Cytidines in tRNAs that are required intact by ATP/CTP:tRNA nucleotidyltransferases from *Escherichia coli* and *Saccharomyces cerevisiae*

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ABSTRACT

Individual species of tRNA from *Escherichia coli* were treated with hydrazine/3 M NaCl to modify cytidine residues. The chemically modified tRNAs were used as substrate for ATP/CTP:tRNA nucleotidyltransferases from *E. coli* and yeast, with [α-^{32}P]ATP as cosubstrate. tRNAs that were labeled were analyzed for their content of modified cytidines. Cytidines at positions 74 and 75 were found to be required chemically intact for interaction with both enzymes. C56 was also required intact by the *E. coli* enzyme in all tRNAs, and by the yeast enzyme in several instances. C61 was found to be important in seven of 14 tRNAs with the *E. coli* enzyme but only in four of 13 tRNAs with that from yeast. Our results support a model in which nucleotidyltransferase extends from the 3'end of its tRNA substrate across the top of the stacked array of bases in the accepter- and ψ-stems to the corner of the molecule where the D- and ψ-loops are juxtaposed.

INTRODUCTION

The enzyme ATP/CTP:tRNA nucleotidyltransferase catalyzes ligation of CMP or AMP onto shortened tRNAs to restore the 3'terminal sequence CpCpA. It is an important repair enzyme in bacteria (1) and an essential biosynthetic enzyme in eukaryotes, where the CpCpA sequence is added posttranscriptionally (2). It is an especially interesting enzyme in that it is highly specific for tRNAs, yet does not discriminate among tRNAs differing in their amino acid specificity or source of origin (3,4). Accordingly, one would anticipate that it recognizes highly conserved aspects of tRNA structure.

In addition, this enzyme faithfully restores the CpCpA sequence in the presence of ATP and CTP without a nucleic acid template, suggesting an ordered set of binding sites for donor nucleoside triphosphates and acceptor substrate near its active site (4–6). Elucidation of structural features of the tRNA substrate that are required for interaction should advance our understanding of the basis for specificity underlying protein-RNA interactions and for regulation of enzymatic activity by RNA structure.

Aspects of tRNA structure required intact for interaction with nucleotidyltransferases from *Escherichia coli*, yeast and rabbit liver have recently been characterized by identifying chemically modified adenosines, guanosines and uridines that interfered with ^{32}P-labeling, when [α-^{32}P]ATP was used as cosubstrate (7,8). Previous studies had implicated binding sites on the rabbit liver enzyme for residues near the 3'end (5,6), especially cytidines at positions 74 and 75, numbered according to Sprinzl *et al.* (9). In order to determine whether these or other cytidines were required intact by the *E. coli* and yeast enzymes, we characterized effects of modifying cytidines in tRNAs with hydrazine/3 M NaCl on the interaction with nucleotidyltransferases from these two organisms.

MATERIALS AND METHODS

Buffers were prepared using deionized distilled water (Barnstead NANOpure II) and reagent grade chemicals, and were stored at 4°C. Purified isoacceptor species of tRNA were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) or Subriden RNA (Rolling Bay, WA). Following labeling with ^{32}P, contaminating species were resolved on 8 M urea, 20% polyacrylamide gels (acrylamide:N-N'-methylene bisacrylamide, 19:1) in 100 mM Tris-Borate, pH 8.3, 2.5 mM EDTA (TBE), and were analyzed separately. [α-^{32}P]ATP (>3000 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, CA). Snake venom phosphodiesterase (46 U/mg) was purchased from Worthington Chemical (Freehold, NJ). Anhydrous hydrazine and aniline were prepared according to Perrin *et al.* (10).

E. coli ATP/CTP:tRNA nucleotidyltransferase (1.2 U/ml; 1 unit is defined as the amount of enzyme required to add 1 μmol of [α-^{32}P]ATP to tRNA in 1 h at 37°C as described in Spacciapoli *et al.* (7)) was prepared according to Carre *et al.* (11) as the eluant from the DEAE cellulose column step. Peak fractions of activity were concentrated using a Centricon-30 (Amicon, Danvers, MA), an equal amount of glycerol was added, and the preparation was stored at −20°C. The homologous enzyme from *Saccharomyces cerevisiae* (0.9 U/ml) was kindly provided by Dr. Jacek Wower (University of Massachusetts, Amherst, MA) as the eluate from the DEAE column in the procedure of Sternbach *et al.* (12).

Removal of the 3'terminal adenosine (or CpA, or CpCpA) was achieved by digestion with snake venom phosphodiesterase (7).

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Missing cytosines were restored to the 3'end by incubating 5 μg of phosphodiesterase-treated tRNA in 20 μL 50 mM Tris-Glycine, pH 8.5, 5mM $MgCl_2$, 8mM DTT, 25μM CTP (CCA Buffer) and adding 1 μL of either *E. coli* or yeast nucleotidyltransferase at 37°C for 30 min. The mixture was then extracted with an equal volume of Tris-EDTA saturated phenol, and the RNA was precipitated with four volumes of 95% ethanol, resuspended in 200 μL of 0.3 M Na-acetate, reprecipitated with four volumes of ethanol, rinsed with ethanol, dried and resuspended in 5 μL of deionized distilled water.

Modification of unlabeled tRNAs with hydrazine/3 M NaCl was performed according to Peattie (13), except the incubation time was shortened to 10 min. Care was taken to use distilled anhydrous hydrazine to minimize reaction with uridines. Labeling of the modified material was carried out according to Spacciapoli *et al.* (7), except that CTP was ommitted. Labeled samples both modified and untreated were loaded onto a 20% polyacrylamide sequencing gel (8 M urea/TBE) and electrophoresis was continued at 25 mA (T=45°C) until the xylene cyanole tracking dye had migrated 26 cm. Labeled material was recovered from the gel by slicing bands and eluting overnight (7).

Unmodified ^{32}P-labeled control samples, together with 10 μg carrier tRNA, were treated with the chemical modifier exactly as described above. Three quarters of the control material and all of the experimental sample were reisolated on another 20% polyacrylamide gel. One quarter of the control material was retained for a non gel-purified control. After recovery from the gels, all samples were treated with aniline-acetate (13) to cleave the tRNA at modified bases.

Cleavage products were divided into aliquots such that their contents of radioactivity were within 10% of each other, as judged using a Bioscan 2000 radiation detector. They were loaded onto a 20% sequencing gel and electrophoresis was carried out at 25 mA for approximately 2, 5 or 7 h. Autoradiograms were exposed at −70°C for 12 hours to 2 weeks, using Kodak X-Omat film.

RESULTS

In order to identify cytidines in tRNAs that were required chemically intact for interaction with the enzyme ATP/CTP:tRNA nucleotidyltransferase, fourteen tRNAs, lacking their 3'-terminal adenosine residue, were treated with hydrazine/3 M NaCl (13) and then used as substrate for the enzyme from *E. coli*; thirteen tRNAs were used as substrate for the homologous enzyme from yeast.

Chemically modified tRNAs were labeled with ^{32}P using [α-^{32}P]ATP as cosubstrate. The content of modified bases in tRNAs that became ^{32}P-labeled was then characterized by treatment with aniline, which cleaves the RNA chain at chemically modified residues (13). Aniline-induced, ^{32}P-labeled cleavage products were resolved on sequencing gels. Bands that were absent, or those of reduced intensity relative to control material that was labeled prior to modification, were identified on autoradiograms of the gels (Figs. 1 and 2).

Hydrazine-induced modification of cytidines 74 and 75, located adjacent to the 3'teminal adenosine, resulted in complete inhibition of both enzymes in all tRNAs examined (Tables I and II, Figs. 1 and 2). To ensure that the absence of modified cytidines in the labeled tRNAs was not due to restoration of missing cytidines during the labeling reaction, two precautions were taken. All tRNAs were incubated with CTP and nucleotidyltransferase following removal of the terminal adenosine to restore missing

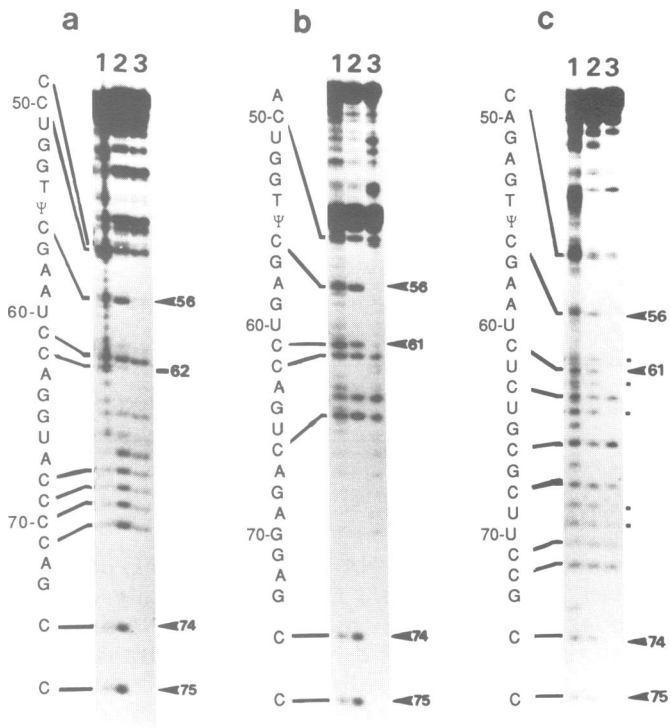

Figure 1. Autoradiogram identifying cytidines in $tRNA^{Gln}$(NUG), $tRNA^{Asn}$ and $tRNA^{Ser}$ required for interaction with nucleotidyltransferase from *E. coli*. $tRNA^{Gln}$(NUG) (a), $tRNA^{Asn}$ (b) and $tRNA^{Ser}$ (c) were treated with hydrazine/3 M NaCl. Unmodified control samples (lanes 1 and 2) and modified experimental samples (lanes 3) were labeled with ^{32}P using *E. coli* nucleotidyltransferase. Control samples were then modified. Non gel-purified controls (lanes 1), gel-purified controls (lanes 2), and experimental samples were treated with aniline and the resulting fragments were resolved on 20% polyacrylamide sequencing gels run for 2.5 h at 25 mA constant current. The sequence from residue number 49 to the 3'end and the location of cytidines are indicated on the left. Arrows indicate nucleotides, numbered according to Sprinzl *et al.* (9), that interfered with the labeling reaction. A solid bar indicates the position of a band that was absent as a result of gel purification. Dots at positions 60, 62, 64, 69 and 70 in $tRNA^{Ser}$ indicate instances where uridines in the control, but not in the experimental sample, had reacted, possibly due to traces of moisture in the control sample. Separate experiments using 50% hydrazine to modify uridines at these positions have shown that they were not required for interaction with the enzyme (Spacciapoli *et al.*, 1989 and data not shown).

cytidines that might have been removed by treatment with the phosphodiesterase. Secondly, CTP was ommitted during the labeling reaction.

Chemically altered C56, situated in the ψ-loop, adjacent to the purine at position 57 and base paired to the guanosine at position 19, dramatically affected the interaction with *E. coli* nucleotidyltransferase in virtually all tRNAs (Table 1, Fig. 1). This result is in accord with the observation that residues 57 and 19 were also required intact for interaction with the enzyme from *E. coli* (7).

In contrast, a modified C56 interfered with the enzyme from yeast to a significantly reduced extent in most tRNAs and not at all in $tRNA^{Phe}$ (Table II, Fig. 2). This correlates with the reduced frequency of interference with the yeast enzyme by modified purines at positions 19 and 58 (7).

The only other modified cytidine we observed to be inhibitory

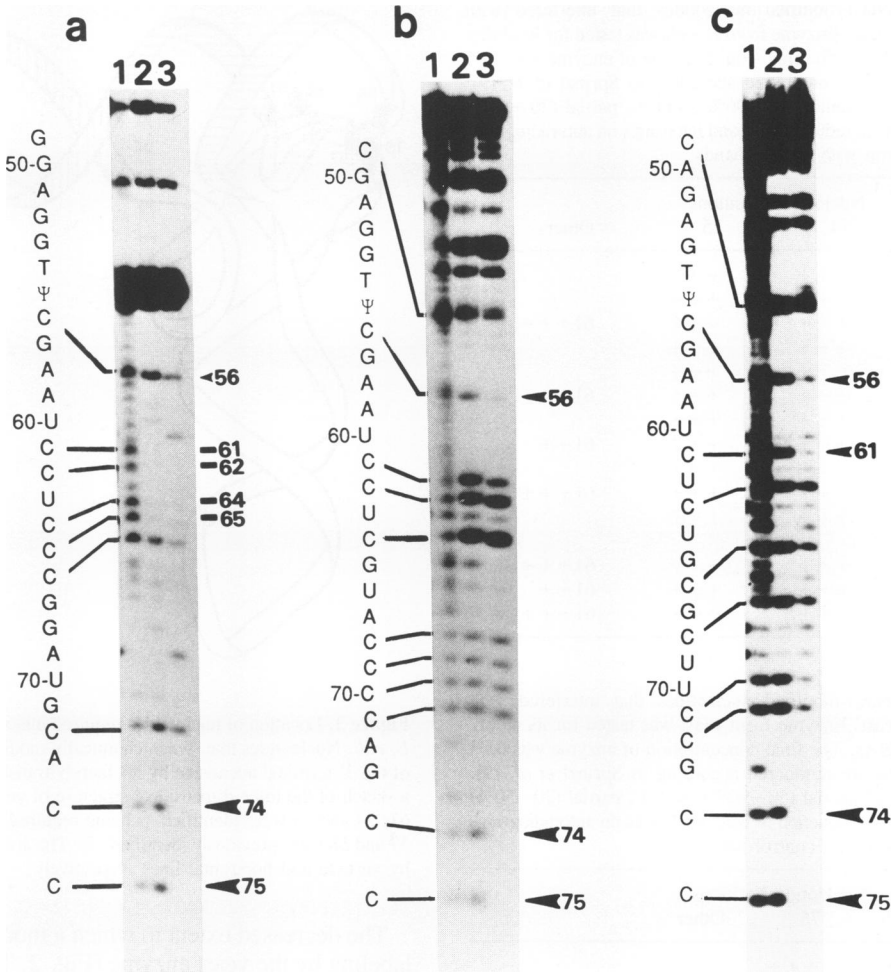

Figure 2. Autoradiogram identifying cytidines in $tRNA^{Arg}$, $tRNA^{Gln}$(CUG) and $tRNA^{Ser}$ required for interaction with nucleotidyltransferase from yeast. $tRNA^{Arg}$ (a), $tRNA^{Gln}$(CUG) (b) and $tRNA^{Ser}$ (c) were treated with hydrazine/3 M NaCl. Unmodified control samples (lanes 1 and 2) and modified experimental samples (lanes 3) were labeled with ^{32}P using yeast nucleotidyltransferase. Control samples were then modified. Non gel-purified controls (lanes 1), gel-purified controls (lanes 2), and experimental samples were treated with aniline and the resulting fragments were resolved on 20% polyacrylamide sequencing gels run for 2.5 h at 25 mA constant current. The sequence from residue number 49 to the 3'end and the location of cytidines are indicated on the left. Arrows indicate nucleotides, numbered according to Sprinzl *et al.* (9), that interfered with the labeling reaction. A solid bar indicates the position of a band that was absent as a result of gel purification.

in more than one tRNA was the invariant C61, located at the loop-proximal end of the ψ-stem (Figs. 1 and 2, Tables I and II). A chemically altered cytidine at this position affected *E. coli* nucleotidyltransferase in seven of 14 tRNAs and the yeast enzyme in four of 13 tRNAs.

DISCUSSION

Interaction of modified tRNAs with nucleotidyltransferase

In the presence of ATP, nucleotidyltransferase faithfully restores AMP to tRNAs lacking the 3'terminal adenosine residue. Accordingly, one would expect the cytidine at position 75, located at the AMP accepting site on the enzyme, to play an essential role in binding of the tRNA. In our set of experiments one would predict that modification and subsequent excision of C75 with hydrazine/3 M NaCl (13) would interfere with restoration of AMP by the enzyme and thereby result in exclusion of such modified tRNAs from the population of labeled molecules. Therefore, it was gratifying to note that all tRNAs containing a modified cytidine at postion 75 were excluded (Figs. 1 and 2, Tables I and II).

In addition, exclusion of tRNAs containing a chemically altered C74 is consistent with the notion that *E. coli* and yeast nucleotidyltransferases have separate binding sites for C75 and C74. This follows from our experimental design in which treatment with the chemical modifier was carried out using single hit kinetics. As a result, most of the tRNA remained intact and small fragments did not predominate following cleavage with aniline (Figs. 1 and 2, see later in Discussion). A chemically altered residue at position 74 was therefore sufficient to prevent ligation of AMP onto an intact C75, implicating a binding site for C74 that was distinct from the AMP accepting site occupied by C75. Alternatively, we cannot exclude the possibility that stacking of cytidines 74 and 75 was required for proper alignment of the tRNA relative to the AMP donor site.

Our results are at least consistent with the model of recognition involving binding sites for both C74 and C75 by the homologous enzyme from rabbit liver, proposed as a result of detailed kinetic

Table I. Hydrazine/3 M NaCl-modified nucleotides that interfered with nucleotidyltransferase from *E. coli*. Enzyme from E. coli was tested for its ability to label chemically treated tRNAs. The final concentration of enzyme was 0.05 units/mL. Nucleotide positions are numbered according to Sprinzl *et al.* (9). +++, total (>90%); ++, substantial (50–90%); and +, partial (20–50%) extents of exclusion; inferred from reduction of band intensities on autoradiograms as judged by visual comparison with control bands.

tRNA	Nucleotide Positions			
	56	74	75	Other
Asp_{GUC}	+++	+++	+++	
Arg_{ACG}	++	+++	+++	
Asn_{GUU}	+++	+++	+++	61+++
Gln_{NUG}	+++	+++	+++	
Gln_{CUG}	+++	+++	+++	
Gly_{GCC}	+++	+++	+++	61+++
Lys_{UUU}	+++	+++	+++	
$fMet_{CAU}$	+++	+++	+++	61++
Met_{CAU}	+++	+++	+++	
Phe_{GAA}	+++	+++	+++	61+++
Phe_{GAA} (yeast)	+	+++	+++	
Ser_{UGA}	+++	+++	+++	61+++
Trp_{CCA}	+++	+++	+++	61++
Val_{VAC}	+++	+++	+++	61+++

Table II. Hydrazine/3 M NaCl-modified nucleotides that interfered with nucleotidyltransferase from yeast. Enzyme from yeast was tested for its ability to label chemically treated tRNAs. The final concentration of enzyme was 0.03 units/mL. Nucleotide positions are numbered according to Sprinzl *et al.* (9). +++, total (>90%); ++, substantial (50–90%); and +, partial (20–50%) extents of exclusion; inferred from reduction of band intensities on autoradiograms as judged by visual comparison with control bands.

tRNA	Nucleotide Positions			
	56	74	75	Other
Asp_{GUC}	+	+++	+++	
Arg_{ACG}	+	+++	+++	
Asn_{GUU}	++	+++	+++	
Gln_{NUG}	++	+++	+++	
Gln_{CUG}	++	+++	+++	
Lys_{UUU}	+	+++	+++	65+++
$fMet_{CAU}$	+++	+++	+++	61++,65–69+,71+
Met_{CAU}	+	+++	+++	
Phe_{GAA}	none	+++	+++	
Phe_{GAA} (yeast)	++	+++	+++	
Trp_{CCA}	+++	+++	+++	61+
Ser_{UGA}	++	+++	+++	61++
Val_{VAC}	+++	+++	+++	61+++

studies using model substrates (5,6). We have shown previously that the rabbit liver enzyme is very similar to that from yeast with regards to its requirements for unmodified purines in the ψ-loop (8), and therefore it was not surprising that these enzymes behaved similarly with respect to recognition of the 3'end.

The central role of C56 in mediating interaction with the enzyme from *E. coli* is in good agreement with the importance of intact purines at positions 57 and 58 (7), also located in the ψ-loop. It also agrees well with the observation that G19, to which C56 is base paired in the tertiary structure of yeast $tRNA^{Phe}$ (14), is required intact by the *E. coli* enzyme (7). Our model of interaction between *E. coli* nucleotidyltransferase and its tRNA substrate is that the enzyme recognizes the 3'end and simultaneously extends across the top of the stacked array of bases in the acceptor- and ψ-stems to interact with the corner of the molecule, where the D- and ψ-loops are juxtaposed (Fig. 3).

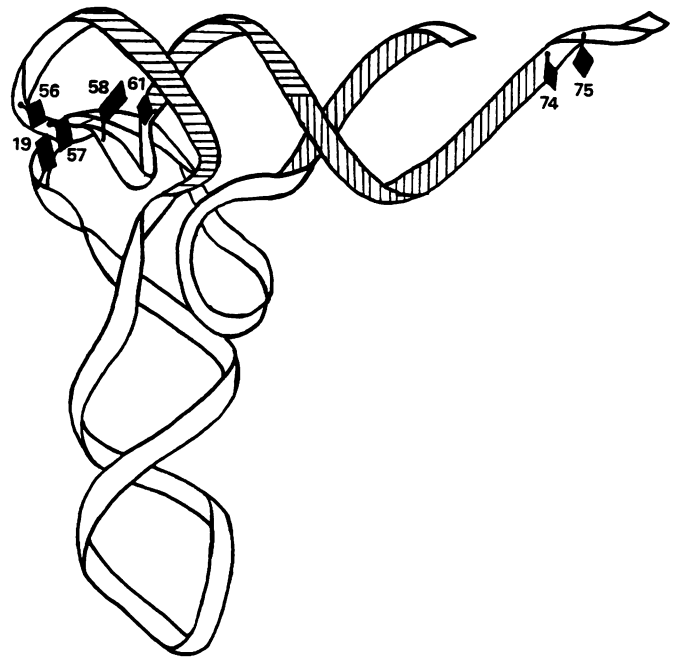

Figure 3. Location of nucleotides required intact by nucleotidyltransferase from *E. coli*. Nucleotides that, when chemically modified, interfered with restoration of the 3'-terminal adenosine by nucleotidyltransferase are depicted (in black) on a sketch of the three dimensional structure of yeast $tRNA^{Phe}$ (14). Cytidines 56, 61, 74 and 75 were identified as being required intact in this study; purines 19, 57 and 58 were previously identified (7). The acceptor- and ψ-stems are indicated by verticle and horizontal lines respectively.

The decreased extent to which a modified C56 interfered with labeling by the yeast enzyme (Fig. 2, Table II) agrees well with our suggestion that the yeast enzyme also interacts with the corner of the tRNA, but to a significantly reduced extent relative to the bacterial nucleotidyltransferase. That is, a carbethoxylated purine at position 57 dramatically inhibited the yeast enzyme, comparable to the situation with *E. coli* nucleotidyltransferase, yet a modified G19 or A58 was not required intact in nearly as many tRNAs (7).

Exclusion of several tRNAs containing a chemically altered C61 from labeled material (Figs. 1 and 2, Tables I and II) further supports the notion that both enzymes interact with the corner of the tRNA, because C61 is an invariant nucleotide located adjacent to A58 in the stacked array of bases extending from the corner towards the 3'end of a tRNA (14, see Fig. 3). Failure of this base to affect the interaction in all tRNAs may reflect subtle structural differences among the tRNAs examined. However, we cannot exclude the possibility that slight variations in conditions of labeling existed, and that under less favorable conditions C61 might prove to be required intact in additional instances.

Aspects of the technique that warrant caution

Gel purification of modified tRNAs

Two types of controls were always included: samples treated with the chemical modifier after labeling and then cleaved with aniline without further purification (Figs. 1 and 2, lanes 1); and samples treated with the chemical modifier after labeling, purified on an 8 M urea-containing gel, and then cleaved with aniline (Figs. 1 and 2, lanes 2). The reason for the second type of control was that purification of chemically modified material on 8 M urea-containing gels can lead to exclusion of material from the main

band on the gel due to the presence of a modified base in a region of the molecule that maintains residual secondary structure during electrophoresis (15). Therefore, gel-purified controls were required to identify bands that were absent from the experimental samples as a result of purification on gels. The chemically modified experimental samples had been gel purified following the labeling reaction to separate intact tRNA from contaminating species, degradation products, and unincorported label.

As a specific example, a band corresponding to the cytidine at position 62 in $tRNA^{Gln}$(NUG) was absent in both samples that had been reisolated on gels following treatment with hydrazine/3 M NaCl (Fig. 1a, lanes 2 and 3). Therefore, we cannot say whether modification of the cytidine at this position in $tRNA^{Gln}$(NUG) affected interaction with nucleotidyltransferase. Similarly, bands corresponding to positions 61, 62, 64 and 65 in $tRNA^{Arg}$ were absent from all gel-purified samples (Fig. 2a, lanes 2 and 3). In contrast, bands corresponding to cytidines at positions 56, 74 and 75 were present in gel-purified controls, but absent from experimental samples (Fig. 1, lanes 2 and 3), indicating that modification of these cytidines had interfered with the interaction.

Hypermodified bases

tRNAs containing a hypermodified uridine at position 47, located adjacent to the 7-methyl guanine at residue 46, exhibited an increased reactivity towards hydrazine/3 M NaCl (Fig. 2a). As a result, the large amount of fragments generated by cleavage with aniline was not consistent with the requirement for single-hit kinetics and thereby prevented identification of nucleotides located on the 5′ side of this position that may have interfered with the enzymatic reaction. In one instance, $tRNA^{Asn}$, the base at position 47 was also hyperreactive towards hydrazine, but has been reported to be an unmodified uridine (9). We suspect that the tRNA used in our experiments was obtained from a strain that contained a modified residue at this site.

Lack of specificity in the hydrazine/3 M NaCl reaction

The reaction of hydrazine/3 M NaCl with tRNAs that were not gel purified following modification occasionally appeared to be nonspecific (Fig. 2c, lane 1). The specificity for cytidines was remarkably improved when the sample was gel purified (Fig. 2c, lane 2), perhaps as a result of removing residual hydrazine during the purification process. In rare instances, the hydrazine/3 M NaCl reaction did not discriminate between cytidines and uridines (Fig 1c, lane 2). The presence of small amounts of moisture, perhaps as a result of condensation on the tubes just prior to adding the anhydrous hydrazine, could account for this lack of specificity.

Effects on binding versus those on the rate of catalysis

Using the damage-selection approach alone, we cannot distinguish between effects of chemically altered bases on binding of the modified substrate to the enzyme from those on the rate of catalysis. To address this question we have constructed mutant tRNAs that contain altered nucleotides at sites which we have identified as being required intact by nucleotidyltransferase. We intend to determine how affinity for the enzyme and the rate of the reaction are affected by such alterations.

CONCLUSIONS

Given the precautions outlined above, the damage-selection experimental approach has enabled us to identify purines (7) and cytidines (this paper) in tRNAs that are required intact for recognition by nucleotidyltransferase. Our results suggest that the enzyme extends from the corner of the molecule, where the D- and ψ-loops are joined by tertiary base pairs, across the top of the tRNA to the 3′end, where the enzyme catalyzes attachment of CMP or AMP.

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