# Effects of nucleotide substitutions within the T-loop of precursor tRNAs on interaction with ATP/CTP:tRNA nucleotidyltransferases from *Escherichia coli* and yeast

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Recognition of tRNA and tRNA-like substrates by the enzyme ATP/CTP:tRNA nucleotidyltransferase requires chemically intact nucleotides within the T-loop, especially at positions 57 and 58, which are invariant purines among naturally occurring tRNAs. To test the effects of base substitutions at these positions, which are distant from the site of catalysis, we synthesized mutant $tRNA^{Glu}$ molecules. These *in vitro*-synthesized RNAs also contained an extra 33 bases at the 5′ end and lacked post-transcriptionally modified bases. The precursor tRNAs were used as substrates for nucleotidyltransferases from *Escherichia coli* and yeast. Substitution of cytidines at either position 57 or 58 had dramatic inhibitory effects on recognition by both enzymes, including raising the apparent $K_m$ and lowering the apparent $V_{max.}$; substitution of an adenosine at position 57 or a uridine at position 58 inhibited the reaction only slightly by comparison. Our results demonstrate that the identities of nucleotides at positions 57 and 58 are relevant to recognition by nucleotidyltransferase, and that a purine is required at position 57. The extra bases at the 5′ end and the lack of post-transcriptionally modified bases did not substantially inhibit interaction with the enzyme, as judged by the wild-type precursor $tRNA^{Glu}$ acting as an effective substrate for both enzymes in the presence of equal concentrations of appropriate tRNA substrates isolated from *E. coli*.

## INTRODUCTION

ATP/CTP:tRNA nucleotidyltransferase, an important tRNA processing enzyme, catalyses the addition of CMP and AMP on to the 3′ ends of tRNA or tRNA-like structures to add or restore the sequence CpCpA. It has been studied extensively and its purification as well as many of its physical and catalytic properties have been reviewed [1–3]. The enzyme is highly specific for tRNAs, but does not discriminate among tRNAs with regard to their amino acid specificity or source organism [1,4]. Remarkably, the CpCpA sequence is restored without a nucleic acid template, suggesting an ordered set of binding sites on the enzyme for the co-substrates CTP and ATP [1,5]. Interaction of nucleotidyltransferase with tRNAs and tRNA-like structures is therefore an important model system for understanding how the catalytic centre of a single polypeptide enzyme can catalyse multiple sequential reactions, separated in both time and space.

The ability to recognize an entire class of molecules such as tRNAs or tRNA-like structures suggests that the structural features of the tRNA substrates that are recognized by the enzyme are conserved among all tRNAs. Consistent with this notion, chemical interference experiments have demonstrated that nucleotides at the corner of a tRNA's three-dimensional structure, in the D- and T-loops, are required in chemically unmodified form for recognition as substrate by nucleotidyltransferases from *Escherichia coli*, yeast and rabbit liver [6–8]. Specifically, residues 57 and 58 in the T-loop, which are invariant purines among naturally occurring tRNAs [9], appear to be essential for interaction with these enzymes [6–8]. A two-site model for recognition of tRNA by nucleotidyltransferase has been proposed in which the enzyme is thought to extend across the top of the tRNA's three-dimensional structure, extending from the reacting 3′ end of the molecule along the acceptor and T-stems to the non-reacting portion at positions 57 and 58 in the T-loop [6–8].

The chemical modification experiments, while implicating positions 57 and 58 as being important to the interaction, could not determine whether the presence of the bulky alkyl groups added by the modification reaction interfered with the reaction, or whether a required structural feature of the nucleotide was destroyed. From these studies it is not clear whether any nucleotide base will suffice to mediate the interaction as long as it is chemically intact. Furthermore, it is not known whether bases at positions 57 and 58 mediate the interaction in terms of binding affinity alone, or whether they play a role in activating enzymic activity.

Accordingly, to more precisely define the roles played by conserved purines at positions 57 and 58 in the T-loop of tRNAs in mediating interaction with nucleotidyltransferase, we have synthesized *in vitro* wild-type and variant precursor forms of $tRNA^{Glu}$ from *E. coli*. Each of these *in vitro*-synthesized RNAs was tested for its ability to be radioactively labelled by nucleotidyltransferases from *E. coli* and yeast, using CTP and [$\alpha$-$^{32}$P]ATP as co-substrates. In addition, competition assays using unfractionated tRNAs were used to assess their competency as substrates. A comparison of kinetic parameters among the wild-type and variant precursor tRNAs (p-tRNAs) confirms the importance of individual nucleotides and local structure within the T-loop both in terms of determining affinity for the enzyme and in terms of stimulating enzyme activity.

## EXPERIMENTAL

### Measurement of activity

The activity of nucleotidyltransferase was measured by incorporation of $^{32}$P-labelled AMP in 50 mM glycine/NaOH buffer, pH 9.4, containing 5–10 mM $MgCl_2$, 1–5 mM dithiothreitol (DTT), 50 $\mu$M CTP and 500 $\mu$M [$\alpha$-$^{32}$P]ATP (0.7 mCi/ml). Purified *E. coli* $tRNA^{Phe}$ (Boehringer Mannheim Biochemicals,

Abbreviations used: DTT, dithiothreitol; p-tRNA, precursor tRNA.

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Indianapolis, IN, U.S.A.), unfractionated *E. coli* tRNA (from which the 3′-terminal CpCpA residues had been removed by treatment with snake venom phosphodiesterase [6]), or *in vitro*-transcribed p-tRNAs were used as substrates. The substrate was present in over 100-fold excess relative to the enzyme.

The mixtures (15–200 $\mu$l) were incubated at 37 °C for 10–60 min and the reaction was terminated by the addition of cold 2.5 % trichloroacetic acid containing 20 mM sodium pyrophosphate. Acid-precipitable radioactivity was recovered on Whatman GF/C glass-fibre filters; the filters were rinsed with 2.5 % trichloroacetic acid and ethanol, and radioactivity on the filters was measured using a Tri-Carb 1900 CA liquid scintillation counter.

Alternatively, small reaction volumes (15 $\mu$l) using the same reaction conditions as described above were incubated for 30 min (*E. coli* enzyme) or 60 min (yeast enzyme) prior to loading on to 12 % polyacrylamide gels (acrylamide/*N*,*N*′-methylenebisacrylamide; 19:1, w/w) in 8 M urea/100 mM Tris-borate, pH 8.3/2.5 mM EDTA. Autoradiograms were exposed at −70 °C for 1–3 days.

### Isolation of nucleotidyltransferases

The enzyme was isolated from *E. coli* MRE600 as described by Gillis [10]. Briefly, cells were lysed by grinding with alumina in 25 mM Tris/HCl, pH 7.4/10 mM $MgCl_2$/2 mM EDTA/1 mM DTT/10 % (v/v) glycerol. Following treatment with DNase I, the sample was subjected to centrifugation at 30000 ***g*** for 20 min, and then 150000 ***g*** for 2.5 h. Following a 25–50 % ammonium sulphate saturation cut of the supernatant, the resulting precipitate was resuspended in 20 mM $KH_2PO_4/K_2HPO_4$, pH 6.8/1 mM $MgCl_2$/0.1 mM EDTA/1 mM DTT/10 % (w/v) glycerol, dialysed against the same buffer, and loaded on to a 2 cm × 20 cm column of DEAE-cellulose (Whatman). The enzyme was eluted with a 250 ml linear gradient (20–200 mM) of potassium phosphate in the same buffer.

The two fractions containing the peak of activity were pooled and dialysed against the 20 mM $KH_2PO_4/K_2HPO_4$ buffer, and loaded on to a 2 cm × 20 cm column of P11 phosphocellulose (Whatman), also equilibrated in the same buffer. The enzyme was eluted using a 0–0.3 M linear gradient of KCl. Peak fractions of activity were pooled, concentrated by dialysis against buffer containing 18 % polyethylene glycol, made to 50 % (v/v) glycerol, and stored at −20 °C.

The yeast enzyme was isolated from 250 g of Baker's yeast grown to late logarithmic phase in 15 litres of yeast extract (1 %)/peptone (2 %)/dextrose (2 %) medium [11]. The cells were ground with sea sand and resuspended in 0.2 M Tris/HCl, pH 8.5/20 mM $MgCl_2$/0.1 M $NH_4Cl$/1 mM EDTA/1 mM PMSF/1 mM benzamidine/10 % (v/v) glycerol. Following centrifugation at 20000 ***g*** for 20 min, the supernatant was treated with DNase I and the 35–55 % ammonium sulphate saturation cut was resuspended and dialysed against 30 mM potassium phosphate, pH 7.3/1 mM EDTA/0.1 mM DTT/1 mM PMSF/1 mM benzamidine/10 % (v/v) glycerol.

The sample was passed through a DEAE-cellulose column (4.5 cm × 45 cm) in the same buffer and peak fractions of activity in the flow through were loaded on to a phosphocellulose column (1.5 cm × 50 cm) in 50 mM sodium phosphate, pH 7.2/1 mM EDTA/1 mM DTT. After washing the column with 0.2 M KCl in the same buffer, the enzyme was eluted with 0.4 M KCl. Peak fractions were dialysed to remove the KCl and loaded on to a column (1 cm × 10 cm) of Affi-Gel Blue. The enzyme was eluted with 1 M KCl, concentrated using Centricon microconcentrators (Amicon, Danvers, MA, U.S.A.) and dialysed against 30 mM potassium phosphate, pH 7.3/1 mM EDTA/0.1 mM DTT/50 % (v/v) glycerol; it was stored at −20 °C.

### Construction of plasmid templates

A detailed description of the cloning procedures is given by Hegg [12]. Briefly, the cloned *rrnB* operon of *E. coli* [13] was digested with *Ava*I to give an 809 bp fragment, which was then cleaved with *Sau*3AI and *Tha*I, and a resulting 189 bp fragment containing the $tRNA^{Glu}$ gene was isolated. This was then subcloned into pTZ18U (United States Biochemical Corp., Cleveland, OH, U.S.A.) to give the recombinant construct pDH3. The inserted gene was then excised using the flanking *Eco*RI and *Hin*dIII restriction sites and cloned into M13mp18 so that single-stranded DNA could be used for oligonucleotide-directed mutagenesis. The synthetic mutant primer was fully degenerate at sites corresponding to positions 57 and 58 in the T-loop, following the numbering scheme of Sprinzl et al. [14]. Mutagenesis was performed using a kit from Amersham Corp. (Arlington Heights, IL, U.S.A.). Mutant genes were then recovered from the M13 constructs by digesting *in vitro*-synthesized double-stranded DNA with *Eco*RI and *Hin*dIII. The recovered fragments were then ligated to pTZ18U (previously digested with *Eco*RI and *Hin*dIII) and used to transform JM109 competent cells; recombinant plasmids from the resulting transformants were then isolated and mutations were confirmed by dideoxy sequencing [12].

### *In vitro* transcription and purification of transcripts

Wild-type or mutant plasmids were linearized using *Bsa*HI and transcribed using T7 RNA polymerase. The *in vitro* transcriptions were carried out using kits from Promega Corp. (Madison, WI, U.S.A.), Epicenter Technologies (Madison, WI, U.S.A.) or Ambion Inc. (Austin, TX, U.S.A.). T7 RNA polymerase was obtained from the commercial kits or was prepared from *E. coli* BL21/pAR1219 as described by Li [15]. Following digestion of the DNA template with RNase-free DNase I, the reaction was extracted with phenol/chloroform and the RNA was precipitated with ethanol, rinsed with ethanol, dried, and resuspended in 10 mM Tris/HCl, pH 8.0/1 mM EDTA. Small fragments and unincorporated nucleotides were removed by spun-column chromatography through Sephadex G-50. Concentrations were determined by measuring the absorbance at 260 nm, assuming RNA at a concentration of 40 $\mu$g/ml has an absorbance of 1.0. Aliquots of the p-tRNAs, adjusted to equal concentrations, were analysed on denaturing polyacrylamide gels to verify their concentration and integrity.

## RESULTS

### *In vitro* transcription of substrates

The wild-type sequence in the T-loop of p-tRNAs used in this study is $G^{57}A^{58}$; single mutants that were isolated and tested as substrates include $A^{57}$, $C^{57}$, $U^{58}$ and $C^{58}$; the doubled mutants were $A^{57}U^{58}$ and $U^{57}C^{58}$ (Figure 1). All the p-tRNAs lacked the entire 3′-terminal CpCpA sequence. When the transcripts were examined on denaturing polyacrylamide gels, less than 5 % of the material was degraded. In most cases there were two resolvable bands that migrated in the size range of full-length products. They either differed in size by fewer than four nucleotides, or they were electrophoretic conformers. They were both substrates for the enzymes (see below).

The p-tRNAs also contained an extra 33 bases on the 5′ end of the tRNA. This was done to test whether the extra bases would interfere with the *E. coli* enzyme but not that of the yeast.

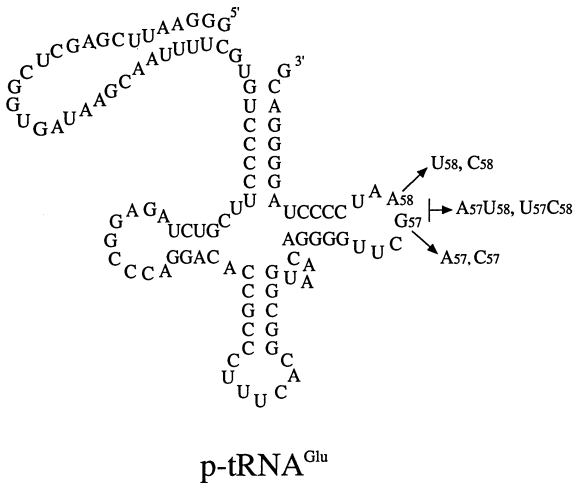


**Figure 1 Cloverleaf model of the secondary structure of p-tRNA$^{Glu}$**

Single and double nucleotide substitutions present in the mutant p-tRNAs are indicated by arrows. All constructs lacked the 3′-terminal CpCpA sequence.

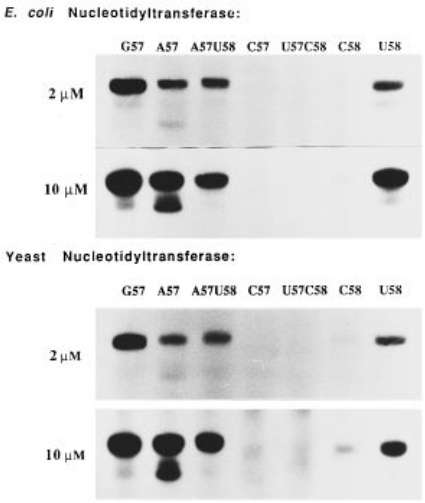


**Figure 2 Autoradiogram of polyacrylamide gel showing p-tRNAs labelled with $^{32}$P using nucleotidyltransferases from *E. coli* and yeast**

Synthetic p-tRNA$^{Glu}$ substrates were incubated with enzyme and [$\alpha$-$^{32}$P]ATP at two different RNA substrate concentrations (2 $\mu$M and 10 $\mu$M). At each concentration, samples were run side by side on the same gel and the exposure times were identical; however, the autoradiograms at different concentrations were exposed for slightly different lengths of time.

The *E. coli* enzyme would not be expected to repair p-tRNAs *in vivo*, because the CpCpA sequence is encoded in the gene; in contrast, the yeast enzyme might add the CpCpA sequence on to p-tRNAs *in vivo* because the CpCpA addition is done post-transcriptionally.

### Relative substrate activities as determined using the gel assay

Following incubation of unfractionated tRNAs or p-tRNAs (wild type and mutant; both of which lacked the entire CpCpA sequence) with CTP, [$\alpha$-$^{32}$P]ATP and nucleotidyltransferase, the reaction mixture was loaded on to a denaturing polyacrylamide gel and the labelled products were visualized by autoradiography.

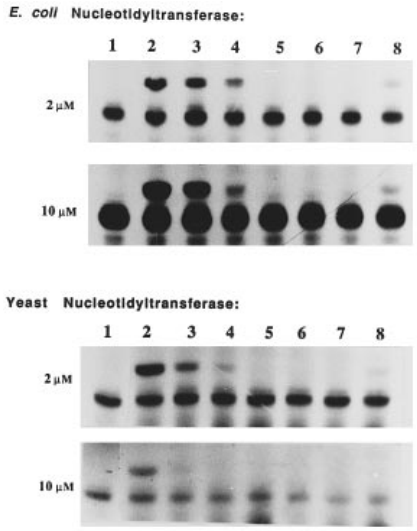


**Figure 3 Autoradiogram of polyacrylamide gel showing p-tRNAs labelled with $^{32}$P using nucleotidyltransferase from *E. coli* and yeast in the presence of an equal concentration of unfractionated tRNAs from *E. coli***

Unfractionated tRNAs lacking the 3′-terminal bases (lane 1) and p-tRNA$^{Glu}$ synthetic substrates together with tRNAs (lane 2, wild type; lane 3, $A^{57}$; lane 4, $A^{57}U^{58}$; lane 5, $C^{57}$; lane 6, $U^{57}C^{58}$; lane 7, $C^{58}$; and lane 8, $U^{58}$) were incubated with enzyme and [$\alpha$-$^{32}$P]ATP at two different RNA substrate concentrations (2 $\mu$M and 10 $\mu$M). The labelled p-tRNA is the slowest-migrating band. At each concentration, samples were run side by side on the same gel and the exposure times were identical; however, the autoradiograms at different concentrations were exposed for slightly different lengths of time.

A side-by-side comparison of substrates at equal concentrations (Figure 2) illustrates that the wild-type p-tRNA was the most effective substrate for both enzymes; the $A^{57}$ was nearly comparable with the wild type, especially at high concentrations; the $U^{58}$ and $A^{57}U^{58}$ variants were somewhat less effective; and the $C^{57}$ and $U^{57}C^{58}$ variants could not be detectably labelled using this assay by either the *E. coli* or yeast enzyme (Figure 2). The $C^{58}$ mutant was labelled to a small extent by both enzymes, but was still a very poor substrate relative to the wild type.

Competition assays were carried out using p-tRNAs and unfractionated tRNA that had been treated with snake venom phosphodiesterase to remove the 3′-terminal residues (Figure 3). The wild-type p-tRNA$^{Glu}$ and the $A^{57}$ variant were similar in their abilities to act as substrates for both enzymes to unfractionated tRNA. The $A^{57}U^{58}$ and $U^{58}$ p-tRNAs were labelled, but to a somewhat lesser extent. The extra 33 bases on the 5′ end and the lack of post-transcriptionally modified bases in the wild-type, $A^{57}$, $A^{57}U^{58}$ and $U^{58}$ p-tRNAs did not appear to inhibit their ability to act as substrates with either enzyme. The $C^{57}$, $U^{57}C^{58}$ and $C^{58}$ p-tRNAs did not effectively compete with unfractionated tRNA for either the *E. coli* or yeast enzyme.

The labelled unfractionated tRNAs appear as one diffuse band, but were resolved into many bands when analysed on 20% gels run for longer periods of time. For all p-tRNAs that were substrates, both of the two nearly full-length p-tRNAs were labelled by each enzyme (Figure 3).

### Apparent kinetic parameters determined using the trichloroacetic acid assay

More complete substrate titrations using the wild-type and mutant p-tRNAs were carried out in the presence of nucleotidyltransferase from both *E. coli* and yeast. The concentrations

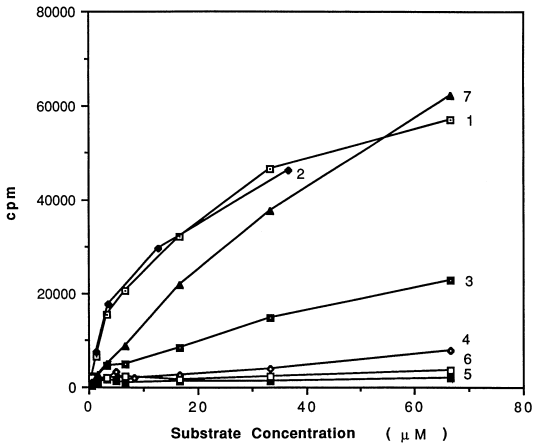


**Figure 4 Substrate titrations of p-tRNAs using *E. coli* nucleotidyltransferase**

$^{32}$P-labelled trichloroacetic acid-precipitable material was collected on filters and counted, following incubation of p-tRNA at various concentrations with enzyme and [$\alpha$-$^{32}$P]ATP for 30 min. The curves are numbered as follows: 1, wild type; 2, $A^{57}$; 3, $A^{57}U^{58}$; 4, $C^{57}$; 5, $U^{57}C^{58}$; 6, $C^{58}$; and 7, $U^{58}$.

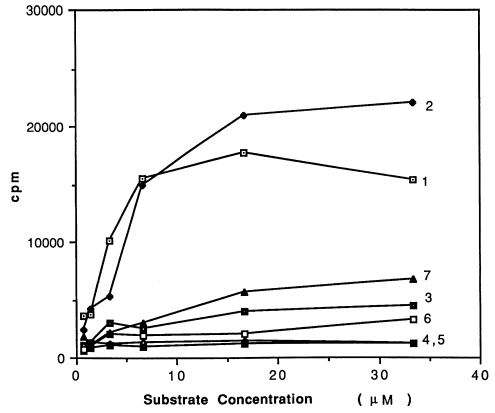


**Figure 5 Substrate titrations of p-tRNAs using yeast nucleotidyltransferase**

$^{32}$P-labelled trichloroacetic acid-precipitable material was collected on filters and counted, following incubation of p-tRNA at various concentrations with enzyme and [$\alpha$-$^{32}$P]ATP for 60 min. The curves are numbered as follows: 1, wild type; 2, $A^{57}$; 3, $A^{57}U^{58}$; 4, $C^{57}$; 5, $U^{57}C^{58}$; 6, $C^{58}$; and 7, $U^{58}$.

of CTP (50 $\mu$M) and ATP (500 $\mu$M) were well above the reported apparent $K_m$ values [2]. Accordingly, these conditions were used to estimate values for apparent $K_m$ and $V_{max.}$ for the p-tRNA substrates. Initial velocities were estimated by measuring acid-precipitable radioactivity after incubation for 30 min (*E. coli*) or 60 min (yeast); incorporation was linear with time over these periods. Incorporation of $^{32}$P is plotted against substrate concentrations in Figures 4 and 5. Precursor tRNA substrate concentrations above 40 $\mu$M led to substantial inhibition of the yeast enzyme.

Judging from the shape of the curves in Figures 4 and 5, the wild-type ($G^{57}$) and $A^{57}$ p-tRNAs were the best substrates for both enzymes. The $U^{58}$ p-tRNA appeared to have a somewhat higher $K_m$ for both enzymes; its $V_{max.}$ was relatively low with the yeast enzyme, but high with that from *E. coli*. The $A^{57}U^{58}$ variant had a slightly higher $K_m$ for the *E. coli* enzyme and lower $V_{max.}$ for both enzymes, relative to those of the wild-type p-tRNA. The

**Table 1 Estimates of apparent $K_m$ and $V_{max.}$ values for wild-type and mutant p-tRNAs**

Kinetic parameters were not determined (nd) for the yeast enzyme using $tRNA^{Phe}$. In some cases the activities were too low to permit making reliable estimates of kinetic parameters (–). All assays were carried out in a final volume of 15 $\mu$l.

| | *E. coli* | | Yeast | |
|---|---|---|---|---|
| Substrate | $K_m$ ($\mu$M) | $V_{max.}$ ($\mu$mol·h$^{-1}$) | $K_m$ ($\mu$M) | $V_{max.}$ ($\mu$mol·h$^{-1}$) |
| $tRNA^{Phe}$ | 5 | 0.14 | nd | nd |
| Wild type | 8 | 0.2 | 3 | 0.03 |
| $A^{57}$ | 10 | 0.2 | 6 | 0.05 |
| $A^{57}U^{58}$ | 14 | 0.08 | 2 | 0.008 |
| $U^{58}$ | 40 | 0.3 | 8 | 0.02 |
| $C^{58}$ | $>100$ | 0.06 | – | $<0.006$ |
| $C^{57}$ | – | $<0.03$ | – | – |
| $U^{57}C^{58}$ | – | – | – | – |

$C^{58}$ variant was a poor substrate for both enzymes; the $C^{57}$ p-tRNA was also a poor substrate for the *E. coli* enzyme and was not detectably labelled at all by nucleotidyltransferase from yeast. We could not detect substrate activity for the $U^{57}C^{58}$ p-tRNA with either enzyme.

Eadie–Hofstee plots were used to estimate values for apparent $K_m$ and $V_{max.}$ for all but the $C^{58}$ variant with the yeast enzyme and the $C^{57}$ and $U^{57}C^{58}$ variants with both enzymes. Upper limit estimates were made for the apparent $V_{max.}$ of the $C^{57}$ p-tRNA with the *E. coli* enzyme and the $C^{58}$ p-tRNA with the yeast enzyme, but the activities were too low to permit making reliable estimates of the apparent $K_m$. Three separate titrations were conducted with the *E. coli* enzyme and two with that from yeast. Average values for apparent $K_m$ and $V_{max.}$ are presented in Table 1.

## DISCUSSION

### Selected base substitutions within the T-loop were dramatically inhibitory

Nucleotide substitutions were made in the T-loop of $tRNA^{Glu}$ to help elucidate the roles of nucleotides at positions 57 and 58, located at a considerable distance from the reacting 3′ end, in mediating interaction with nucleotidyltransferases from *E. coli* and yeast. Previous chemical modification interference assays had identified these bases as being required intact [6–8], but such experiments could not determine whether the identity of each individual nucleotide is important. In addition, the heterogeneous nature of randomly modified tRNAs prevented determination of the effects of altering an individual nucleotide base on kinetic parameters of the enzyme–substrate interaction.

Single or double mutations within the T-loop, such as the $C^{57}$, $C^{58}$ and $U^{57}C^{58}$ p-tRNAs, exhibited dramatic inhibition of labelling by nucleotidyltransferases from both *E. coli* and yeast. This demonstrates that the identities of individual nucleotides at positions 57 and 58 are relevant to the interaction with nucleotidyltransferase, and that purines are generally preferred over pyrimidines. This may in part explain why bases at these positions are universally purines among naturally occurring tRNAs. We speculate that conserved nucleotides at the corner of the tRNA's L-shaped tertiary structure may also mediate interactions with other tRNA-processing enzymes, which recognize all tRNAs regardless of their amino acid specificity.

There are several possible reasons why both enzymes failed to

recognize C57 or U57C58 p-tRNAs, both of which have a pyrimidine at position 57 in place of the universally conserved purine found in tRNAs *in vivo* [9]. One straightforward explanation of why a purine is required for interaction with nucleotidyltransferase is that H-bonding with a purine's N7 at position 57 is involved in recognition. However, it is also possible that replacement of a purine with a pyrimidine disrupts the stacking of nearby bases. A third possibility is that $C^{57}$ can alter the nature of the tertiary interactions at the corner of the p-tRNA's L-shaped three dimensional structure. For example, $C^{57}$ may participate in a tertiary base pair with $G^{19}$, along with $C^{56}$ base pairing with $G^{18}$. Such a change in tertiary base pairing would most certainly distort the local conformation within the T-loop. In any of the above cases, the essential role of structure within the T-loop to recognition by nucleotidyltransferase is confirmed.

The observed effects of base substitutions at position 58 are not easily explained. A uridine at position 58 had a less dramatic effect than a cytidine at this position, suggesting that neither the amino group at C6 nor the N7 in the conserved adenosine at this position are absolutely required for formation of H-bonds. Base pairing by itself between positions 54 and 58 does not seem to be decisive, because a $U^{54}$–$C^{58}$ pair in the $C^{58}$ p-tRNA can be proposed as feasibly as a $U^{54}$–$U^{58}$ base pair in the $U^{58}$ p-tRNA. However, the exact geometries of the two proposed base pairings may differ sufficiently so that the local conformations and overall patterns of H-bond donors/acceptors within the T-loop are quite different between the $U^{58}$ and $C^{58}$ variants.

Disruption of stacking cannot solely explain the dramatic inhibitory effects of a cytidine at position 58, because one would expect an equal disruption of stacking in the $U^{58}$ variant, which was a very reasonable substrate. Furthermore, the $C^{58}$ p-tRNA was a detectable, although poor, substrate for both enzymes. Therefore it is unlikely that the overall structure of the $C^{58}$ p-tRNA had been grossly distorted.

The effects of a cytidine residue at position 58 appeared to have a more pronounced inhibitory effect when the enzyme from *E. coli* was used, relative to that seen with yeast enzyme (Figure 2). This is consistent with the suggestion that chemical modifications at position 58 inhibit the reaction to a lesser extent and in fewer tRNAs with the yeast enzyme than they do with the enzyme from *E. coli* [6].

The effects of base substitutions on the kinetics of the interaction with nucleotidyltransferase were to raise the apparent $K_m$ and to lower the apparent $V_{max.}$ in most cases (Table 1). Consistent with the elevated values for $K_m$ in general, none of the variant p-tRNAs was labelled as extensively as the wild-type p-tRNA in the presence of equal concentrations of mature tRNA (Figure 3). Elevated values of the apparent $K_m$ suggest that such base substitutions at positions 57 and 58 reduced the affinity of the substrate for the enzyme. It was particularly significant that the apparent $V_{max.}$ for the $A^{57}U^{58}$ p-tRNA, whose apparent $K_m$ was near that of the wild-type p-tRNA for both enzymes, was lowered, suggesting that this variant may have been unable to activate the enzyme, despite its ability to bind.

Activation of nucleotidyltransferase by non-reacting parts of the substrate has been proposed previously based on the ability of non-full-length tRNAs to stimulate the rate of incorporation of CMP on to the model substrate cytidine by the enzyme from rabbit liver [16]. Our data measure the rate of incorporation of AMP, but may also reflect the rate of ligation of CMP, because all our substrates, including the wild-type construct, lacked the entire CpCpA sequence.

### The 33 extra bases on the 5′ end and the absence of post-transcriptionally modified bases did not substantially inhibit recognition by the enzyme

The 33-base 5′ tail and the lack of post-transcriptionally modified bases in the wild-type p-$tRNA^{Glu}$ did not detectably interfere with labelling by nucleotidyltransferase from either *E. coli* or yeast. This is most directly apparent from the results of competition assays in which the wild-type p-$tRNA^{Glu}$ was as effectively labelled as mature tRNA at equal concentrations (Figure 4). The apparent lack of effect by the 5′ tail was somewhat surprising given its proximity to the site of ligation of the CMP and AMP residues catalysed by nucleotidyltransferase. However, it is consistent with the lack of effects of chemical modifications near the 5′ end in previous studies [6–8]. It was also somewhat surprising to note that its presence did not affect interaction with the enzyme from *E. coli*, despite the likelihood that this enzyme would not interact with p-tRNAs *in vivo*. In contrast, the lack of inhibition by the extra bases on the yeast enzyme was not surprising, because p-tRNAs may be substrates for this enzyme *in vivo*.

The absence of inhibition due to the lack of post-transcriptionally modified bases is consistent with the ability of nucleotidyltransferase to label tRNA-like structures, such as those at the 3′ end of plant viral RNAs, which lack most of the modified bases normally found in a tRNA [17]. It is also consistent with tRNAs synthesized *in vitro* being appropriate substrates for other enzymes such as aminoacyl tRNA synthetases and RNase P [18,19].

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