with the target site U54 (which is normally modified to either T or s2T) (10). Taking these facts into consideration together with our results, it is supposed that 2-thiolation activity at position 54 requires the canonical T loop structure formed by the conserved U54-A58 interaction together with the conserved residue C56.
s^2T Biosynthesis in Thermus thermophilus tRNA

Fig. 8. Effects of mutations at each position in the T loop on 2-thiolation at position 54 and T arm sequences of T. thermophilus tRNAs. A, the 2-thiolation pattern obtained from this study (Fig. 7 and Table I). Only data for mutants that could be thiolated are included. The bases shown as gray circles are strictly required for 2-thiolation; those shown as white circles are not strictly required. The mutant U55C is partially thiolated. B, T loop consensus sequence derived from sequences of 11 species of T. thermophilus tRNAs contained in the tRNA Compilition Data base (4). Positions 54, 55, 56, and 58 (gray circles) are completely conserved, whereas positions 57 and 60 are conserved as a purine and a pyrimidine, respectively. Only position 59 is not conserved. The dashed line between U54 and A58 depicts the reverse Hoogsteen base pair.

DISCUSSION

Although almost 30 years have passed since we first found s^2T54 in tRNAs from T. thermophilus (3), there has been no report during that time on the biosynthetic pathway of the s^2T modification or on the identification of a putative s^2T-thiolase, the key enzyme presumed to be responsible for the thermal adaptation of protein synthesis. In the present study, we first attempted to detect s^2T thiolation activity in T. thermophilus cells by utilizing an E. coli-T. thermophilus shuttle vector (11), into which the T. thermophilus tRNA^{Asp}_{s} gene with an altered sequence (tRNA^{Asp}_{s}^{54}) was introduced. This strategy enabled us to successfully express the non-native tRNA^{Asp}_{s} in T. thermophilus cells and identify s^2T54, thereby demonstrating that s^2T-thiolase activity could in fact be detected in T. thermophilus cells.

Several approaches have been employed to elucidate recognition mechanisms of tRNA modification enzymes toward tRNA. If the relevant enzyme(s) can be purified or a recombinant enzyme is available, it may be possible to reconstruct the modification reaction in vitro using unmodified tRNA as a substrate. There has been much work in this area, especially with methyltransferases (31). Almost all known methyltransferases utilize S-adenosylmethionine as a common methyl donor, and methylation can be detected from the radioactivity of the ^{3}C- or ^{3}H-labeled methyl group. If an enzyme(s) cannot be purified, its characterization in vivo may be possible, as was done, for example, by microinjecting labeled-tRNA into the cytoplasm of the Xenopus oocyte (32). However, this approach does not always reflect the in vivo situation, particularly when heterologous tRNA species are examined.

Because the putative s^2T-thiolase in T. thermophilus cells has never been identified or isolated, we developed our in vivo characterization method involving expression of the non-native tRNA^{Asp}_{s}. Although artificial, the tRNA^{Asp}_{s} gene was normally expressed to give a mature tRNA product with the full sequence and nucleoside residue modifications expected (Fig. 3C). Although both s^2T54 and T54 were detected by postlabeling, only s^2T54 was detected in the LC/MS analysis (Fig. 4), indicating the preponderance of the 2-thioribothymidine modification in tRNA^{Asp}_{s}. The native tRNA^{Asp}_{s} from T. thermophilus HB8 was reported to possess 50% s^2T54 when cultured at 70 °C (20). The apparent difference in the degree of 2-thiolation at position 54 in the native and non-native tRNAs^{Asp}_{s} may arise from dissimilarities in the amounts of tRNA expressed, the altered sequence of the constructed tRNA, and/or the fact that the host strains were different (tRNA from strain TTY1, a derivative of strain HB27, has a higher molar content of s^2T than strain HB8 (33)).

The combination of APM gel electrophoresis and Northern hybridization proved to be a simple and sensitive method for the systematic analysis of 2-thiolation at position 54 in the series of tRNA mutants that we constructed. Using this method, we found that 2-thiolation precedes the processing of the tRNA precursor. Although we do not know whether 5-methylation of U54 occurs in the precursor form, it has been shown that m^5U is synthesized in the tRNA^{Tyr}_{s} precursor with a 5'-leader sequence when it is expressed in E. coli (34) and that m^5U occurs in intron-containing tRNA^{Pho}_{s} and tRNA^{Tyr}_{s} precursors from Saccharomyces cerevisiae (35). As Grosjean et al. have speculated (32), this 5'-methyl modification might stabilize tRNA precursors to promote the subsequent processing and other modification steps. We have shown here that T. thermophilus precursor tRNAs are also thiolated at residue 54, which probably occurs together with methylation at position 5 of U54 in the early stages of tRNA processing. Our finding is consistent with the evidence for mesophilic tRNAs. In T. thermophilus, the 2-thiolation apparently contributes particularly to the structural stabilization of precursor tRNAs at higher temperatures.

Our experiments with a series of structural mutants enabled us to identify the s^2T (54)-thiolase recognition elements in tRNA. The determinants for 2-thiolation were ascertained to be conserved T loop residues, and the conformation of the T loop formed with these residues. The U54-A58 reverse Hoogsteen base pair is especially important for 2-thiolation. The residues necessary for recognition by the s^2T-thiolase (Fig. 8A) were revealed to be broader than those making up the T. thermophilus tRNA T loop consensus sequence (Fig. 8B). This raises the possibility that almost all tRNA species can be recognized and modified to s^2T54 and is consistent with the fact that 2-thiolation of T54 occurs in 68% of unfractonated tRNAs in T. thermophilus HB27 cultured at 80 °C (33).

Two distinct modification steps are involved in s^2T synthesis, 5-methylation and 2-thiolation. Because we have never detected 2-thiouridine at position 54 either in the present work or in many previous studies (2, 36), 5-methylation of U54 most probably occurs before its 2-thiolation. This raises the question whether 2-thiolation of U54 requires 5-methylation. The modification enzyme for 5-methylation of U54, known as tRNA(m^5)U54-methyltransferase (RUMT), is encoded in trmA in E. coli (37) and in TRM2 in S. cerevisiae (38). The recognition pattern of the E. coli RUMT has been investigated (39, 40). It is most likely that the T. thermophilus counterpart of RUMT has similar characteristics to the E. coli enzyme because the two species are both eubacteria. If this is the case, most of the s^2T-thiolase recognition sites would be shared with RUMT, because it is known that RUMT strictly recognizes the conserved T loop structure and that D loop-T loop interaction is not necessary for this recognition (39, 41), which is exactly what we observed in this study. On the other hand, a distinct difference in the recognition mechanisms of these two modification enzymes is evident with respect to residue 57. We found that in T. thermophilus the G57C mutation caused no reduction in 2-thiolation (Figs. 7B and 8), whereas the same mutation resulted in a drastic decrease in E. coli RUMT recognition activity to about 4% of the k_{cat}/K_{m} of the wild-type tRNA (39). This difference in sensitivity of the mutation at residue 57 may mean that 2-thiolation and 5-methylation of U54 are independent modifications. It has been reported that in E. coli tRNAs, C-5 modification and 2-thiolation in the biosynthesis of 5-methylamino-methyl-2-thiouridine at position 34 are independent processes (42–44). The same may be true for 5-methylation and 2-thio-
vation of U54. Another possibility is that the apparent difference in the recognition mechanisms of s^2'^T-thiolase and RUMT may arise from variances between in vivo and in vitro experiments. In any event, further investigations, including the use of a deletion strain of the trnA gene in *T. thermophilus* cells, are needed to elucidate this.

A clearer picture of the s^2'^T-thiolase recognition mechanism and temperature-dependent control of the T to s^2'^T ratio in tRNA is likely to emerge when s^2'^T-thiolase is isolated and the 2-thiolation reaction is reconstituted in vitro, which are the goals of work currently in progress.

Acknowledgment—We thank Takeo Suzuki for analysis of tRNA-modified nucleosides by LC/MS.

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