Probing structural differences between native and *in vitro* transcribed *Escherichia coli* valine transfer RNA: evidence for stable base modification-dependent conformers

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ABSTRACT

Structural differences between native (modified) and *in vitro* transcribed (unmodified) *Escherichia coli* tRNA^{Val} were explored by comparing their temperature-absorbance profiles as a function of magnesium ion concentration and by probing their solution conformation with single- and double-strand-specific endonucleases. *In vitro* transcribed tRNA^{Val} has a less ordered structure as monitored by thermal melting profiles; its T_m is appreciably lower than that of native tRNA^{Val} at all Mg^{2+} concentrations. Structure probing experiments with nuclease S1 and ribonuclease V1 show that the unmodified tRNA^{Val} transcript is more susceptible to nuclease attack at low Mg^{2+} concentrations, particularly in the D- and T-loops, indicative of at least a partial disruption of D-loop/T-loop interactions. These experiments also provide evidence for temperature-dependent alternative conformations of the anticodon loop of native tRNA^{Val}. Modified nucleosides are essential for the stability of these conformers; they cannot be detected in the unmodified *in vitro* transcript. The observations suggest that post-transcriptional modifications in tRNA allow the adoption of unique conformations and act to stabilize those that are biologically active.

INTRODUCTION

Recent development of methods for *in vitro* transcription of synthetic tRNA genes by bacteriophage (T7 or SP6) RNA polymerase (1) has made relatively large quantities of tRNA available for biochemical and biophysical studies. The transcripts have structures and properties similar, though not identical, to native tRNAs (1–3). They lack all the modified bases found in native tRNA, but are readily recognized by their cognate synthetases and efficiently aminoacylated, with kinetics similar to those of native tRNA (1, 2).

Comparison of native tRNAs and *in vitro* transcripts has revealed subtle conformational differences, especially at low Mg^{2+} concentrations. Thermal denaturation profiles indicate

that *in vitro* transcribed tRNA^{Phe} has a less stable, more flexible structure than native tRNA^{Phe} (1). The rate of lead ion-catalyzed cleavage of tRNA at U17 (4) has been shown to be a sensitive probe of tRNA tertiary structure in the region of T-loop/D-loop interaction (5). A twofold slower rate of Pb^{2+} cleavage of the *in vitro* transcript of yeast tRNA^{Phe} compared with that of native tRNA^{Phe} is indicative of an increased flexibility of the unmodified transcript (5). Cleavage of the unmodified yeast tRNA^{Phe} transcript by photoactivated bis(phenanthroline)(phenanthrenequinone diimine)rhodium(III) [$Rh(phen)_2phi^{3+}$] differs from that of fully modified tRNA^{Phe}, particularly in the anticodon stem and loop, where additional cleavage sites are observed in the transcript (6). Base modifications protect *Escherichia coli* tRNA^{Gln} from glutamyl-tRNA synthetase-induced cleavage in the anticodon loop and the D-loop and stem (7), presumably because of conformational differences between native tRNA^{Gln} and the unmodified *in vitro* transcript. Furthermore, proton NMR studies (8) have detected local structural differences between the yeast tRNA^{Phe} transcript and the native tRNA at low Mg^{2+} concentrations; evidence for a second G-U base pair in the transcript was reported.

Our ^{19}F NMR studies with fully active 5-fluorouracil-substituted *E. coli* tRNA^{Val} also provide evidence for structural differences between native and *in vitro* transcribed molecules. Although the ^{19}F NMR spectra of native and synthetic (Fura)tRNAs, recorded at 10 mM Mg^{2+} , are quite similar, chemical shift changes of resonances corresponding to FU47 and FU29, FU33 and FU34 were noted (2). These suggest structural differences in the variable loop region and in the anticodon stem and loop and may be due to the absence of m⁷G46 and m⁶A37 in the *in vitro* transcript; these are the only two modified bases present in native (Fura)tRNA^{Val} prepared from *E. coli* cells (9). At low Mg^{2+} concentrations, the ^{19}F NMR spectrum of *in vitro* transcribed (Fura)tRNA^{Val} differs markedly from that of the native fluorine-substituted tRNA (2). This is especially true in the high field region, where resonances in the spectrum of the transcript are poorly resolved and exhibit extensive peak broadening, an indication that the secondary or tertiary structure of the transcript is less stable at low divalent cation concentrations.

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To examine the role(s) of modified nucleosides in the stability, structure and function of tRNA in greater detail we have used a number of physical, chemical and enzymatic means to probe conformational differences between native (modified) and *in vitro* transcribed (unmodified) *E. coli* tRNA^{Val}. In this paper, we describe evidence for temperature-induced, base modification-dependent conformational changes in the anticodon loop.

MATERIALS AND METHODS

Materials

Construction of the recombinant phagemid pVAL119-21, which contains the wild-type *E. coli* tRNA^{Val} gene linked directly to an upstream T7 promoter, was reported earlier (2). Valine tRNA was transcribed *in vitro* with T7 RNA polymerase in the presence of GMP to produce a tRNA with a 5'-terminal monophosphate (2). The transcript, after purification by HPLC, could be aminoacylated by purified valyl-tRNA synthetase to approximately 1200 pmol/A₂₆₀, and migrated as a single band on 15% denaturing polyacrylamide gels with the same mobility as native tRNA^{Val}. Previous studies (2) had shown that the RNase T1 digestion pattern of the transcript is identical to that of the native tRNA. T7 RNA polymerase was prepared from *E. coli* BL21/pAR1219 (10). Native tRNA^{Val} was obtained from Subriden RNA and further purified by denaturing gel electrophoresis. Transfer RNA was 5'-³²P-labeled with T4 polynucleotide kinase and (γ -³²P)ATP (DuPont-New England Nuclear) after dephosphorylation with bacterial alkaline phosphatase (11), and the labeled tRNA was purified by gel electrophoresis. Ribonuclease T1 was from Boehringer Mannheim. Bethesda Research Laboratories was the source of bacterial alkaline phosphatase and T4 polynucleotide kinase. Nuclease S1 and ribonuclease V1 (FPLC-pure grade) were from Pharmacia/LKB.

Temperature-absorbance profiles

Comparative melting curves for native tRNA^{Val} and *in vitro* transcribed tRNA^{Val} were determined on a Gilford Response^T spectrophotometer equipped with a Thermoset. Temperature was increased at the rate of 1°C/min, and absorbance was recorded between 20°C and 90°C. Experiments comparing the thermal stability of native tRNA and an *in vitro* transcript were always run in parallel. Measurements were made in 50 mM sodium-cacodylate buffer (pH 7.2) with different concentrations of Mg²⁺. To ensure effective control of Mg²⁺ concentration, tightly bound divalent cations were first removed by heating the tRNA to 80°C in 10 mM EDTA. The Mg²⁺ concentration was then adjusted to the desired level by addition of MgCl₂.

Structure mapping of tRNA

³²P end-labeled tRNA was subjected to nuclease cleavage under conditions that result in a limited scission of tRNA molecules to avoid secondary cleavages which would make interpretation of results difficult. Primary cleavages were identified by digesting tRNA with increasing amounts of enzyme. RNA fragments detected at the lowest enzyme concentration were considered to arise from primary cleavages. Levels of nuclease used in probing reactions were roughly half those that give rise to the first detectable secondary cleavages. *E. coli* tRNA^{Val} is partially cleaved in the absence of nucleases, usually at a YpA sequence as previously described for other RNAs by Witzel (12); e.g., at positions 9, 14, 26, 35, 37 and 66 (Figs. 2, 4, 5 and 6).

Structural probing reactions were carried out at various Mg²⁺ concentrations, with tightly bound Mg²⁺ first removed as described earlier. Preceding the probing reactions, labeled tRNA was diluted with unlabeled carrier tRNA to a final concentration of 8 μ M, and the tRNA was renatured by heating at 55°C (low-temperature renaturation) or 65–70°C (high-temperature renaturation) for 1–2 min and then slowly cooled to 37°C.

Limited digestion with nuclease S1 and RNase V1

Limited nuclease S1 hydrolysis was carried out in a reaction mixture (10 μ L) containing 2 μ g labeled tRNA (20,000 cpm), 50 mM Tris-Cl, pH 7.2, 0–10 mM MgCl₂, 1 mM ZnCl₂ and 25 units nuclease S1 (13). Incubated control reactions included all ingredients except nuclease S1. After 5 min incubation at 37°C, the reaction was terminated by phenol extraction, the aqueous layer was washed with ether three times, heated briefly to drive off the ether and the reaction products recovered by ethanol precipitation. Pellets were lyophilized to dryness and dissolved in 10 μ L of sequencing dye solution (8 M urea, 20 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol).

Conditions for limited digestion with RNase V1 were similar to those used for nuclease S1 digestion except that 0.15 units (unless otherwise noted) of ribonuclease V1 were added, and digestion was carried out in the absence of Na⁺. Reactions were stopped and prepared for gel electrophoresis as described for nuclease S1 hydrolysis.

Polyacrylamide gel electrophoresis was carried out in 15% denaturing gels (0.4×300×310 mm) prepared in 100 mM Tris-borate buffer, pH 8.3, 2mM EDTA and 8.3 M urea, and the cleavage products were visualized by autoradiography at –70°C. Films were analyzed with a digital scanner (Mirror Technologies) using Adobe Photoshop software. Assignment of cleavage positions was made by comparison with a partial RNase T1 digest (14) and an alkaline hydrolysis ladder; cleavage sites are designated by the nucleotide that donates the phosphate; e.g.,

Figure 1. Normalized ultraviolet absorbance melting curves of native (modified) tRNA^{Val} (lower set of curves; closed circles) and *in vitro* transcribed (unmodified) tRNA^{Val} (upper set of curves; open circles) at 0, 2, 5 and 10 mM MgCl₂, in 50 mM sodium cacodylate, pH 7.2.

cleavage between the first and second nucleotides from the 5' end is designated as cleavage at p2.

RESULTS

Thermal stability of native and *in vitro* transcribed tRNA^{Val}

The lower ultraviolet light absorption (hypochromicity) of folded, highly structured RNA compared with that of its constituent nucleotides is largely a reflection of the extent of base stacking in the polynucleotide (15). Comparison of the absorbance increase at 260 nm resulting from complete alkaline hydrolysis of fully modified native *E. coli* tRNA^{Val} and *in vitro* transcribed tRNA^{Val}, which lacks all base modifications, revealed that native tRNA^{Val} has a hypochromicity (17.7%) nearly twice that of the *in vitro* transcript (10.1%), an indication that it has a more ordered, base-stacked structure than the *in vitro* transcript.

This conclusion is supported by comparison of the temperature-absorbance profiles of native and *in vitro* transcribed tRNA^{Val} as a function of Mg²⁺ concentration (Fig. 1). In the absence of divalent cations, the temperature-absorbance profile of native tRNA^{Val} (closed circles) exhibits a biphasic transition with T_m values of 47°C and 70°C. The *in vitro* transcript (open circles) does not display the low-temperature transition in the absence of Mg²⁺; however, a biphasic transition is observed at low Mg²⁺ concentration (2 mM; Fig. 1). Both native and synthetic

tRNAs show increasing thermal stability as Mg²⁺ concentration increases, but the melting profiles of native tRNA^{Val} are sharper and have a higher T_m under all conditions studied.

Structure mapping of native and *in vitro* transcribed tRNA^{Val}

To characterize the structural differences between native and *in vitro* transcribed tRNA^{Val}, the solution structure of 5'-end-labeled tRNA was probed by limited nuclease digestion over a range of Mg²⁺ concentrations. For these experiments, the tRNAs were renatured by heating at temperatures above 70°C to facilitate folding into their native conformation.

Limited nuclease S1 digestion. Comparison of the patterns of limited digestion by the single-strand-specific nuclease S1 reveals significant structural differences between native tRNA^{Val} and *in vitro* transcribed tRNA^{Val}. The *in vitro* transcript is much more readily hydrolyzed in the D-loop and stem and in the T-loop than is native tRNA^{Val}. Strong cleavage in the D-loop of the *in vitro* transcript occurs at p14 and p16–20 (Fig. 2B; see tRNA^{Val} sequence in Fig. 3), whereas only relatively minor cleavages are observed in the D-loop of native tRNA^{Val}, at p17–p20 (Fig. 2A). Furthermore, although the D-stem of native tRNA^{Val} is resistant to nuclease S1 hydrolysis (Fig. 2A), substantial cleavage occurs on the 5' side of the D-stem (p12–p13) of the *in vitro* transcript (Fig. 2B), suggesting instability and/or partial melting of the D-stem of *in vitro* transcribed tRNA^{Val}. In native tRNA^{Val}, the T-loop is protected from nuclease S1 hydrolysis (Figure 2A), but the *in vitro* transcript is cleaved in the T-loop primarily at p54 (Fig. 2B).

Both *in vitro* transcribed and native tRNA^{Val} are readily hydrolyzed in the anticodon loop at all concentrations of Mg²⁺

Figure 2. Structural mapping of native and *in vitro* transcribed tRNA^{Val}, renatured at 70°C (high-temperature), by limited nuclease S1 digestion at various Mg²⁺ concentrations. Autoradiograph of cleavage products of 5'-labeled tRNA^{Val} separated by electrophoresis on a 15% polyacrylamide-8M urea gel. A) Native tRNA^{Val}. B) *In vitro* transcribed tRNA^{Val}. Lanes 1 and 2 are RNase T1 and alkaline hydrolysis ladders, respectively; lane 3 is a control incubated in the absence of nuclease S1 (20 mM Mg²⁺). Lanes 4–7 correspond to nuclease S1 digestions with: (4), no added Mg²⁺; (5) 1 mM Mg²⁺; (6) 10 mM Mg²⁺; (7) 20 mM Mg²⁺. Cleavages are designated by the number of the nucleotide that contributes the 5-PO₄ to the phosphodiester bond cleaved. Position labels on the left indicate the tRNA sequence; numbers on the right designate positions cleaved by the probe.

Figure 3. Nucleotide sequence (cloverleaf structure) of *E. coli* tRNA^{Val} with modified nucleosides (abbreviation, position): 4-thiouracil (s⁴U, 8), dihydrouridine (D, 17), uridine-5-oxyacetic acid (cmo⁵U, 34), N⁶-methyl adenine (m⁶A, 37), 7-methylguanosine (m⁷G, 46), ribothymidine (T, 54), and pseudouridine (ψ, 55). *In vitro* transcribed tRNA^{Val} is identical in sequence except that no bases are modified.

Figure 4. Structural mapping of native and *in vitro* transcribed tRNA^{Val}, renatured at 70°C (high-temperature), by limited RNase V1 cleavage at various Mg²⁺ concentrations. Autoradiograph of cleavage products of 5'-labeled tRNA^{Val} separated by electrophoresis on a 15% polyacrylamide-8M urea gel. A) Native tRNA^{Val}. B) *In vitro* transcribed tRNA^{Val}. Lanes 1 and 2 are RNase T1 and alkaline hydrolysis ladders, respectively. Lane 3 is an unincubated control; lane 4 is a control incubated in the absence of RNase V1 (20 mM Mg²⁺). Lanes 5–8 show the products of RNase V1 digestion with: (5), no added Mg²⁺; (6) 1 mM Mg²⁺; (7) 10 mM Mg²⁺; (8) 20 mM Mg²⁺.

(Fig. 2). Strong cleavage of native tRNA^{Val} occurs at p34 and p35 (Fig. 2A); minor cleavages are also evident throughout the 3' side of the anticodon loop, from p36 to p39. The unmodified *in vitro* transcript is cleaved strongly at p34 and p35 and less strongly at p36, p37 and p38; no cleavage is observed at p39 (Fig. 2B).

A variable degree of spontaneous hydrolysis is observed at p37 of *in vitro* transcribed tRNA^{Val} (Fig. 2B, lane 3); in some experiments no cleavage at this position is evident. The nuclease S1 digestion pattern, however, remains unchanged regardless of the extent of splitting at p37, effectively ruling out the possibility that the fragments observed in lanes 4–7 of Fig. 2B are the products of secondary cleavages.

Limited RNase V1 digestion. Fewer conformational differences between native tRNA^{Val} and the *in vitro* transcript are revealed by structural mapping with double-strand-specific RNase V1 at different Mg²⁺ concentrations (Fig. 4). The native tRNA is cleaved more intensely than the unmodified *in vitro* transcript in the acceptor stem at p63, p66 and p69 and in the anticodon stem at p29, p30 and p31; native tRNA^{Val}, but not the transcript is cleaved at p28. Both the native tRNA and the *in vitro* transcript are hydrolyzed at p8, in the region between the acceptor- and the D-stems. Differences in the RNase V1 cleavage patterns of the two tRNAs are observed in the P-10 and variable loop regions. The transcript, but not native tRNA^{Val}, is cleaved weakly at p10, and weak cleavages are observed at p43 and p44 in native tRNA^{Val}, but not in the transcript (compare Figs. 4A and B).

Figure 5. Structural mapping of native and *in vitro* transcribed tRNA^{Val}, renatured at 55°C (low-temperature), by limited nuclease S1 digestion at various Mg²⁺ concentrations. Autoradiograph of cleavage products of 5'-labeled tRNA^{Val} separated by electrophoresis on a 15% polyacrylamide-8M urea gel. A) Native tRNA^{Val}. B) *In vitro* transcribed tRNA^{Val}. Lanes 1 and 2 are RNase T1 and alkaline hydrolysis ladders, respectively; lane 3 is a control incubated in the absence of nuclease S1 (20 mM Mg²⁺). Lanes 4–8 correspond to nuclease S1 digestions with: (4), no added Mg²⁺; (5) 1 mM Mg²⁺; (6) 5 mM Mg²⁺; (7) 10 mM Mg²⁺; (8) 20 mM Mg²⁺.

Figure 6. Structural mapping of native and *in vitro* transcribed tRNA^{Val}, renatured at 55°C (low-temperature), by limited RNase V1 cleavage, at varying Mg²⁺ concentrations. Autoradiograph of cleavage products of 5'-labeled tRNA^{Val} separated by electrophoresis on a 15% polyacrylamide-8M urea gel. A) Native tRNA^{Val}. B) *In vitro* transcribed tRNA^{Val}. Lane 1 is an unincubated control. Lanes 2 and 3 correspond to RNase T1 and alkaline hydrolysis ladders. Lane 4 is a control incubated in the absence of RNase V1 (10 mM Mg²⁺). Lanes 5–7 show the products of RNase V1 digestion with: (5) 1 mM Mg²⁺; (6) 5 mM Mg²⁺; (7) 10 mM Mg²⁺.

Temperature-induced conformers of fully modified native tRNA^{Val}

During these studies, which were performed with tRNA renatured at 70°C, we noticed that the 260 nm absorbance of native tRNA^{Val} renatured by heating to temperatures above 65°C is reproducibly 3–4% lower than that of tRNA renatured at 55°C (results not shown). Renaturation temperature has little effect on the absorbance of *in vitro* transcribed tRNA^{Val}. The structural basis for the temperature-dependent differences in hypochromicity was determined by nuclease mapping studies comparing the solution structure of native tRNA^{Val} renatured at 55°C (low temperature or l-form) with that renatured at 70°C (high-temperature or h-form), over a range of Mg²⁺ concentrations. The results provide evidence for base modification-dependent alternative stable anticodon loop conformations in *E. coli* tRNA^{Val}.

Comparison of the patterns of limited nuclease S1 digestion of 5-end-labeled native tRNA^{Val} renatured at 55°C (Fig. 5A) with tRNA renatured at 70°C (Fig. 2A) reveals that the anticodon loop of the h-form is less susceptible to hydrolysis. At all Mg²⁺ concentrations, native tRNA^{Val}, renatured at both 55°C and 70°C, is cleaved strongly at p34 and p35 in the anticodon loop (Figs. 5A and 2A). However, the low-temperature form of the tRNA is also strongly hydrolyzed at p37 and p39 (Fig. 5A, lanes 4–8), whereas the h-form is only weakly cleaved at p36 and p37 and shows little or no hydrolysis at p38 or p39 (Fig. 2A, lanes 4–7). The nuclease S1 cleavage pattern of *in vitro* transcribed tRNA^{Val} is not affected by the temperature used to renature the tRNA (compare Figs. 2B and 5B).

Results of limited RNase V1 digestion of native tRNA^{Val} renatured at 55°C are shown in Fig. 6A. The major difference from the cleavage pattern of the h-form of native tRNA^{Val} is the hydrolysis at p8 (the band just below the strong spontaneous cleavage at p9), which is readily detected in tRNA^{Val} heated to 70°C (Fig. 4A), but is not evident in the cleavage pattern of the l-form (Fig. 6A). Ribonuclease V1 hydrolysis of *in vitro* transcribed tRNA^{Val} renatured at 55°C (Fig. 6B) differs only slightly from that of the tRNA renatured at 70°C (Fig. 4B). The variable loop of the low-temperature form is weakly cleaved at p42 and p43 (Fig. 6B, lanes 5–7), whereas no such splitting is detected in the high-temperature form (Fig. 4B, lanes 5–8).

DISCUSSION

The ready availability of tRNAs transcribed *in vitro* by phage RNA polymerases has stimulated studies of tRNA structure and function. Although *in vitro* transcripts are readily recognized and aminoacylated by their cognate aminoacyl-tRNA synthetases, they lack modified nucleosides, and other significant differences with native tRNAs have become evident (see Introduction). To determine how well results obtained with *in vitro* transcripts apply to native tRNAs, it is important to understand these structural differences.

The global structure of *in vitro* transcribed tRNA^{Val} is less ordered than that of the native, fully modified molecule as shown by the appreciably lower *T*_m of the former at all Mg²⁺ concentrations (Fig. 1). Previous studies contrasting native and *in vitro* transcribed yeast tRNA^{Phe} (1) and tRNA^{Asp} (3) have also emphasized the greater flexibility of the unmodified molecules. These differences between fully modified native tRNA and the

unmodified *in vitro* transcript suggest that base modifications help to stabilize tRNA structure.

Nuclease probing experiments designed to identify the structural elements of the *in vitro* transcript most affected by the absence of modified nucleosides, show that the differences between *in vitro* transcribed and native tRNA^{Val} are greatest in the D- and T-loops. The unmodified transcript is strongly cleaved by nuclease S1 in both the D- and T-loops, whereas native tRNA^{Val} is cleaved to only a limited degree in the D-loop and not at all in the T-loop (Figs. 2 and 5). The greater accessibility of the D- and T-loops of the *in vitro* transcribed tRNA to nuclease probes indicates that base modifications play a significant role in stabilizing D-loop/T-loop interactions, and their absence results in at least a partial disruption of the tertiary structure in this corner of the tRNA molecule.

Subtler differences between the structures of native and *in vitro* transcribed tRNA^{Val} were seen in the pattern of RNase T1 digestion (results not shown). Both native tRNA^{Val} and the *in vitro* transcript are cleaved in the T-loop. The unmodified *in vitro* transcribed tRNA^{Val} is cleaved at p52, p53 and p54; native tRNA^{Val} is cleaved at p54 (although not as extensively), but not at p52 or p53 (refer to tRNA^{Val} sequence in Fig. 3). Cleavage at p53 in the transcript suggests some disruption of the T-stem, particularly at low Mg²⁺ concentrations. Such a structural change is consistent with our earlier observation (2) that the resonance corresponding to FU64 in the ¹⁹F NMR spectrum of *in vitro* transcribed FURA-substituted tRNA^{Val}, is split at low Mg²⁺ concentrations, signifying an exchange of the fluorine nucleus of FU64 between two magnetically distinct environments and suggesting that the T-stem of *in vitro* transcribed (FURA)tRNA^{Val} exists in alternate conformations in slow exchange on the NMR time scale. RNase T1 cleavage at p53 may be a measure of the nuclease sensitivity of one of these conformations. Hydrolysis in the T-loop of native tRNA^{Val} by RNase T1 and the lack of cleavage there by nuclease S1 is presumably due to probe size differences: RNase T1 is a much smaller enzyme (*M*_r = 11,000) than nuclease S1 (*M*_r = 32,000).

Native and *in vitro* transcribed tRNA^{Val} differ only slightly in cleavage patterns by ribonuclease V1, which is double-strand specific but is also able to recognize and cleave base-stacked single-stranded regions of RNA (16). The unmodified transcript seems to have a less ordered structure at the end of the anticodon stem and in the variable region: p28, cleaved in the native tRNA, is not cleaved in the transcript; weak cleavages occur at p43 and p44 of native tRNA^{Val}, whereas *in vitro* transcribed tRNA^{Val} is resistant to hydrolysis at these positions (Fig. 4).

Overall, these results indicate that the standard tertiary structure of unmodified *in vitro* transcribed tRNA^{Val} is partly disrupted. This is reflected in a lower stability of helical stems, a decrease in base stacking and disturbance of the normal interaction between the D- and T-loops. Disruption of the tertiary D-loop/T-loop interactions of unmodified tRNA is the most significant effect observed in other studies that compare the structure of native and *in vitro* transcribed tRNAs. The D- and T-loops of the *in vitro* transcript of tRNA^{Asp} are more accessible to methylation by dimethyl sulfate under native (10 mM MgCl₂), semidenaturing (1 mM EDTA) or denaturing (1 mM EDTA, 90°C) conditions than those of native yeast tRNA^{Asp} (3). Lead-ion-induced cleavage of *in vitro* transcripts of yeast tRNA^{Phe} and tRNA^{Asp} is less effective than that observed for the native modified tRNAs, reflecting disruption of D-loop/T-loop interactions (1, 3). ¹H NMR of *in vitro* transcribed tRNA^{Phe} also

indicates disturbance of tertiary interactions between the D- and T-loops (8).

Comparison of the nuclease S1 (Fig. 5) and RNase V1 (Fig. 6) digestion patterns of native tRNA^{Val} renatured at 55°C with those of the tRNA renatured by heating to 70°C (Figs. 2 and 4) provides direct evidence for the existence of alternative conformational states of the tRNA: a low-temperature form (the l-conformer) and a high-temperature form (the h-conformer). Existence of multiple conformational states of transfer RNA is well documented, and, further, such conformational variability may be essential for function (reviewed in 17, 18). Occurrence of alternative conformational states in native *E. coli* tRNA^{Val} was previously inferred from fluorescence polarization anisotropy (19) and methyl proton NMR (20) studies.

The two conformers of native tRNA^{Val} differ from one another primarily in the anticodon loop. Strong nuclease S1 cleavage of the h-conformer is limited to p34 and p35 (Fig. 2), whereas strong nuclease S1 cleavage of the l-conformer occurs at p37 and p39, as well as at p34 and p35 (Fig. 5). These results suggest that the conformation of the anticodon loop in the h-conformer resembles that in the crystal structure of tRNA^{Phe} (21, 22), where 3 stacking of the nucleotides renders the anticodon loop relatively resistant to nuclease S1 hydrolysis (23, 24). The observed cleavage at p34 and p35 in the h-conformer may be due to decreased stacking of the anticodon bases as a result of the modification of U34 to cmo⁵U34, providing the backbone flexibility needed for nuclease S1 activity.

The nature of the structural differences between the h- and l-conformers is not clear, although the 3–4% greater hypochromicity of the h-conformer suggests differences in the degree of base stacking. It is unlikely that the differences involve a shift in anticodon loop conformation from a 3' to a 5' stack (such a shift would throw bases m⁶A37 and A38 out of the stacked array, making p37 and p39 accessible to nuclease S1) because this places p34 and p35 in the middle of the base stack and should inhibit nuclease S1 cleavage at these positions in the l-conformer. Contrary to this expectation, the enzyme readily cleaves the l-conformer at p34 and p35 (Fig. 5).

Modified nucleosides appear essential for the stability of these conformers because parallel experiments probing the structure of *in vitro* transcribed tRNA^{Val} fail to provide evidence for the existence of stable conformational isomers of the unmodified tRNA (compare Figs. 2B & 5B; 4B & 6B). The modified nucleoside, m⁶A, at position 37 in native tRNA^{Val} could play an important role in this stabilization by promoting strong base-stacking interactions with A38. Absence of the modified m⁶A37 in *in vitro* transcribed tRNA^{Val} would then account for the failure to observe conformational isomers in unmodified tRNA.

The importance of base modifications on anticodon loop structure was recently demonstrated in studies with deoxy analogs of the anticodon arm of yeast tRNA^{Phe}. A magnesium-induced conformational transition in the anticodon loop was shown to be dependent on the presence of a dm⁵C residue in the anticodon stem (25, 26). The base modification promotes site-specific Mg²⁺ binding in the upper part of the hairpin (26) that induces structural alterations in the anticodon loop (25).

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REFERENCES

1. Sampson, J. and Uhlenbeck, O.C. (1988) *Proc. Natl. Acad. Sci. USA* **83**, 1033–1037.
2. Chu, W.-C. and Horowitz, J. (1989) *Nucleic Acids Res.* **30**, 7241–7252.
3. Perret, V., Garcia, A., Puglisi, J., Grosjean, H., Ebel, J.P., Florentz, C. and Giege, R. (1990) *Biochimie* **72**, 735–744.
4. Brown, R.S., Dewan, J.C. and Klug, A. (1985) *Biochemistry* **24**, 4785–4801.
5. Behlen, L.S., Sampson, J.R., DiRenzo, A.B. and Uhlenbeck, O.C. (1990) *Biochemistry* **29**, 2515–2523.
6. Chow, C.S., Behlen, L.S., Uhlenbeck, O.C. and Barton, J.K. (1992) *Biochemistry* **31**, 972–982.
7. Beresten, S., Jahn, M. and Söll, D. (1992) *Nucleic Acids Res.* **20**, 1523–1530.
8. Hall, K.B., Sampson, J., Uhlenbeck, O.C. and Redfield, A.G. (1989) *Biochemistry* **25**, 5794–5801.
9. Horowitz, J., Ou, C.-N., Ishaq, M., Ofengand, J. and Bierbaum, J. (1974) *J. Mol. Biol.* **88**, 301–312.
10. Grodberg, J. and Dunn, J.J. (1988) *J. Bacteriol.* **170**, 1245–1253.
11. Silberklang, M., Gillum, A.M. and RajBhandary, U.L. (1979) *Methods Enzymol.* **59**, 58–109.
12. Witzel, H. (1963) *Prog. Nucleic Acid Res.* **2**, 221–258.
13. Theobald, A., Springer, M., Grunberg-Manago, M., Ebel, J.P. and Giege, R. (1988) *Eur. J. Biochem.* **182**, 445–450.
14. Donis-Keller, H., Maxam, A., and Gilbert, W. (1977) *Nucleic Acids Res.* **4**, 2527–2538.
15. DeVoe, H. (1969) *Ann. N.Y. Acad. Sci.* **158**, 298–307.
16. Lowman, H.B. and Draper, D.E. (1985) *J. Biol. Chem.* **261**, 5396–5403.
17. Crothers, D.M. and Cole, P.E. (1978) In Altman, S. (ed.), *Transfer RNA*. MIT Press, Cambridge, pp. 196–247.
18. Rigler, R. and Wintermeyer, W. (1983) *Annu. Rev. Biophys. Bioeng.* **12**, 475–505.
19. Thomas, J.C., Schurr, J.M., Reid, B.R., Ribeiro, N.S. and Hare, D.R. (1984) *Biochemistry* **23**, 5414–5420.
20. Reid, B.R. (1981) *Annu. Rev. Biochem.* **50**, 969–996.
21. Kim, S.-H., Suddath, F.L., Quigley, G.J., McPherson, A., Wang, A.H., Seeman, N.C. and Rich, A. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 4970–4974.
22. Robertus, J.D., Ladner, J.E., Finch, J.T., Rhodes, D., Brown, R.S., Clark, B.F.C. and Klug, A. (1974) *Nature* **25**, 546–551.
23. Wrede, P., Wurst, R., Vournakis, J. and Rich, A. (1979) *J. Biol. Chem.* **254**, 9608–9616.
24. Woo, N., Roe, B. and Rich, A. (1980) *Nature* **286**, 346–351.
25. Guenther, R.H., Hardin, C.C., Sierzputowska-Gracz, H., Dao, V. and Agris, P.F. (1992) *Biochemistry* **31**, 11004–11011.
26. Dao, V., Guenther, R.H. and Agris, P.F. (1992) *Biochemistry* **31**, 11012–11019.