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* tRNAVal 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 tRNAVal has a less ordered structure as monitored by thermal melting profiles; its Tm is appreciably lower than that of native tRNAVal at all Mg2+ concentrations. Structure probing experiments with nuclease S1 and ribonuclease V1 show that the unmodified tRNAVal transcript is more susceptible to nuclease attack at low Mg2+ 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 tRNAVal. 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 Mg2+ concentrations. Thermal denaturation profiles indicate that in vitro transcribed tRNAVal has a less stable, more flexible structure than native tRNAVal (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 Pb2+ cleavage of the in vitro transcript of yeast tRNAVal compared with that of native tRNAVal is indicative of an increased flexibility of the unmodified transcript (5). Cleavage of the unmodified yeast tRNAVal transcript by photoactivated bis(phenanthroline)(phenanthrenequinone diimine)rhodium(III) [Rh(phen)2phi2+] differs from that of fully modified tRNAVal, particularly in the anticodon stem and loop, where additional cleavage sites are observed in the transcript (6). Base modifications protect *Escherichia coli* tRNAVal from glutaminyl-tRNA synthetase-induced cleavage in the anticodon loop and the D-loop and stem (7), presumably because of conformational differences between native tRNAVal and the unmodified in vitro transcript. Furthermore, proton NMR studies (8) have detected local structural differences between the yeast tRNAVal transcript and the native tRNA at low Mg2+ concentrations; evidence for a second G-U base pair in the transcript was reported.

Our 19F NMR studies with fully active 5-fluorouracil-substituted *E. coli* tRNAVal also provide evidence for structural differences between native and in vitro transcribed molecules. Although the 19F NMR spectra of native and synthetic (FUra)tRNAs, recorded at 10 mM Mg2+, 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 m8A37 in the in vitro transcript; these are the only two modified bases present in native (FUra)tRNAVal prepared from *E. coli* cells (9). At low Mg2+ concentrations, the 19F NMR spectrum of in vitro transcribed (FUra)tRNAVal 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.
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 tRNAVal. 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 tRNAVal 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 tRNAVal. 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 tRNAVal was obtained from Subrifen 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 (11), and the labeled tRNA was purified by gel electrophoresis as described for nuclease S1 hydrolysis.

Polyacrylamide gel electrophoresis was carried out in 15% denaturing gels (0.4 x 300 x 310 mm) prepared in 100 mM Tris-borate buffer, pH 8.3, 2 mM 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.,

Limited digestion with nuclease S1 and RNase V1

Limited nuclease S1 hydrolysis was carried out in a reaction mixture (10 µL) containing 2 µg 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 µL 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 x 300 x 310 mm) prepared in 100 mM Tris-borate buffer, pH 8.3, 2 mM 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.,

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 µM, 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.

Temperature–absorbance profiles

Comparative melting curves for native tRNAVal and in vitro transcribed tRNAVal were determined on a Gilford Response 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 tRNAVal 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).

Figure 1. Normalized ultraviolet absorbance melting curves of native (modified) tRNAVal (lower set of curves; closed circles) and in vitro transcribed (unmodified) tRNAVal (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 tRNAVal
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 tRNAVal and in vitro transcribed tRNAVal, which lacks all base modifications, revealed that native tRNAVal 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 tRNAVal as a function of Mg2+ concentration (Fig. 1). In the absence of divalent cations, the temperature-absorbance profile of native tRNAVal (closed circles) exhibits a biphasic transition with Tm 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 tRNAVal are sharper and have a higher Tm under all conditions studied.

Structure mapping of native and in vitro transcribed tRNAVal
To characterize the structural differences between native and in vitro transcribed tRNAVal, 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 tRNAVal and in vitro transcribed tRNAVal. The in vitro transcript is much more readily hydrolyzed in the D-loop and stem and in the T-loop than is native tRNAVal. Strong cleavage in the D-loop of the in vitro transcript occurs at p14 and p16–20 (Fig. 2B; see tRNAVal sequence in Fig. 3), whereas only relatively minor cleavages are observed in the D-loop of native tRNAVal, at p17–p20 (Fig. 2A). Furthermore, although the D-stem of native tRNAVal 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 tRNAVal. In native tRNAVal, the T-loop is protected from nuclease S1 hydrolysis (Fig. 2A), but the in vitro transcript is cleaved in the T-loop primarily at p54 (Fig. 2B).

Both in vitro transcribed and native tRNAVal are readily hydrolyzed in the anticodon loop at all concentrations of Mg2+.
Figure 4. Structural mapping of native and in vitro transcribed tRNA\textsuperscript{Val}, renatured at 70°C (high-temperature), by limited RNase V1 cleavage at various Mg\textsuperscript{2+} concentrations. Autoradiograph of cleavage products of 5'-labeled tRNA\textsuperscript{Val} separated by electrophoresis on a 15% polyacrylamide-8M urea gel. A) Native tRNA\textsuperscript{Val}. B) In vitro transcribed tRNA\textsuperscript{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 VI (20 mM Mg\textsuperscript{2+}). Lanes 5–8 show the products of RNase VI digestion with: (5), no added Mg\textsuperscript{2+}; (6) 1 mM Mg\textsuperscript{2+}; (7) 10 mM Mg\textsuperscript{2+}; (8) 20 mM Mg\textsuperscript{2+}.

(Fig. 2). Strong cleavage of native tRNA\textsuperscript{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\textsuperscript{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\textsuperscript{Val} and the in vitro transcript are revealed by structural mapping with double-strand-specific RNase V1 at different Mg\textsuperscript{2+} 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\textsuperscript{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\textsuperscript{Val}, is cleaved weakly at p10, and weak cleavages are observed at p43 and p44 in native tRNA\textsuperscript{Val}, but not in the transcript (compare Figs. 4A and B).

Figure 6. Structural mapping of native and in vitro transcribed tRNA\textsuperscript{Val}, renatured at 55°C (low-temperature), by limited RNase V1 cleavage, at varying Mg\textsuperscript{2+} concentrations. Autoradiograph of cleavage products of 5'-labeled tRNA\textsuperscript{Val} separated by electrophoresis on a 15% polyacrylamide-8M urea gel. A) Native tRNA\textsuperscript{Val}. B) In vitro transcribed tRNA\textsuperscript{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 VI (10 mM Mg\textsuperscript{2+}). Lanes 5–7 show the products of RNase V1 digestion with: (5) 1 mM Mg\textsuperscript{2+}; (6) 5 mM Mg\textsuperscript{2+}; (7) 10 mM Mg\textsuperscript{2+}.
Temperature-induced conformers of fully modified native tRNAVal

During these studies, which were performed with tRNA renatured at 70°C, we noticed that the 260 nm absorbance of native tRNAVal 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 tRNAVal. The structural basis for the temperature-dependent differences in hypochromicity was determined by nuclease mapping studies comparing the solution structure of native tRNAVal 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 tRNAVal.

Comparison of the patterns of limited nuclease S1 digestion of 5-end-labeled native tRNAVal 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 tRNAVal, 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 tRNAVal is not affected by the temperature used to renature the tRNA (compare Figs. 2B and 5B).

Results of limited RNase V1 digestion of native tRNAVal renatured at 55°C are shown in Fig. 6A. The major difference from the cleavage pattern of the h-form of native tRNAVal is the hydrolysis at p8 (the band just below the strong spontaneous cleavage at p9), which is readily detected in tRNAVal 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 tRNAVal 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 aminocacylated by their cognate aminocayl-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 tRNAVal is less ordered than that of the native, fully modified molecule as shown by the appreciably lower Tm of the former at all Mg²⁺ concentrations (Fig. 1). Previous studies contrasting native and in vitro transcribed yeast tRNA^Phe (1) and tRNA^Amp (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 tRNAVal 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 tRNAVal 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 tRNAVal were seen in the pattern of RNase T1 digestion (results not shown). Both native tRNAVal and the in vitro transcript are cleaved in the T-loop. The unmodified in vitro transcribed tRNAVal is cleaved at p52, p53 and p54; native tRNAVal is cleaved at p54 (although not as extensively), but not at p52 or p53 (refer to tRNAVal 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 tRNAVal, 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)tRNAVal 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 tRNAVal 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 (Mn = 11,000) than nuclease S1 (Mn = 32,000).

Native and in vitro transcribed tRNAVal 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 tRNAVal, whereas in vitro transcribed tRNAVal is resistant to hydrolysis at these positions (Fig. 4).

Overall, these results indicate that the standard tertiary structure of unmodified in vitro transcribed tRNAVal 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^Amp 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^Amp (3). Lead-ion-induced cleavage of in vitro transcripts of yeast tRNA^Phe and tRNA^Amp 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 tRNAVal 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 tRNAVal was previously inferred from fluorescence polarization anisotropy (19) and methyl proton NMR (20) studies.

The two conformers of native tRNAVal 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 cmo^5^ (25). 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 m^6^A37 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 tRNAVal fail to provide evidence for the existence of stable conformational isomers of the unmodified tRNA (compare Figs. 2B & 5B; 4B & 6B). The modified nucleoside, m^6^A, at position 37 in native tRNAVal could play an important role in this stabilization by promoting strong base-stacking interactions with A38. Absence of the modified m^6^A37 in in vitro transcribed tRNAVal 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 dm^3^C residue in the anticodon stem (25, 26). The base modification promotes site-specific Mg^2+^ binding in the upper part of the hairpin (26) that induces structural alterations in the anticodon loop (25).

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