The Role of Posttranscriptional Modification in Stabilization of Transfer RNA from Hyperthermophiles†

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ABSTRACT: The influence of posttranscriptional modification on structural stabilization of tRNA from hyperthermophilic archaea was studied, using *Pyrococcus furiosus* (growth optimum 100 °C) as a primary model. Optical melting temperatures (T_m) of unfractionated tRNA in 20 mM Mg^{2+} are 97 °C for *P. furiosus* and 101.5 °C for *Pyrodictium occultum* (growth optimum, 105 °C). These values are ~20 °C higher than predicted solely from G-C content and are attributed primarily to posttranscriptional modification. Twenty-three modified nucleosides were determined in total digests of *P. furiosus* tRNA by combined HPLC–mass spectrometry. From cells cultured at 70, 85, and 100 °C, progressively increased levels of modification were observed within three families of nucleosides, the most highly modified forms of which were N^4-acetyl-2′-*O*-methylcytidine (ac^4Cm), N^2,N^2,2′-*O*-trimethylguanosine (m_2^2Gm), and 5-methyl-2-thiouridine (m^5s^2U). Nucleosides ac^4Cm and m_2^2Gm, which are unique to the archaeal hyperthermophiles, were shown in earlier NMR studies to exhibit unusually high conformational stabilities that favor the C3′-*endo* form [Kawai, G., et al. (1991) *Nucleic Acids Symp. Ser. 21*, 49–50; (1992) *Nucleosides Nucleotides 11*, 759–771]. The sequence location of m^5s^2U was determined by mass spectrometry to be primarily at tRNA position 54, a site of known thermal stabilization in the bacterial thermophile *Thermus thermophilus* [Horie, N., et al. (1985) *Biochemistry 24*, 5711–5715]. It is concluded that selected posttranscriptional modifications in archaeal thermophiles play major stabilizing roles beyond the effects of Mg^{2+} binding and G-C content, and are proportionally more important and have evolved with greater structural diversity at the nucleoside level than in the bacterial thermophiles.

The discovery, cultivation, and phylogenetic classification of hyperthermophiles in earlier years [reviewed by Stetter (1992)] has raised substantial questions concerning structural stabilization of macromolecules in these organisms, which grow optimally in the range 80–105 °C. Transfer RNA, because of its highly conserved structure and the corresponding wealth of knowledge concerning secondary and tertiary structural features in tRNA from mesophiles, constitutes a useful target for understanding mechanisms of stabilization at the molecular (mononucleotide) level. In addition, current knowledge of the role of posttranscriptional modification of tRNA in relation to structure–function relationships (Björk et al., 1987; Björk, 1992), including cellular adaptation to stress (Buck & Ames, 1984), suggests that such modifications may be important for structural stabilization of RNA from high-temperature organisms. This thesis is supported by earlier work in two areas. First, studies of the effects of cultivation temperature in the bacterial thermophiles *Bacillus stearothermophilus* (optimal growth, 57 °C; Agris et al., 1973) and *Thermus thermophilus* (75 °C; Watanabe et al., 1976) have demonstrated greater levels of tRNA modification in each organism as a function of increasing temperature. The most important of these earlier studies have demonstrated that, at position U-54 of the T-loop, methylation to produce m^5U results in a 6 °C melting temperature (T_m) increase in *Escherichia coli* $tRNA^{Met}$ (Davanloo et al., 1979), while thiolation of m^5U-54 to form m^5s^2U (s^2T) in *T. thermophilus* $tRNA^{Ile}$ correlates with a 2.9 °C increase in T_m (Horie et al., 1985).[1] Second, it is clear that posttranscriptional modifications in the archaeal thermophiles have in part followed different routes of evolutionary development than in the bacterial thermophiles, producing an unusually large number of tRNA nucleosides unique to the archaeal domain, most of which are found specifically in tRNAs from the hyperthermophiles (Edmonds et al., 1991), across both major archaeal kingdoms, the Euryarchaeota and the Crenarchaeota (Woese et al., 1990).

We presently report results of a detailed study of nucleoside modification patterns in the unfractionated tRNA from the

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[1] Abbreviations: m^5U or T, 5-methyluridine or ribosylthymine; Ψ, pseudouridine; $m^1Ψ$, 1-methylpseudouridine; s^2C, 2-thiocytidine; m^5C, 5-methylcytidine; m^1A, 1-methyladenosine; Cm, 2′-*O*-methylcytidine; I, inosine; Um, 2′-*O*-methyluridine; m^1I, 1-methylinosine; m^1G, 1-methylguanosine; Gm, 2′-*O*-methylguanosine; ac^4C, N^4-acetylcytidine; m^2G, N^2-methylguanosine; m^5s^2U (or s^2T), 5-methyl-2-thiouridine; m_2^2G, N^2,N^2-dimethylguanosine; Am, 2′-*O*-methyladenosine; t^6A, N^6-[(threonyl)carbamoyl]adenosine; ac^4Cm, N^4-acetyl-2′-*O*-methylcytidine; m^6A, N^6-methyladenosine; hn^6A, N^6-[(3-hydroxynorvalyl)carbamoyl]adenosine; m_2^2Gm, N^2,N^2,2′-*O*-trimethylguanosine; ms^6hn^6A, 2-methylthio-N^6-[(3-hydroxynorvalyl)carbamoyl]adenosine; mimG, 3-(β-D-ribofuranosyl)-4,9-dihydro-4,6,7-trimethyl-9-oxoimidazo[1,2*a*]purine; mnm^5s^2U, 5-[(methylamino)methyl]-2-thiouridine; mcm^5s^2U, 5-[(methoxycarbonyl)methyl]-2-thiouridine; s^2U, 2-thiouridine; s^2Um, 2-thio-2′-*O*-methyluridine; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; t_R, chromatographic retention time; LC/MS, directly combined high-performance liquid chromatography–mass spectrometry; M, molecule; MH^+, protonated molecule; BH_2^+, fragment ion corresponding to the protonated free base derived from a nucleoside containing the base moiety B.

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extremely thermophilic archaeon *Pyrococcus furiosus*, which exhibits a growth optimum at 100 °C (Fiala & Stetter, 1986). Melting temperatures were measured for transfer RNAs from *P. furiosus*, and for comparison from the most thermophilic known organism, *Pyrodictium occultum* (optimal growth, 105 °C; Stetter, 1982), to establish whether overall tRNA stability is significantly greater than that estimated from G-C content. Modified nucleosides in hydrolysates of unfractionated tRNA from *P. furiosus* grown at 70, 85, and 100 °C were determined by directly combined liquid chromatography–mass spectrometry, a method capable of providing a rigorous census of modifications at the nucleoside level (Pomerantz & McCloskey, 1990). Only a single isoaccepting tRNA sequence has been reported from archaeal hyperthermophiles, $tRNA^{Met}$ from *Sulfolobus acidocaldarius* (Kuchino et al., 1982), in contrast to 31 tRNA gene sequences (Steinberg et al., 1993). No X-ray crystallographic or NMR data are yet available. In spite of the paucity of current information concerning details of structure in hyperthermophile tRNA, the goal was to establish whether levels of specific posttranscriptional modifications change in response to changes in the temperature of cell growth. As we describe, three families of modifications emerge whose abundances change systematically with cell culture temperature, and all of them have previously documented roles in molecular stabilization.

MATERIALS AND METHODS

Cells. Pyrococcus furiosus, type strain DSM 3638, was grown at 70, 85, and 100 °C in 300-L batch cultures (fermentor HTE, Bioengineering, Wald, Switzerland) in the presence of sulfur as described (Fiala & Stetter, 1986). In the late logarithmic growth phase, cultures were rapidly cooled to 4 °C by using a heat exchanger, and cells were harvested by flow-through centrifugation (Cepa 41G, Padberg, Lahr, Germany). Cell masses were stored under liquid nitrogen. *Pyrodictium occultum*, strain DSM 2709, was cultured as earlier described (Edmonds et al., 1991).

Isolation of tRNA. Transfer RNA was extracted from *P. furiosus* and *P. occultum* cells as described by Kuchino et al. (1987). Final purification of tRNA was accomplished by tube gel electrophoresis (3.5 × 100 mm, 8% polyacrylamide, 7 M urea), using an Applied Biosystems (San Jose, CA) Model 230A micropreparative electrophoresis system. Transfer RNA was precipitated from 0.5× TBE buffer by the addition of 2.0 vol of absolute EtOH, stored overnight at −20 °C, and pelleted by centrifugation. Alternatively, tRNA was purified by treating the crude tRNA isolate with DNase I. One hundred micrograms of crude DNA-containing tRNA was treated with 50 μg of DNase I in 20 mM Tris-HCl and 20 mM $MgCl_2$, pH 7.0, in a total volume of 100 μL. The reaction mixture was incubated at 37 °C for 2 h. The tRNA was then recovered by anion-exchange chromatography with a Nucleogen DEAE 60–7 column (4 × 125 mm) and pre-column (4 × 30 mm) installed on a Beckman (Berkeley, CA) Model 332 liquid chromatograph with a Beckman Model 160 UV monitor with detection at 254 nm. A linear gradient of 0.025 M TEAB, pH 6.4, and 20% CH_3CN to 1.0 M TEAB, pH 7.8, and 20% CH_3CN was used as the eluant.

Transfer RNA Melting Experiments. Melting curves were measured in 10 mM sodium cacodylate buffer, pH 7.2, in the presence or absence of 20 mM $Mg(OAc)_2$. Thermal denaturation profiles of *P. furiosus* tRNA were also acquired in the presence of 14 mM CDTA (no added $Mg(OAc)_2$) to ensure the absence of divalent cations. The buffers were degassed by saturation with helium to prevent bubble formation (Felsenfeld & Sandeen, 1962). Optical melting transitions were measured with an Aviv 118DS spectrophotometer coupled to an IBM PC, capable of UV absorbance measurements at temperatures up to 120 °C. The temperature was increased from 25 to 120 °C at a rate of 0.5 °C/min. The tRNA samples were allowed to equilibrate at 25 °C for 15–20 min before the thermal denaturation profiles were obtained. Absorbance was monitored at 258 nm. Transfer RNA samples were measured in tightly capped Teflon-stoppered Hellma QS 119 spectrophotometer cells of 1-cm path length. A computer-generated differential thermal transition plot (dA/dT) was automatically calculated for each melting profile. The T_m was taken as the peak of the differential thermal transition plot for each sample.

Enzymatic Digestion of Transfer RNA. Transfer RNA was hydrolyzed to nucleosides using nuclease P_1, venom phosphodiesterase, and bacterial alkaline phosphatase as previously reported by Crain (1990). Nine nanomoles of electrophoretically purified *P. furiosus* tRNA (100 °C cell culture) was completely digested to oligonucleotides by RNase T_1 (8800 units) for 30 min at 37 °C, as described by Warrington (1974).

Directly Combined Liquid Chromatography–Mass Spectrometry (LC/MS). Analysis of nucleosides in tRNA digests was carried out with a mass spectrometer consisting of a noncommercial quadrupole mass analyzer, with a thermospray HPLC interface (Vestec Corp., Houston, TX), controlled by a Vector/One data system (Teknivent Corp., St. Louis, MO). The mass spectrometer was directly coupled to a Beckman 322M liquid chromatograph with a Waters 440 dual-wavelength (254 and 280 nm) UV monitor placed in series between the chromatograph and the mass spectrometer. HPLC separations were made using a Supelcosil LC-18S column, 4.6 × 250 mm (Supelco, Bellefonte, PA), and a 3-cm Brownlee Spheri-5 C_{18} precolumn (Applied Biosystems, Foster City, CA), thermostated at 31 °C. The HPLC gradient elution system of Buck et al. (1983), with 0.25 M ammonium acetate (pH 6.0) and acetonitrile, was used with minor modifications in the gradient profile (Pomerantz & McCloskey, 1990). Thermospray ionization mass spectra were acquired every 1.7 s during the 35-min chromatographic separation. The instrument, procedures, and interpretation of data for characterization of nucleosides in RNA hydrolysates have been described in detail (Edmonds et al., 1985; Pomerantz & McCloskey, 1990).

Fractionation of Oligonucleotides. The oligonucleotide mixture resulting from RNase T_1 digestion of *P. furiosus* 100 °C tRNA was resolved using anion-exchange HPLC, as described (Kowalak et al., 1993). Specific DEAE oligonucleotide pools were further fractionated by reversed-phase HPLC (Kowalak et al., 1993), prior to measurement of mass spectra.

Molecular Weight and Composition Analysis of Oligonucleotides by Electrospray Mass Spectrometry. RNase T_1 hydrolysis fragments were dissolved in H_2O and diluted with CH_3OH to 1:9 (v/v) at a final sample concentration of 18 pmol/μL (assuming 1 mol equiv of oligonucleotide per mole of tRNA and quantitative recovery from two chromatographic steps). Samples were continuously infused, at flow rates of 1–2 μL/min, into the electrospray ion source of a Vestec 201 mass spectrometer (Vestec Corp., Houston, TX), as described by Kowalak et al. (1993). Ion profiles of selected regions of interest were obtained from single 8-s mass spectral scans acquired in the calibration mode of a Teknivent (St. Louis, MO) Vector/One data system. Procedures for the calculation of oligonucleotide molecular weights from electrospray mass

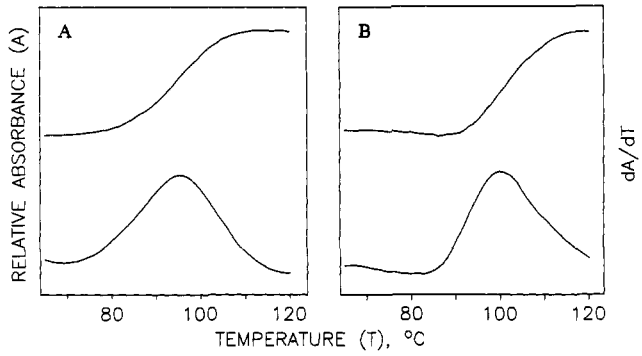

FIGURE 1: Optical melting profiles and corresponding computer-generated differential melting transitions (dA/dT) from unfractionated tRNA (20 mM Mg^{2+}, 10 mM Na^{+}). (A) *Pyrococcus furiosus* (100 °C culture). (B) *Pyrodictum occultum* (105 °C culture).

spectra, and conversion of the resulting values to nucleotide compositions, have been described (Kowalak et al., 1993).

RESULTS

Thermal Melting of tRNA from Pyrococcus furiosus and Pyrodictium occultum. *P. furiosus* was selected to study the effects of cell culture temperature on tRNA modification because the organism adapts well to an unusual range of lower culture temperatures. Unfractionated tRNA isolated from cells cultured at optimum growth temperatures (*P. furiosus*, 100 °C; *P. occultum*, 105 °C) was melted in the presence of 20 mM Mg^{2+}, giving the thermal denaturation curves shown in Figure 1. Both tRNA isolates produced smooth monophasic transitions, with a midpoint of 96.9 °C for *P. furiosus* and 101.5 °C for *P. occultum*.

In the presence of Mg^{2+}, melting profiles of *P. furiosus* tRNA from 85 and 70 °C cultures were indistinguishable from that shown in Figure 1A, with T_m's ~97 °C (data not shown). In the absence of Mg^{2+}, the melting curves (Figure 2) undergo significant and reproducible changes as a function of cell culture temperature. All three curves are clearly multiphasic, and separate thermal transitions become increasingly cooperative as culture temperatures increase. The 85 and 100 °C cultures show major transitions at 74 and 91 °C, with denaturation of the most stable subdomain at 105 °C.

Determination of Posttranscriptional Modification in P. furiosus tRNA by LC/MS. The identities of posttranscriptionally modified nucleosides in enzymatic hydrolysates of *P. furiosus* tRNA from three cell cultures, grown at 70, 85, and 100 °C, respectively, were established by LC/MS. The HPLC chromatogram corresponding to the LC/MS analysis of *P. furiosus* 100 °C tRNA, the optimum growth temperature (Fiala & Stetter, 1986), is presented in Figure 3A. The nucleoside identities indicated were established from HPLC retention times relative to cataloged values (Pomerantz & McCloskey, 1990) and from continuously recorded mass spectra. The ratio of UV absorbances at two wavelengths (A_{254}/A_{280}) was used as an additional criterion for nucleoside identification if the HPLC peak in question was homogeneous (on the basis of mass spectral data). In particular, alignment of characteristic mass spectral ion profiles with UV detection peak profiles, as shown in panel A *vs* panel B of Figure 3, constitutes the principal means of peak assignment and does not require chromatographic separation of each nucleoside component because of the selectivity afforded by mass as a detection parameter. All assignments made in Figure 3A were corroborated in this fashion. Selected ion profiles used for identification of the nucleosides found to be of principal interest (see following section) are shown in Figure 3B.

Twenty-three modified nucleosides were identified in the 100 °C culture tRNA. Four ribonucleosides were found which are unique to the archaea (Edmonds et al., 1991): 1-methylpseudouridine (Pang et al., 1987), N^4-acetyl-2′-*O*-methylcytidine and N^2,N^2,2′-*O*-trimethylguanosine (Edmonds et al., 1987), and 3-(β-D-ribofuranosyl)-4,9-dihydro-4,6,7-trimethyl-9-oxoimidazo[1,2-*a*]purine (McCloskey et al., 1987). Interestingly, 5-methyl-2-thiouridine, m^5s^2U (s^2T), was also found. This constitutes the second known occurrence of this modified residue (or of m^5U) in archaeal tRNA; the first occurrence was in tRNA from the closely related (A.- L. Reysenbach, L. Achenbach-Richter, and N. R. Pace, in preparation) organism *Thermococcus* sp. (Edmonds et al., 1991).

Analysis of tRNA hydrolysates from 85 and 70 °C cell cultures (data not shown) produced HPLC profiles similar to that shown in Figure 3A, with experimentally indistinguishable G-C content (71%) measured using UV detection, suggesting that no change in the proportion of G-C-rich tRNAs occurs as a function of culture temperature. However, marked changes were observed in the relative abundances of some modified nucleosides. One nucleoside was found in 85 and 70 °C tRNAs for which only a trace was observed at 100 °C (not marked in Figure 1A), 5-methyluridine, m^5U (or ribosyl thymine, T): t_R, 14.9 min; MH^+ 259. The ratio of peak heights for pseudouridine and 2′-*O*-methylcytidine, both of which are abundant and completely resolved from other components, was found to be constant in each of the three data sets and was unchanged with respect to the overall modification patterns, implying that the molar content of these residues is insensitive to growth temperature. Using UV detection, the absolute peak height of pseudouridine was used to calculate a correction factor to normalize the peak heights of modified residues within each data set. The normalized peak heights from the modified nucleosides were then compared among the 100, 85, and 70 °C data sets. On the basis of these criteria, the relative abundances of seven modified nucleosides were found to change with increasing growth temperature as shown in Table 1: ac^4C, ac^4Cm, m^2G, m_2^2G, m_2^2Gm, m^5U, and m^5s^2U.

Overall, the data show increasing levels of modification within three families of related nucleosides, leading to the most highly modified forms, ac^4Cm, m_2^2Gm, and m^5s^2U (structures in Figure 4), at the highest culture temperature. The progressive steps of modification that are implied are C → ac^4C → ac^4Cm, G → m^2G → m_2^2G → m_2^2Gm, and U → m^5U → m^5s^2U, for which changes in the relative abundances of the precursors C, G, and U (all present in large molar excess) cannot be reliably measured. Clearly, alternate intermediate pathways may exist, such as Cm → ac^4Cm, an issue which the experiment was not designed to address.

Using weighed amounts of authentic nucleosides, the molar proportions of ac^4Cm, m_2^2Gm, and m^5s^2U relative to A were determined from HPLC chromatographic peak heights, using UV detection. The nucleosides used for this purpose were ac^4C (the same chromophore as ac^4Cm), m_2^2G (for m_2^2Gm), and m^5s^2U. By similar means the molar proportions of the three nucleosides were measured relative to adenosine, which was determined to be 18.4 mol % in unfractionated *P. furiosus* tRNA. The overall molar quantities were then estimated as 1.7% ac^4Cm, 0.72% m_2^2Gm, and 0.77% m^5s^2U in tRNA from the 100 °C cell culture.

To some extent, sequence locations of the temperature-sensitive residues can be inferred from published data, although the need for additional tRNA sequences (rather than gene

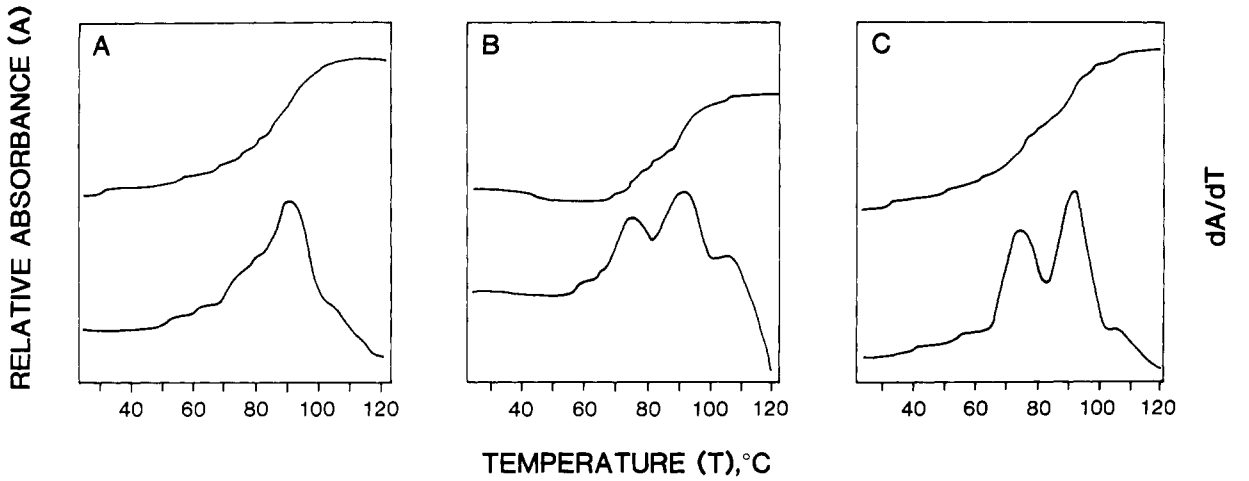

FIGURE 2: Optical melting profiles and corresponding computer-generated differential melting transitions (dA/dT) from unfractionated *Pyrococcus furious* tRNA (without Mg^{2+}; 10 mM Na^+) from cells cultured at (A) 70, (B) 85, and (C) 100 °C.

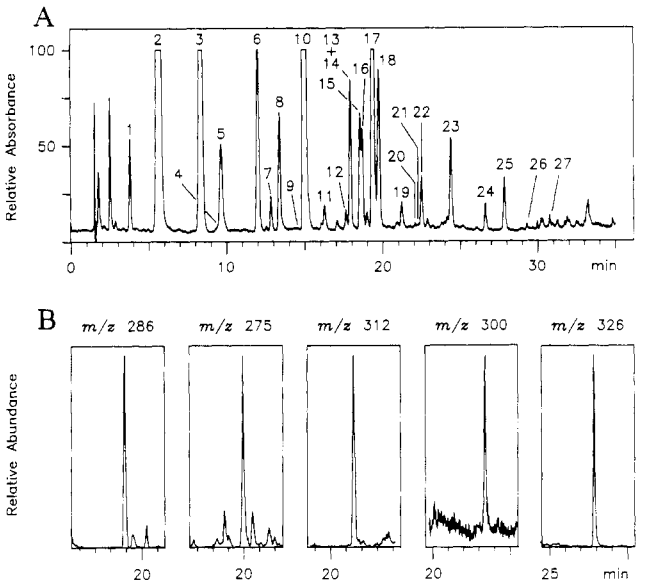

FIGURE 3: Determination of nucleosides from *Pyrococcus furiosus* tRNA (100 °C culture). (A) Separation and characterization of nucleosides from an enzymatic digest of unfractionated tRNA using combined reversed-phase HPLC–mass spectrometry, with UV detection at 280 nm. Nucleoside identities established from chromatographic retention times and thermospray mass spectra: 1, Ψ; 2, C; 3, U; 4, $m^1\Psi$; 5, s^2C; 6, m^5C; 7, m^1A; 8, Cm; 9, I; 10, G; 11, Um; 12, m^1I; 13, m^1G; 14, Gm; 15, ac^4C; 16, m^2G; 17, A; 18, m^5s^2U; 19, m_2^2G; 20, Am; 21, t^6A; 22, ac^4Cm; 23, m^6A; 24, hn^6A; 25, m_2^2Gm; 26, ms^2hn^6A; 27, mimG. Unnumbered peaks were shown from the corresponding mass spectra not to be nucleosides. (B) Ion chromatograms reconstructed from mass spectra, for selective detection using MH^+ ions: ac^4C (*m/z* 286), m^5s^2U (*m/z* 275), m_2^2G (*m/z* 312), ac^4Cm (*m/z* 300), and m_2^2Gm (*m/z* 326). The 6-s offset in time between UV detection (panel A) and mass detection (panel B) is due to the transit time of the eluant between the UV detector and the mass spectrometer.

Table 1: Relative Abundances of Temperature-Responsive Nucleosides from *P. furiosus* tRNA

	cell culture temperature (°C)		
modification	70	85	100
ac^4C	0.08	0.30	1.00
ac^4Cm	0.86	0.87	1.00
m^2G	1.00	0.76	0.75
m_2^2G	1.00	0.57	0.39
m_2^2Gm	0.30	0.74	1.00
m^5U	1.00	0.17	0.04
m^5s^2U	0.65	0.96	1.00

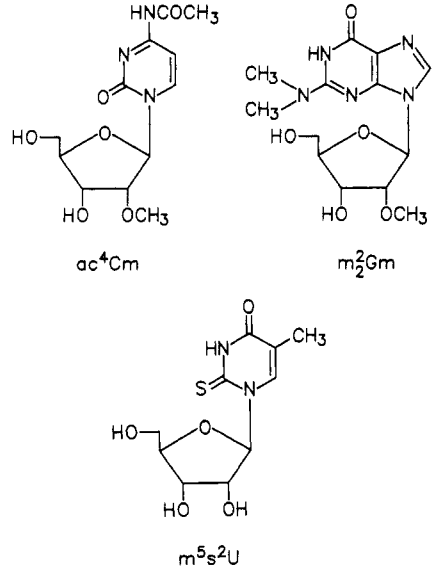

FIGURE 4: Structures of nucleosides from *P. furiosus* tRNA found to increase in relative abundance as a function of cell culture temperature.

sequences) from the archaeal hyperthermophiles is obvious. The sequence position of m^5s^2U was judged to be of particular importance because of earlier studies linking m^5s^2U-54 to tRNA stability in the bacterium *Thermus thermophilus* (growth optimum, 72 °C) [reviewed in Yokoyama et al. (1987)]. However, the rarity of m^5s^2U (or of m^5U) in archaeal tRNA (Edmonds et al., 1991) led to caution in assigning m^5s^2U specifically to the T-loop position 54, at which the presence of m^5U is nearly ubiquitous in eukaryotes and bacteria (Steinberg et al., 1993). Other sequence locations were considered plausible, notably position 34 in the anticodon, a common site for conformational stabilization through post-transcriptional modification (Yokoyama et al., 1985). Several precedents supported the anticodon as a possible site: s^2U (the unmethylated form of m^5s^2U) is known in tRNA from all phylogenetic domains only at the first position of the anticodon [*Drosophila melanogaster* $tRNA^{Glu}$ (Altweg & Kubli, 1980)]. Furthermore, other derivatives of m^5s^2U (e.g., mnm^5s^2U and mcm^5s^2U) are found at the first position of the anticodon in tRNAs that code for lysine, glutamine, and glutamic acid (Ajitkumar & Cherayil, 1988).

The strategy employed to establish the location of m^5s^2U was similar to that of Watanabe et al. (1983), involving the

digestion of unfractionated tRNA using RNase T_1, with subsequent isolation and characterization of m^5s^2U-containing oligonucleotides. Several factors were used to predict that m^5s^2U-bearing nucleotides would be in high abundance. The abundance of m^5s^2U in Figure 3A was estimated from HPLC peak height (UV detection) to constitute about 0.77 mol % of the nucleoside population, suggesting that most tRNAs contain this modification [compared with 0.52% in unfractionated *T. thermophilis* tRNA (Watanabe et al., 1976)]. Cm is completely conserved at position 56 in all reported archaeal tRNA sequences, compared with universal absence of modification at this position in bacteria and eukaryotes (Gupta, 1985; Steinberg et al., 1993), providing a required marker in a T-loop-derived RNase T_1 fragment. Finally, G is nearly ubiquitous at position 53 and is in one-third of the hyperthermophile gene sequences at position 57 (Steinberg et al., 1993), providing a reasonable population of potential RNase T_1 cut sites in the unfractionated *P. furiosus* tRNAs.

Analysis of m^5s^2U in RNase T_1 Oligonucleotides from Pyrococcus furiosus 100 °C tRNA. To establish the sequence location of m^5s^2U in *P. furiosus* tRNA, electrophoretically purified unfractionated tRNA from the 100 °C cell culture was digested with RNase T_1 and the resultant oligonucleotide mixture was fractionated by DEAE anion-exchange chromatography (data not shown) for analysis by mass spectrometry (Kowalak et al., 1993). LC/MS analysis of nuclease P_1–alkaline phosphatase digests of each major anion-exchange fraction showed that m^5s^2U (t_R, 20.0 min; MH^+ *m/z* 275; BH_2^+ *m/z* 143) occurred principally in the oligonucleotide tetramer fraction, with appreciable amounts in other fractions (data not shown). The tetranucleotide pool was then further separated into 10 fractions by reversed-phase HPLC (data not shown) to reduce the complexity of the mixture for subsequent analysis of each fraction by electrospray ionization mass spectrometry.

The electrospray mass spectrum of HPLC fraction 8 from the anion-exchange tetramer pool is shown in Figure 5A. Ions in Figure 5 at *m/z* 661.35 (centroid) and 440.5 (estimate, not centroided) are assigned to the $(M - 2H)^{2-}$ and $(M - 3H)^{3-}$ ions of an oligonucleotide with M_r determined from the $(M - 2H)^{2-}$ ion as 1324.70. Extensive calculations for molecular mass versus subunit composition for oligonucleotides generated by RNase T_1 hydrolysis show that the base composition can usually be directly determined by measurement of molecular weight within ±0.01% (Pomerantz et al., 1993). Interestingly, there are no allowable base compositions for an RNase T_1 oligonucleotide of M_r 1324.7 with consideration of zero through three methyl groups. However, if the replacement of an oxygen atom by sulfur is considered [a modification common in bacterial thermophiles (Watanabe et al., 1976)], then a single composition is allowed: $CU_2Gp + 2CH_2 - O + S$ [M_r (calc) 1324.9]. This derived composition and net modification is consistent with the oligonucleotide $m^5s^2U\Psi CmGp$, although several other isomers are possible (following section).

LC/MS Analysis of the Oligonucleotide of M_r 1324.7. To confirm the composition concluded from the measured mass of the oligonucleotide, it was enzymatically hydrolyzed to nucleosides. The chromatogram corresponding to the reversed-phase LC/MS analysis of the resultant hydrolysate, Figure 5B, shows the presence of Ψ, Cm, G, and m^5s^2U. Therefore, of the three posttranscriptional modification units inferred from accurate molecular mass data, one methyl resides specifically at O-2′ of cytidine, and the second, at C-5 of uridine, with replacement of O by S at position O^2 in the same molecule (m^5s^2U). Pseudouridine, the only posttranscriptional

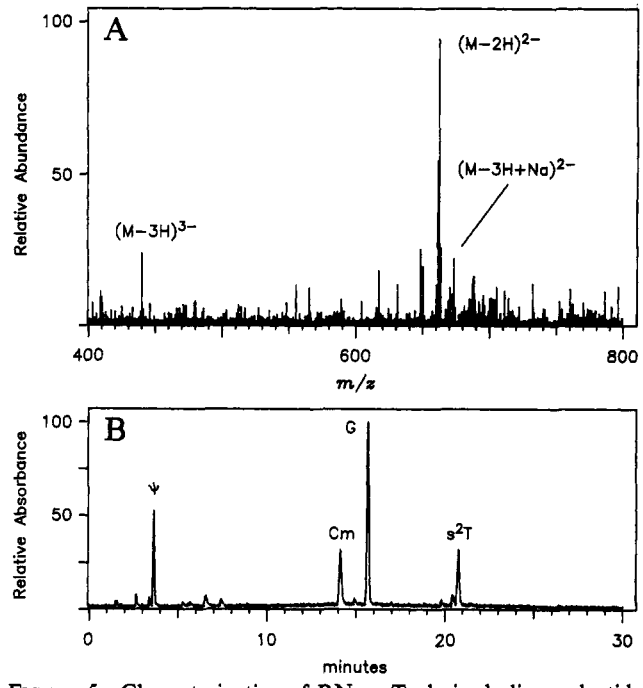

FIGURE 5: Characterization of RNase T_1-derived oligonucleotide from unfractionated *P. furiosus* tRNA as 54-s^2TpΨpCmpGp-57. (A) Negative ion electrospray mass spectrum. M_r determined from ions resulting from deprotonation, *m/z* 661.35 $(M - 2H)^{2-}$, 1324.7; M_r calculated for composition $CU_2Gp + 2CH_2 - O + S$, 1324.9. (B) Analysis of nucleosides by LC/MS following digestion with nuclease P_1 and alkaline phosphatase.

modification in RNA not represented by a change in mass (U → Ψ), is thus observed in Figure 5B but is not detected by mass measurement of the intact oligonucleotide. Differentiation of isomers (Cm *vs* m^5C, m^5s^2U *vs* s^2Um, etc.) is made on the basis of differences in HPLC retention time, which have been previously cataloged (Pomerantz & McCloskey, 1990). While these findings imply that m^5s^2U occurs principally at position 54, it does not rigorously exclude its presence in the anticodon or other sites in some isoaccepting tRNAs.

DISCUSSION

The thermal melting temperatures of unfractionated tRNAs from *P. furiosus*, 97 °C, and *P. occultum*, 101 °C (Figure 1), are unprecedented for natural RNAs and reveal exceptional intrinsic thermal stabilities. For comparison, unfractionated tRNAs from *E. coli* melt at 75 °C (Watanabe et al., 1980), and those from *T. thermophilus*, at 85.8 °C (Watanabe et al., 1976). An estimate of the contribution of posttranscriptional modification to thermal stability can be obtained by comparison of these values with those expected from the %G-C content of tRNA stems, using T_m data compiled by Watanabe et al. (1980) from isoaccepting mesophile tRNAs, shown by the solid line in Figure 6. T_m values observed for *P. furiosus* and *P. occultum* are approximately 14 and 18 °C, respectively, higher than expected from the combined effect of G-C content and levels of modification common to the bacterial mesophiles (i.e., the solid line in Figure 6). Previously measured differences between completely unmodified and native tRNA in *E. coli* (Derrick & Horowitz, 1993) and yeast (Sampson & Uhlenbeck, 1988) are 4–5 °C, shown graphically in Figure 6. (Similar results were reported for yeast $tRNA^{Asp}$ (Perret et al., 1990) although with a one GC base pair difference between modified and unmodified tRNAs.) Therefore, the total stabilization conferred by modification in the archaeal

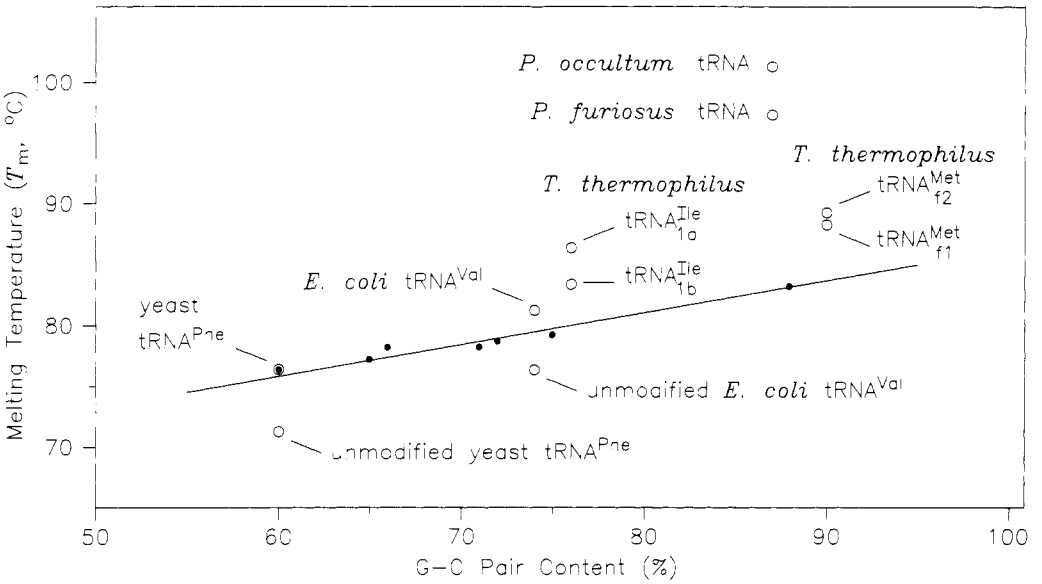

FIGURE 6: Relationship between T_m and %G-C content from tRNA stem regions. The solid line was derived from previously compiled values (solid circles) for seven isoaccepting tRNAs from yeast and mesophilic bacteria [for the listing of individual data points, see Watanabe et al. (1980)]. Archaeal hyperthermophiles: unfractionated *P. occultum* tRNA, T_m 101.5 °C; unfractionated *P. furiosus* tRNA, T_m 97 °C. Bacterial thermophile *T. thermophilus* cultured at 65°C: tRNA$^{Met}_{f2}$, T_m 89 °C; tRNA$^{Met}_{f1}$, T_m 88 °C (Watanabe et al., 1980); tRNA$^{Ile}_{1a}$, T_m 86.2 °C; tRNA$^{Ile}_{1b}$, T_m 83.3 °C (Horie et al., 1985). Comparison of wild-type tRNAs with unmodified *in vitro* tRNA transcripts: *E. coli* tRNA^{Val}, T_m 81 °C; unmodified tRNA^{Val}, T_m 76 °C (Derrick & Horowitz, 1993); yeast tRNA^{Phe}, T_m 76 °C; unmodified tRNA^{Phe}, T_m 71 °C (Sampson & Uhlenbeck, 1988). Abscissa values of ~88% G-C for *P. furiosus* and *P. occultum* are estimated as the mean calculated from the 31 reported archaeal hyperthermophile tRNA gene sequences (Steinberg et al., 1993), and one tRNA sequence (Kuchino et al., 1982).

hyperthermophiles is around 20 °C, compared with approximately 10 °C in the case of the bacterial thermophile *T. thermophilus* (estimated from Figure 6).

The influence of cell culture temperature on T_m is particularly striking in the absence of Mg^{2+} (Figure 2) a condition which releases the tRNA from its native conformation and permits observation of the denaturation of individual helical domains (Cole et al., 1972; Stein & Crothers, 1976; Johnston & Redfield, 1981). Multiphasic transitions are observed which become more cooperative with increasing growth temperature (Figure 2), which we interpret as due to increased levels of selected forms of posttranscriptional modification as discussed below. The finding that overall T_m remains constant at ~97 °C for the three culture temperatures in the presence of Mg^{2+} suggests that high stability of the tertiary structure prevents melting of the individual helices (Stein & Crothers, 1976); i.e., the intrinsic T_m's of individual helices are lower than that of the tertiary structure even though the helices may be increasingly stabilized by modification. By contrast, in the case of bacterial thermophiles, for which the need for overall tertiary stabilization would be somewhat lower, modest increases in the T_m's of unfractionated tRNA in the presence of Mg^{2+} were observed. Unfractionated *B. stearothermophilus* tRNA exhibited a T_m increase from approximately 72 °C (50 °C culture) to 74 °C (70 °C culture) in 3.3 mM Mg^{2+}, and no significant multiphasic denaturation is apparent in the absence of Mg^{2+} (Agris et al., 1973), while the tRNAs from *T. thermophilus* grown at various temperatures between 50 and 80 °C showed a smooth increase in T_m from 83.3 to 86.4 °C in 10 mM Mg^{2+} (Watanabe et al., 1976). Although a detailed examination of the role of Mg^{2+} was not made in the present study, the melting profiles in Figures 1 and 2C suggest additional differences between the bacterial and archaeal thermophiles, with regard to the influence of Mg^{2+}. It was earlier concluded that a 35 °C increase in T_m of *T. thermophilus* tRNA in the presence of 10 mM Mg^{2+} compared with its absence, versus a corresponding 24–28 °C increase for *E. coli* tRNA, indicates a greater role for Mg^{2+} in stabilization in *T. thermophilus* than in *E. coli* (Watanabe et al., 1980). However, for *Pyrococcus* the shift is only about 15 °C (Figure 1A *vs* Figure 2C), which we conclude shows that a relatively larger proportional role is played in stabilization by modification than by Mg^{2+} in *Pyrococcus*, compared with *E. coli* and *T. thermophilus*.

In general, modification serves to enhance tRNA stability as shown for example in yeast (Sampson & Uhlenbeck, 1988), in mesophilic bacteria (Davanloo et al., 1979), and in thermophilic bacteria (Watanabe et al., 1980). The most extensive evidence comes from studies of ribose methylation in the anticodon (Kawai et al., 1992a) and of m^5s^2U in *T. thermophilus* (Yokoyama et al., 1987). In the archaeal hyperthermophiles requirements for stabilization are much more stringent because of temperature optima as high as 105 °C and maxima of 110 °C (Stetter, 1986). The unfractionated tRNA of *P. furiosus* contains an exceptional range of posttranscriptional modifications, with 23 modified nucleosides identified by LC/MS (Figure 3). This level of modification is generally characteristic of archaeal hyperthermophiles (Edmonds et al., 1991) and is significantly higher than in archaea having growth optima below about 80 °C (Edmonds et al., 1991). Six nucleoside species were found that are methylated at O-2′ of ribose, a modification that generally results in regional stabilization by restricting the conformational flexibility of the ribose ring (Kawai et al., 1992a).

The analysis by LC/MS of unfractionated tRNA isolated from *P. furiosus* grown at 70, 85, and 100 °C (the optimum for cell growth) revealed that relative abundances of the majority of modified nucleosides were essentially unchanged, with the exception of three families of nucleosides, consisting of ac^4Cm, m^2_2Gm, and m^5s^2U and their less modified counterparts. In each case the change in relative abundance was in the direction of increased modification with temperature of cell growth (Table 1). Analogous results were reported from *B. stearothermophilus*, which exhibits a 3-fold increase of overall ribose methylation (50 → 70 °C cultures) (Agris

et al., 1973), and *T. thermophilus*, in which m^5s^2U increased 1.4-fold, from 0.22 to 0.52 mol % (50 → 80 °C cultures) (Watanabe et al., 1976). All three forms of modification have been shown in previous studies to possess elements of unusual stability. N^4-Acetylation and subsequent 2′-*O*-methylation of cytidine, resulting in ac^4C and ac^4Cm, respectively, independently and additively stabilize the C3′-*endo* conformation of ribose (Kawai et al., 1992b). N^4-Acetylation has been shown to make cytidine conformationally rigid through its electron-withdrawing effect on C-5,6 (Kawai et al., 1992b), while 2′-*O*-methylation induces conformational rigidity of pyrimidines in general, primarily through steric interactions with O^2 of the base and C3′-phosphate (Kawai et al., 1992a). This conformational ridigity effectively stabilizes the A form RNA helical conformation, making ac^4Cm one of the most conformationally stable pyrimidine nucleosides from RNA (Kawai et al., 1992a,b). In RNA, ac^4Cm is known only in the archaeal hyperthermophiles. Its presence was reported in the unfractionated tRNA of seven organisms (Edmonds et al., 1991), and it was recently discovered at position 35 in 5S rRNA of *P. occultum* (Bruenger et al., 1993), which was unexpected because of the otherwise nearly universal absence of modification in 5S rRNA. Among the three temperature-responsive modifications in *P. furiosus* (ac^4Cm, m_2^2Gm, and m^5s^2U), the position of ac^4Cm is most problematic: Cm-34 and ac^4C-34 are, surprisingly, the only reported anticodon modifications in archaea (Steinberg et al., 1993), although the sequence database is derived principally from mesophilic halophiles (Gupta, 1984). Other positions in which Cm is found (positions 4, 13, 32, and 39 in bacteria and eukaryotes; universally at 56 in archaea) and ac^4C is found (position 12 in eukaryotes) (Steinberg et al., 1993) suggest additional possible sites for ac^4Cm. Indeed, the 1.7 mol % level of ac^4Cm occurrence (see Results) indicates that many tRNAs contain more than one ac^4Cm residue.

Similar to that of ac^4Cm, the conformational stability of G is greatly enhanced by multiple methylations. It was reported in a preliminary communication based on NMR data that the modification of G to m_2^2Gm results in an increase in the ratio [C3′-*endo*-*anti*]/[C2′-*endo*-*anti*] from 0.15 to 21 (Kawai et al., 1991), in which conformational stability is conferred primarily by 2′-*O*-methyl–base steric interactions. Qualitatively, the effect of higher temperature (Table 1) is to increase modification in the direction of m_2^2Gm. The lesser effect observed for m^2G may reflect the presence of m^2G at an additional site (or sites) which is not temperature-responsive. In the nonthermophilic archaeal halophiles, 31% of the sequences show m^2G or m_2^2G at position 10 at the head of the D-stem, and 43% at position 26, and nowhere else (Steinberg et al., 1993). Although enhanced stabilization from ribose methylation would be effective at either site, evidence suggestive of position 26 is provided by $tRNA_i^{Met}$ from *Sulfolobus acidocaldarius*, the only reported hyperthermophile sequence (Kuchino et al., 1982), in which m_2^2Gm was hypothesized as the identity of unknown nucleoside N-26, a proposal supported by the rigorous characterization of m_2^2Gm in a hydrolysate of *Sulfolobus solfataricus* tRNA (Edmonds et al., 1987). The crystal structure of yeast $tRNA^{Phe}$ shows that m_2^2G-26 forms the linkage between the D-stem and the anticodon stem (Kim et al., 1974; Robertus et al., 1974). While two models exist for the spatial configuration of m_2^2G-26 (Kim et al., 1974; Robertus et al., 1974), both models imply that the m_2^2G-26 linkage is the hinge that adjusts the angular disposition of the D-stem and the anticodon stem during protein synthesis. Thus, m_2^2Gm presumably serves to stabilize coaxial stacking of the two stems, in which ribose methylation enforces greater stability against thermal motion at the stem junction.

The location of m^5s^2U at position 54 in the T-loop of *P. furiosus* tRNA from mass spectral data (see Results) suggests the likelihood that the well-established role (Yokoyama et al., 1987) of m^5s^2U in thermal stabilization of *T. thermophilus* tRNA operates as well as in *P. furiosus*, and in the related organism in which m^5s^2U occurs, *Thermococcus* sp. (Edmonds et al., 1991). Proton NMR spectra of oligonucleotides containing m^5s^2U demonstrate that the ribose moiety is favored in C3′-*endo*, and that conformational rigidity is due to steric interaction between the 2-thioketo moiety of the pyrimidine ring and the 2′-hydroxyl group of ribose (Yokoyama et al., 1984). Furthermore, the 2-thioketo functionality of m^5s^2U has been shown to stabilize stacking interactions (Scheit & Faerber, 1975), thus conferring regional stability to the T-loop. More recently, two isoleucine isoaccepting tRNAs isolated from *T. thermophilus* were shown to have identical sequences except for the modification at position 54: $tRNA_{1a}^{Ile}$ has m^5s^2U, and $tRNA_{1b}^{Ile}$ has m^5U (Horie et al., 1985). Remarkably, the melting temperatures of the two tRNAs differed by ~3 °C (as shown graphically in Figure 6), implying that the single oxygen → sulfur replacement significantly contributes to the thermostability of the entire $tRNA_{1a}^{Ile}$ molecule (Horie et al., 1985). In unfractionated *T. thermophilus*, m^5s^2U constitutes about 0.49 mol % of the nucleoside population at the wild-type temperature optimum (75 °C) (Watanabe et al., 1976), while in *P. furiosus* (100 °C culture) the level is about 0.77 mol %. However, it is reasonable to assume that the proportional contribution of m^5s^2U to thermostability relative to other modifications may be less in *P. furiosus* because of the overall much higher level of modification in *P. furiosus*.

Ultimately, a more detailed picture will emerge from the availability of tRNA sequences (as opposed to those from tDNA) from the hyperthermophiles and from X-ray crystallographic structures or NMR data. In the meantime, by a combination of the results in the present study from melting experiments and from selective modification changes associated with culture temperature, it is clear that selective posttranscriptional modifications in the archaeal thermophiles play major stabilizing roles above and beyond the well-known effects of magnesium ion binding (Stein & Crothers, 1976) and G-C content of tRNA stem regions (Watanabe et al., 1980). These modifications have evolved to produce much greater structural diversity at the nucleoside level than in the case of bacterial thermophiles, and they are concluded to play a greater proportional role in the thermal stabilization of tRNA.

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