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CCA addition by tRNA nucleotidyltransferase: polymerization without translocation?

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The CCA-adding enzyme repairs the 3′-terminal CCA sequence of all tRNAs. To determine how the enzyme recognizes tRNA, we probed critical contacts between tRNA substrates and the archaeal *Sulfolobus shibatae* class I and the eubacterial *Escherichia coli* class II CCA-adding enzymes. Both CTP addition to tRNA-C and ATP addition to tRNA-CC were dramatically inhibited by alkylation of the same tRNA phosphates in the acceptor stem and TΨC stem–loop. Both enzymes also protected the same tRNA phosphates in tRNA-C and tRNA-CC. Thus the tRNA substrate must remain fixed on the enzyme surface during CA addition. Indeed, tRNA-C cross-linked to the *S.shibatae* enzyme remains fully active for addition of CTP and ATP. We propose that the growing 3′-terminus of the tRNA progressively refolds to allow the solitary active site to reuse a single CTP binding site. The ATP binding site would then be created collaboratively by the refolded CC terminus and the enzyme, and nucleotide addition would cease when the nucleotide binding pocket is full. The template for CCA addition would be a dynamic ribonucleoprotein structure.

Keywords: ATP(CTP):tRNA nucleotidyltransferase/cross-linking/ribonucleoprotein

Introduction

The CCA-adding enzyme [ATP(CTP):tRNA nucleotidyltransferase] catalyzes the synthesis and regeneration of the 3′-terminal CCA sequence of tRNA by adding one nucleotide at a time to the 3′-terminus of tRNAs lacking one, two or all three 3′-terminal nucleotides (Sprinzl and Cramer, 1979; Deutscher, 1982). In organisms which do not encode the 3′-terminal CCA (eukaryotes, some archaea and many eubacteria), CCA addition is an essential step in tRNA biosynthesis. In organisms like *Escherichia coli* where all tRNA genes encode CCA, the CCA-adding enzyme repairs CCA termini depleted by exonucleolytic attack (Zhu and Deutscher, 1987).

All CCA-adding enzymes belong to the nucleotidyltransferase superfamily (Martin and Keller, 1996; Yue *et al.*, 1996). This diverse superfamily includes enzymes that add nucleotides to RNA [poly(A) polymerase], to DNA (terminal deoxynucleotidyltransferase, DNA polymerase β), to protein (glutamine synthase adenylyltransferase) and to antibiotics (streptomycin and kanamycin nucleotidyltransferases) (Holm and Sander, 1995). The CCA-adding enzymes of all three kingdoms, Eubacteria, Eukarya and Archaea, share local sequence homology surrounding the nucleotidyltransferase signature sequence. Nevertheless, only the eubacterial and eukaryotic CCA-adding enzymes exhibit more extensive homology over a 25 kDa region including the active site. This and other features of the signature sequence led us to divide the superfamily into two (and possibly three) subfamilies, the archaeal enzymes belonging to class I and the eubacterial and eukaryotic enzymes belonging to class II (Yue *et al.*, 1996).

Only three enzymes currently are known to add nucleotides to a nucleic acid in a primer-dependent but template-independent fashion: poly(A) polymerase, terminal deoxynucleotidyltransferase and the CCA-adding enzyme. The CCA-adding enzyme is by far the most specific of these, uniquely recognizing tRNA and tRNA-like structures as substrate and adding a precisely defined 3′-terminal CCA sequence. The other two enzymes do not add a defined sequence, and require only that the primer have the correct chemical composition: RNA for poly(A) polymerase and DNA for terminal transferase.

How does the CCA-adding enzyme recognize tRNA and add a unique CCA sequence without using a conventional nucleic acid template? The ability of the CCA-adding enzyme to add CCA to the 3′-termini of all tRNAs regardless of amino acid acceptor specificity, and to viral RNAs with terminal tRNA-like structures (Rao *et al.*, 1989; Giegé 1996), suggests that recognition involves structural features common to all tRNAs. Thus the enzyme might be expected to interact with the ribose–phosphate backbone of tRNA or with the invariant nucleosides. Consistent with this expectation, nucleosides essential for ATP addition to tRNA-CC (lacking the 3′-terminal A) are conserved among all tRNA molecules (Spacciapoli *et al.*, 1989; Hegg and Thurlow, 1990). However, the structural requirements for CCA addition have not been explored.

Ethylnitrosourea (ENU) preferentially alkylates phosphate groups in RNA and DNA, and has been used widely to study RNA structure and protein–nucleic acid interactions (Vlassov *et al.*, 1981, 1983; Garret *et al.*, 1983, 1984; Riehl *et al.*, 1983; Romby *et al.*, 1985; Cavarelli *et al.*, 1993). We have now used ENU to identify phosphates in $tRNA^{Asp}$ of *Bacillus subtilis* that are essential for addition of CTP to tRNA-C, and ATP to tRNA-CC. We took two complementary approaches: footprinting and interference experiments. In the footprinting experiments, 5′-labeled tRNA-C and 3′-labeled tRNA-CC were alkylated by ENU in the presence or absence of the *Sulfolobus shibatae* CCA-adding enzyme. Following mild alkaline cleavage, tRNA phosphates in close contact with the enzyme were visualized as protected positions by denaturing PAGE. In the interference experiments,

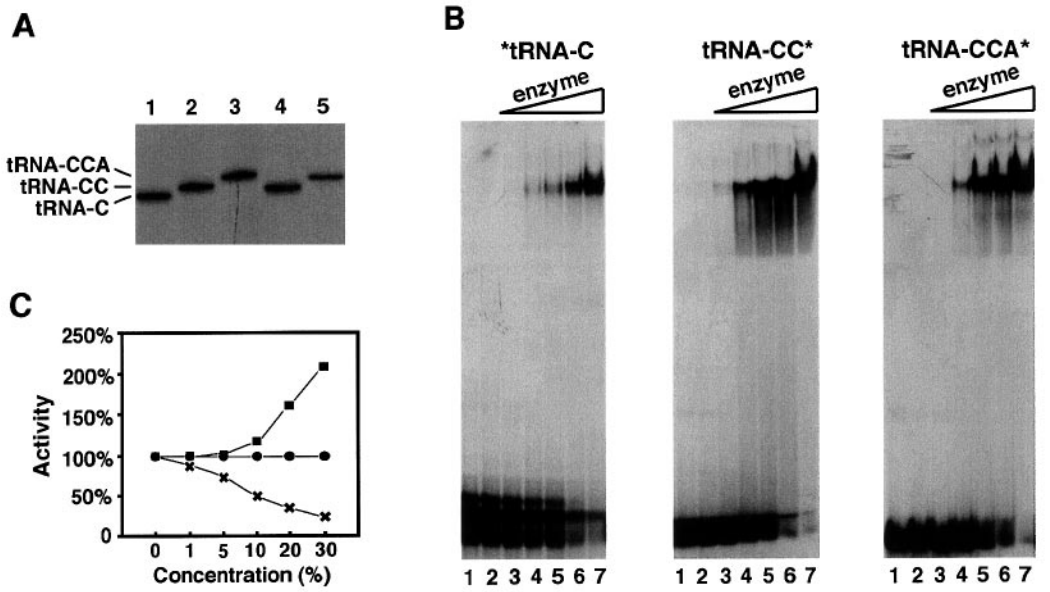

Fig. 1. tRNA substrates and ENU modification conditions. (**A**) 5′-Labeled tRNA-C (lane 1) was converted to tRNA-CC (lane 2) and tRNA-CCA (lane 3) by incubation with the *S.shibatae* CCA-adding enzyme in the presence of CTP alone (lane 2), or CTP and ATP (lane 3). Unlabeled tRNA-C was converted to 3′-labeled tRNA-CC (lane 4) and tRNA-CCA (lane 5) by incubation with the *S.shibatae* enzyme in the presence of [α-^{32}P]CTP (lane 4), or cold CTP and [α-^{32}P]ATP (lane 5). (**B**) Gel mobility shift assays of stable complexes between the *S.shibatae* enzyme and three different tRNA substrates, 5′-labeled *tRNA-C, 3′-labeled tRNA-CC* and 3′-labeled tRNA-CCA*. Binding reactions contained 0 (lane 2), 100 (lane 3), 200 (lane 4), 300 (lane 5), 400 (lane 6) or 500 ng (lane 7) of *S.shibatae* CCA-adding enzyme. No complex formed when the reaction was treated with proteinase K (lane 1). (**C**) Effect of ethanol and ENU-saturated ethanol on CCA addition by the *E.coli* and *S.shibatae* CCA-adding enzymes. Activities were normalized to a control reaction without ethanol and plotted as a function of ethanol or ENU-saturated ethanol concentration: *E.coli* enzyme, ethanol (●); *S.shibatae* enzyme, ethanol (■); *S.shibatae* enzyme, ENU-saturated ethanol (×).

tRNA-C and tRNA-CC were first alkylated by ENU and then incubated with the *S.shibatae* or *E.coli* enzyme in the presence of [α-^{32}P]CTP or [α-^{32}P]ATP. The enzyme could not add to tRNA molecules that were alkylated on phosphates which are critical for activity. Following mild alkaline cleavage and denaturing PAGE, positions at which phosphate alkylation prevented or severely reduced CCA addition were revealed as missing ladder bands.

tRNA consists of two coaxially stacked helices: the 'top half' (also referred to as a 'minihelix') containing the acceptor stem and the TΨC stem–loop; and the 'bottom half' containing the D stem–loop and the anticodon stem–loop. Our data show that both the archaeal and eubacterial CCA-adding enzymes recognize the top half of tRNA, consistent with previous results on CCA addition to minihelix substrates (Li *et al.*, 1997; Shi *et al.*, 1998). More importantly, our data demonstrate that the top half of tRNA remains fixed on the surface of the CCA-adding enzyme during addition of the penultimate C and the 3′-terminal A. Thus, as terminal nucleotide addition proceeds, either the nucleotidyltransferase active site and/or the extreme 3′ end of the top half of tRNA must move. The CCA-adding enzyme contains a single active site for CTP and ATP addition (D.Yue, N.Maizels and A.M. Weiner, in preparation). As such extensive movement of the single active site seems unlikely, we propose that the template for CCA addition is a dynamic RNP structure in which progressive refolding of the growing CCA terminus allows re-use of a single nucleotide-binding pocket.

Results

Preparation of homogeneous end-labeled tRNAs

CCA-adding enzymes can use three different substrates: tRNAs lacking one (tRNA-CC), two (tRNA-C) or all three 3′-terminal nucleotides (tRNA). We used two of these substrates (tRNA-C and tRNA-CC) and the mature product (tRNA-CCA) in our experiments. As it was critical to generate homogeneous, end-labeled tRNA for analysis, we synthesized *B.subtilis* tRNA^{Asp} tRNA-C by *in vitro* transcription, using the specially engineered pmBsDCCA plasmid as template (Oh and Pace, 1994). tRNA-C was synthesized and 5′ end-labeled as described in Materials and methods, and shown to be homogeneous by denaturing PAGE (Figure 1A, lane 1). The 5′ end-labeled tRNA-C was extended by the purified *S.shibatae* enzyme, in the presence of CTP alone or both CTP and ATP, to generate 5′ end-labeled tRNA-CC and tRNA-CCA (Figure 1A, compare lanes 1, 2 and 3). To generate 3′ end-labeled tRNA-CC and tRNA-CCA, unlabeled tRNA-C was incubated with the *S.shibatae* enzyme in the presence of [α-^{32}P]CTP alone, or cold CTP and [α-^{32}P]ATP (Figure 1C, lanes 4 and 5). Only a single band of the expected mobility was observed for each reaction, and the 5′- and 3′-labeled tRNA-CC and tRNA-CCA displayed identical mobilities (Figure 1A, compare lanes 2 and 4 with lanes 3 and 5). Similarly pure substrates could be obtained with the *E.coli* enzyme (data not shown).

The S.shibatae CCA-adding enzyme forms a stable complex with tRNA

Identification of essential tRNA phosphates by footprinting requires formation of a stable complex between the CCA-adding enzyme and the tRNA substrate. To assay for stable complex formation, we performed gel mobility shift assays with the purified *S.shibatae* and *E.coli* CCA-adding enzymes, using labeled tRNAs with different 3′-terminal sequences. The enzymes were incubated with labeled tRNA for 10 min at 25°C in reaction buffer lacking CTP

or ATP, and complexes analyzed by gel mobility shift. Stable complexes were observed with the *S.shibatae* enzyme not only with the true substrates, 5′-labeled tRNA-C and 3′-labeled tRNA-CC, but also with the mature product, 3′-labeled tRNA-CCA (Figure 1B). From the binding data shown, we estimated the K_ds for tRNA-C, tRNA-CC and tRNA-CCA to be 0.71, 0.59 and 0.54 μM, respectively. No complexes were detected with the *E.coli* enzyme (data not shown).

The K_ds for binding of the *S.shibatae* CCA-adding enzyme to tRNA-C and tRNA-CC are similar to those reported for the rabbit liver enzyme; however, the rabbit enzyme binds mature tRNA-CCA ~10-fold less tightly than tRNA-C and tRNA-CC (Deutscher, 1982). Our binding assays (Figure 1B) were carried out at 25°C which is 45°C below the activity optimum for this enzyme (Yue *et al.*, 1996). The observed tight binding of mature tRNA-CCA to the *S.shibatae* enzyme at 25°C might block product release, thus accounting at least in part for the dramatically reduced activity of the enzyme at suboptimal temperatures (Yue *et al.*, 1996). Low temperature also affects catalytic steps other than product release, including both CTP and ATP addition (P.-Y.Shi, N.Maizels and A.M.Weiner, unpublished data).

Intriguingly, although each tRNA species migrated as a single band on denaturing PAGE (Figure 1A), several species were evident on native PAGE (Figure 1B). Here, free tRNA-C migrated as three distinct bands, free tRNA-CC as two bands, and free tRNA-CCA primarily as a single band. Since the three tRNA substrates differed solely by addition of nominally unpaired 3′-terminal nucleotides, these data suggest that addition of each nucleotide progressively stabilizes the conformation of the CCA terminus. This would be consistent with evidence that the discriminator base and the CCA sequence together determine the stability and precise structure of the 3′ end of tRNA (Limmer *et al.*, 1993; Puglisi *et al.*, 1994). Interestingly, only one major tRNA–enzyme complex was observed with each of the three tRNA substrates, and all three conformers of tRNA-C formed the same complex with almost equal efficiency (Figure 1B).

Effect of ethanol and ethylnitrosourea on CCA-adding enzyme activity

To establish that footprinting analysis could produce an accurate picture of interaction between the CCA-adding enzyme and tRNA substrates, it was important to show first that the footprinting reagents did not severely inhibit the activity of the enzyme. ENU will alkylate exposed tRNA phosphates, making them susceptible to cleavage in mild alkali. Like other *N*-nitroso compounds, ENU reacts not only with the phosphodiester backbone of nucleic acids but also with protein thiol and amino groups (Margison and O'Connor, 1978). Moreover, ENU itself must be introduced into the binding reaction as a saturated solution in ethanol, and some enzymes are inhibited by ethanol. To control for these effects, we assayed CCA addition by the *E.coli* and *S.shibatae* enzymes in the presence of ethanol, or ENU-saturated ethanol, and analyzed the products by denaturing PAGE (Figure 1C). CCA addition by the *E.coli* enzyme was unaffected by ethanol concentrations as high as 30%, and added ethanol stimulated the *S.shibatae* enzyme as much as 2-fold. Under all

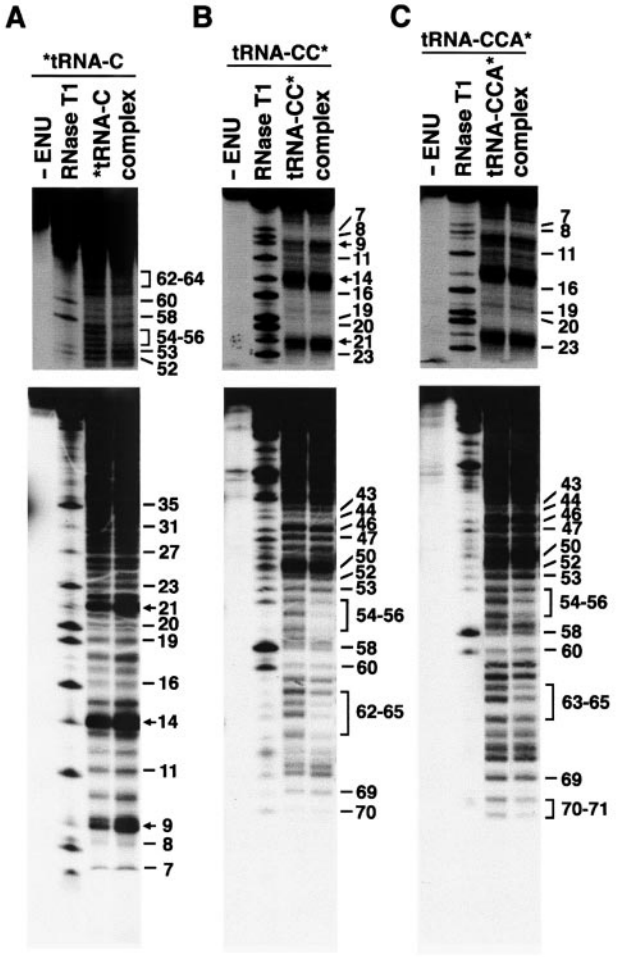

Fig. 2. Alkylation protection footprints of the *S.shibatae* CCA-adding enzyme bound to three different tRNA substrates. (**A**) 5′-Labeled *tRNA-C, (**B**) 3′-labeled tRNA-CC*, (**C**) 3′-labeled tRNA-CCA*. Assays were performed at 64 μg/ml enzyme in order to shift all labeled tRNA into complex, as determined by the gel mobility shift assay (Figure 1B). To resolve both large and small fragments clearly, each sample was subjected to electrophoresis for longer (above) and shorter (below) times. Lanes at the left of each panel contained enzyme and substrate, but were treated with ethanol lacking ENU (–ENU). Fragment sizes were assigned with reference to the RNase T1 ladder. The α-phosphates in tRNA are numbered according to convention (Figure 5). Clusters of protected phosphates are bracketed. Arrows designate phosphates that are more accessible to modification in the presence of enzyme. Asterisks indicate 5′ or 3′ sites of end-labeling.

conditions tested, CCA addition generated a single major labeled tRNA band (data not shown). While addition of ENU-saturated ethanol inhibited the *S.shibatae* enzyme somewhat, the enzyme retained ~30% activity during 15 min incubation in 20% ENU-saturated ethanol. The *S.shibatae* enzyme therefore substantially retains both activity and specificity under ENU-footprinting conditions.

Footprinting of exposed phosphates in the S.shibatae tRNA–enzyme complex

tRNA phosphates that are essential for binding to the *S.shibatae* CCA-adding enzyme were identified by comparing alkylation of tRNAs in the presence or absence of enzyme (Figure 2). Three different tRNA substrates were analyzed: 5′-labeled tRNA-C, 3′-labeled tRNA-CC and 3′-labeled tRNA-CCA. Alkaline cleavage of alkylated

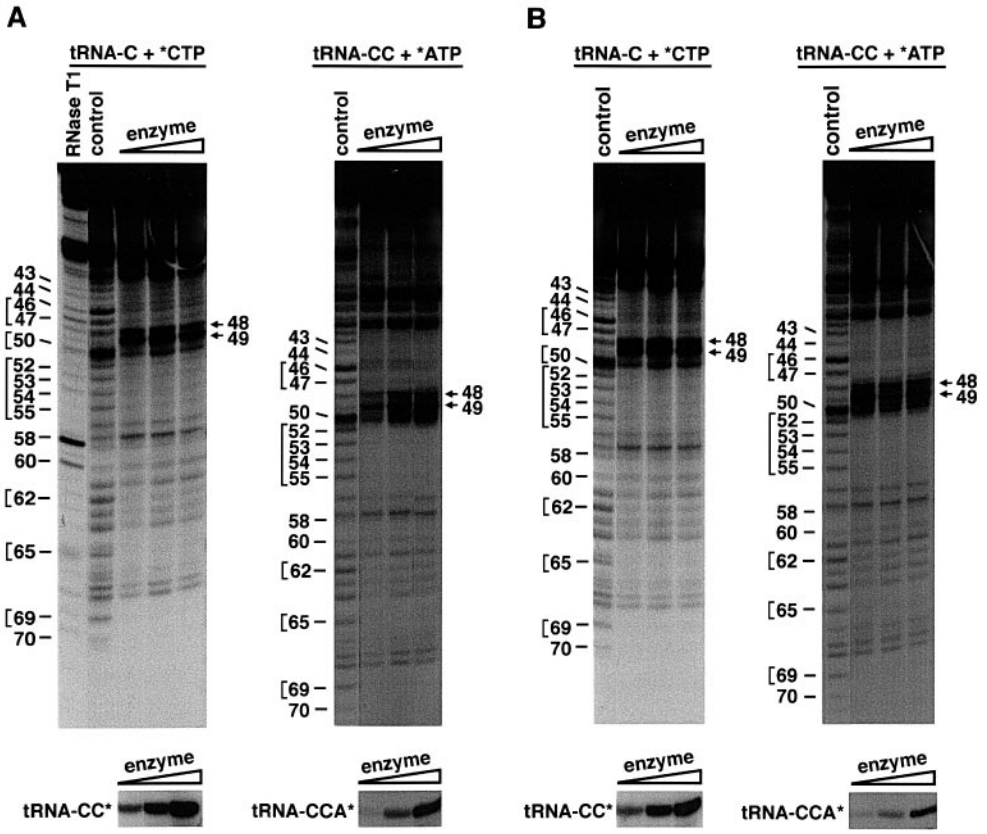

Fig. 3. Identification by alkylation interference of tRNA phosphates essential for addition of the terminal CA. Alkylation interference using the (**A**) *S.shibatae* or (**B**) *E.coli* enzyme for CMP addition to tRNA-C (left panels) and AMP addition to tRNA-CC (right panels). Reactions with the tRNA-C substrate contained [α-^{32}P]CTP, and reactions with the tRNA-CC substrate contained [α-^{32}P]ATP (label indicated by an asterisk). The top part of each panel shows interference data. The bottom part of each panel shows that addition to the tRNA substrate is proportional to added enzyme (5, 15 and 30 ng). Markers were generated by partial RNase T1 digestion. The lanes labeled 'control' contain tRNAs which had been 3′ labeled before alkylation with ENU. Other symbols are as in Figure 2.

phosphoester bonds generated fragments which were resolved by denaturing PAGE. The intensities of the bands visualized in the autoradiogram presumably reflect the accessibility of the corresponding phosphates in tRNA to ENU in the presence or absence of enzyme. Sizes of bands were assigned by reference to an RNase T1 ladder specific for cleavage after G residues. Note that bands derived from alkylated 5′-labeled tRNA migrate more slowly than the corresponding RNase T1 bands due to the presence of a 3′-terminal ethyl group, while bands derived from alkylated 3′-labeled tRNA co-migrate with the corresponding RNase T1 bands.

Binding to the CCA-adding enzyme protected 6–8 phosphates in each substrate from alkylation. In tRNA-C, phosphates 55, 56, 63 and 64 were strongly protected by enzyme, and phosphates 54 and 62 weakly protected (Figure 2A). In tRNA-CC, phosphates 54–56 and 63–65 were strongly protected, and phosphate 62 weakly protected (Figure 2B). In tRNA-CCA, phosphates 55, 56, 63, 64 and 71 were strongly protected, and phosphates 54, 65 and 70 weakly protected (Figure 2C). Interestingly, modification of phosphates 9, 14 and 21 was stronger in the tRNA–enzyme complexes with tRNA-C and tRNA-CC than in the complex with the mature product, tRNA-CCA. This suggests that genuine tRNA substrates may undergo a local conformational change upon binding of the enzyme, rendering certain phosphates more accessible to ENU modification. As anticipated from the absence of a detectable complex in the gel mobility shift assay, no footprint was observed with the *E.coli* enzyme (data not shown). Overall, the *S.shibatae* enzyme protected very similar phosphates in tRNA-C, tRNA-CC and tRNA-CCA. These results are summarized in Figure 5.

Identification of phosphates essential for CA addition by the E.coli and S.shibatae enzymes

Interference experiments were carried out to identify tRNA phosphates that are critical for addition of the terminal CA by the CCA-adding enzyme. The tRNA-C and tRNA-CC substrates were alkylated with ENU, then incubated with either the *E.coli* or *S.shibatae* enzyme in an addition reaction containing either 1 μM [α-^{32}P]CTP alone, for tRNA-C, or both 1 μM [α-^{32}P]ATP and 50 μM CTP, for tRNA-CC. The alkylation patterns of free tRNA-CC and tRNA-CCA were determined by treatment with ENU after addition of the 3′-labeled nucleotide. Essential phosphates were identified as bands which were absent or dramatically reduced when ENU treatment was carried out in the presence of the enzyme. As high levels of enzyme might obscure some effects of alkylation, ENU treatment was carried out at several enzyme concentrations, and products of the addition reactions were analyzed to show that enzyme was not saturating (Figure 3, bottom panels).

Interference analysis identified 10 phosphates in each

substrate which inhibited activity of the *S.shibatae* enzyme. Phosphates at positions 46, 47, 50, 52–55, 62, 65 and 69 interfered with addition to tRNA-C, even at the highest levels (30 ng) of enzyme (Figure 3A, left). A very similar phosphate interference pattern, except for position 50, was observed for addition to tRNA-CC (Figure 3A, right). Intriguingly, alkylation of phosphates 48 and 49 was enhanced dramatically in the interference reactions for both CTP and ATP addition, implying that substrates alkylated at these phosphates reacted preferentially with the enzyme.

Although the *E.coli* enzyme did not form a sufficiently stable tRNA–enzyme complex for footprinting, phosphates essential for addition to both tRNA-C and tRNA-CC were identified readily by interference experiments (Figure 3B). Remarkably, the *E.coli* and *S.shibatae* enzymes required the very same phosphates for C and A addition (Figure 3, compare A and B; results summarized in Figure 5). Thus, although the two enzymes represent different subfamilies of the nucleotidyltransferase superfamily and do not share obvious homology outside the active site signature sequence (Yue *et al.*, 1996), they interact with tRNA in a similar fashion during catalysis.

CCA addition by cross-linked tRNA–enzyme complexes

The footprinting and interference data demonstrated that the enzyme contacts similar regions of the tRNA substrate during addition of CTP to tRNA-C and ATP to tRNA-CC. This implies that the tRNA substrate remains fixed on the surface of the enzyme, at least during the last two steps of CCA addition. If indeed the enzyme does not move along the tRNA substrate, then a covalently cross-linked tRNA–enzyme complex might be expected to remain active in CCA addition.

To test this prediction, we performed UV cross-linking with enzyme and tRNA-C under two sets of conditions, and analyzed the products by denaturing SDS–PAGE (Figure 4). The reactions shown in Figure 4A identify two complexes (complex I and II) formed when 3′ end-labeled tRNA-CC was cross-linked to the *S.shibatae* CCA-adding enzyme by direct 254 nm UV irradiation in the absence of CTP or ATP. No complexes were formed in the absence of enzyme (Figure 4A, lane 2) or when the reaction was not subjected to UV irradiation (Figure 4A, lane 3). The observation that the two complexes have different mobilities upon SDS–PAGE suggests that they are cross-linked at different sites on the enzyme and/or the tRNA substrate.

The same two complexes became labeled (compare Figure 4B, lanes 1 and 2 with 4A, lane 1) when unlabeled tRNA-C was cross-linked to the *S.shibatae* enzyme by direct 254 nm UV irradiation, and then incubated under standard conditions with either [α-^{32}P]CTP and cold ATP, or with [α-^{32}P]ATP and cold CTP. Since both complexes were labeled under both reaction conditions, each labeled complex must contain full-length tRNA-CCA; thus tRNA-C was converted to 3′-labeled tRNA-CC*A and tRNA-CCA*, respectively, under these conditions (an asterisk indicates a labeled nucleotide; Figure 4B, lanes 1 and 2). As expected, no labeled complexes were detected without prior UV irradiation (Figure 4B, lanes 3 and 4). Similar results were obtained with *E.coli* CCA-adding

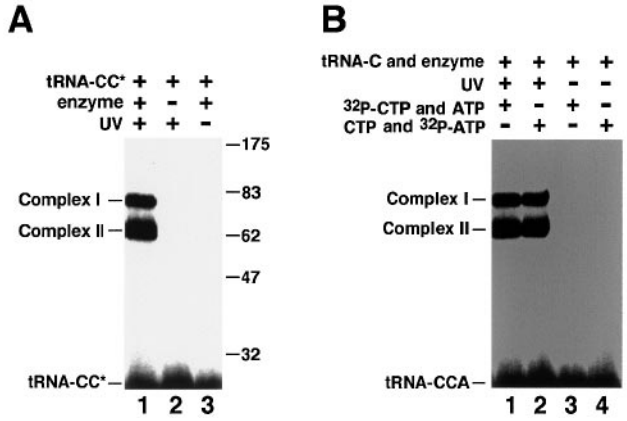

Fig. 4. UV cross-linking of the tRNA–enzyme complex does not inhibit CA addition. (**A**) SDS–PAGE of 3′-labeled tRNA-CC* cross-linked by UV irradiation to the *S.shibatae* enzyme (lane 1); UV cross-linked without enzyme (lane 2); or incubated with enzyme but not UV cross-linked (lane 3). The mobilities of protein molecular weight markers (kDa) are shown on the right. (**B**) SDS–PAGE of unlabeled tRNA-C, cross-linked to the *S.shibatae* CCA-adding enzyme before addition of [α-^{32}P]CTP in the presence of cold ATP (lane 1), or [α-^{32}P]ATP in the presence of cold CTP (lane 2). Identical reactions were performed without UV cross-linking (lanes 3 and 4).

enzyme; in all cases, complexes contained both tRNA and protein as judged by sensitivity to proteinase K and RNase T1 digeston (data not shown).

Criticial phosphates for CCA-adding enzyme binding and activity are located in the top half tRNA minihelix

Figure 5 summarizes the protection and interference data for each of the tRNA molecules analyzed: tRNA-C, tRNA-CC and tRNA-CCA. Remarkably, the same phosphates that interfered with addition (Figure 3) were protected in the enzyme–substrate complex (Figure 2). Nearly all of these phosphates lie within the top half of tRNA, as can be seen in a three-dimensional view (Figure 5B), and many but not all are concentrated in the T-stem–loop. We recently have shown that tDNA top half minihelices are efficient substrates for CCA addition (Shi *et al.*, 1998), and the footprinting and interference results now identify the specific phosphates that are essential to the enzyme. Alkylation of phosphates 48 and 49 in the variable loop dramatically enhanced both C and A addition by the *S.shibatae* (Figure 3A) and *E.coli* enzymes (Figure 3B), but neither phosphate was protected from alkylation by the *S.shibatae* enzyme (Figure 2). Alkylation could enhance activity by removing negative charge which interferes with tRNA binding to the enzyme, or by causing a tRNA conformational change which strengthens binding to the enzyme.

Discussion

We used ENU, a reagent that preferentially alkylates backbone phosphates, to examine the structural features of tRNA that are recognized by the *E.coli* and *S.shibatae* CCA-adding enzymes. We chose these divergent enzymes to ensure the generality of our results. Although both enzymes belong to the nucleotidyltransferase superfamily (Yue *et al.*, 1996), the eubacterial and archaeal CCA-

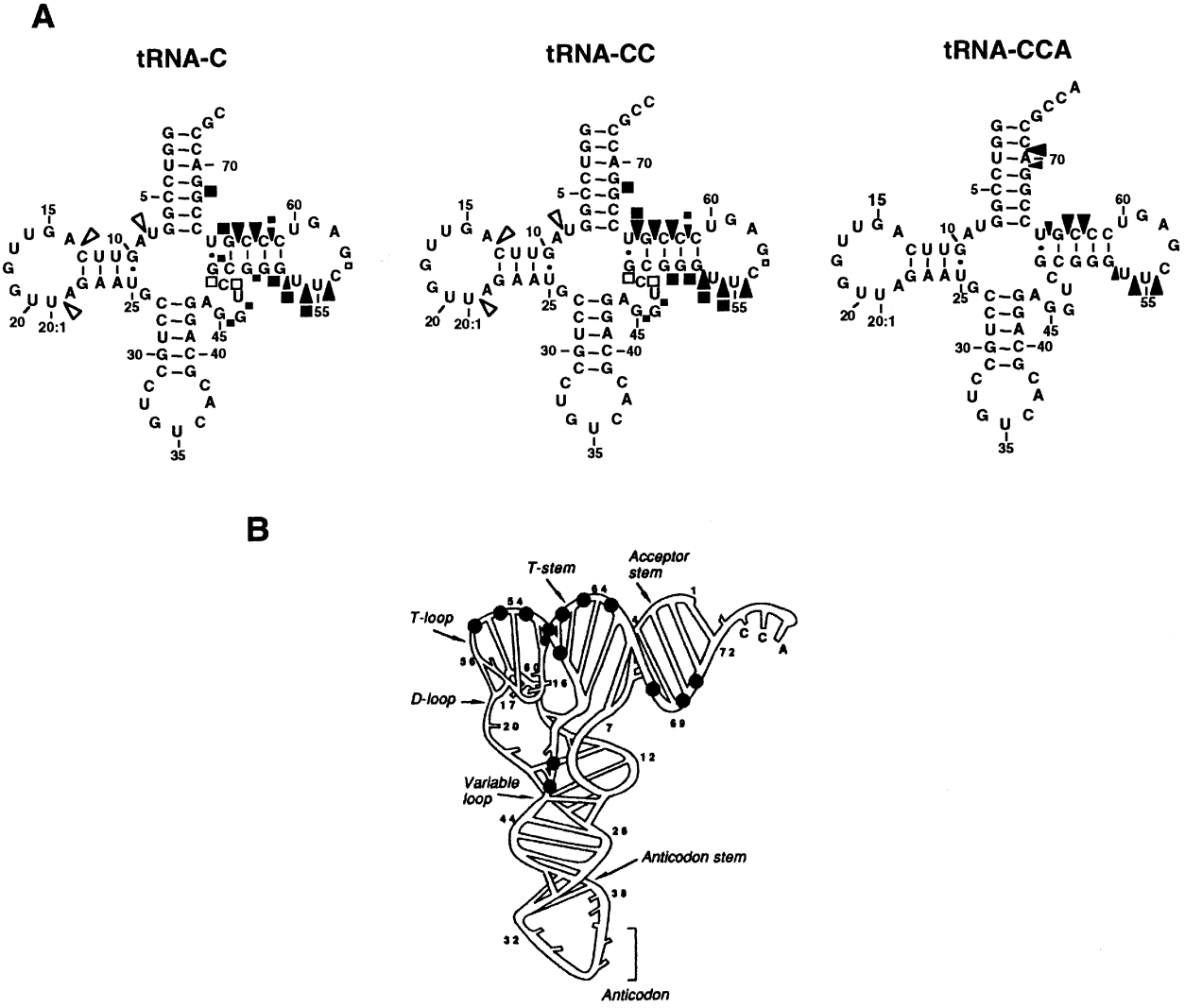

Fig. 5. Summary of alkylation protection and interference analysis. (**A**) Cloverleaf representation of tRNAs, showing phosphates that are protected by enzyme binding or that interfere with nucleotide addition to tRNA-C (left), tRNA-CC (middle) or tRNA-CCA (right). Solid arrowheads indicate phosphates that are protected from ENU alkylation by the *S.shibatae* enzyme. Squares indicate phosphates that interfere with the activity of the *E.coli* and *S.shibatae* enzymes when alkylated. Larger symbols reflect stronger effects, smaller symbols weaker effects. Unfilled arrowheads indicate phosphates at which alkylation is enhanced by enzyme binding. Unfilled squares indicate phosphates at which alkylation dramatically enhances the activity of both the *E.coli* and *S.shibatae* enzymes. (**B**) The tRNA tertiary structure, showing critical phosphates identified by protection and interference experiments.

adding enzymes belong to different subfamilies which apparently share little or no sequence homology outside the active site signature sequence (Martin and Keller, 1996; Yue *et al.*, 1996). ENU has been used previously to examine tRNA tertiary structure (Vlassov *et al.*, 1981), as well as the interaction of tRNAs with cognate aminoacyl-tRNA synthetases (Garret *et al.*, 1983; Vlassov *et al.*, 1983; Romby *et al.*, 1985), with elongation factor Tu (Riehl *et al.*, 1983) and with retroviral reverse transcriptase (Garret *et al.*, 1984). Although data obtained by ENU alkylation of phosphates could, in principle, be compromised by concurrent modification of protein amino or thiol groups (Margison and O'Connor, 1978), this has not proved to be a problem. For example, the ENU footprint of yeast $tRNA^{Asp}$ synthetase correlated well with the co-crystal structure (Romby *et al.*, 1985; Cavarelli *et al.*, 1993). The main limitation of ENU RNA protection and interference experiments is that short cleavage fragments are recovered inefficiently, and thus the first few phosphates nearest the labeled end are difficult to observe (Vlassov *et al.*, 1981; Romby *et al.*, 1985). This prevented us from examining phosphates located near the 5′ and 3′ ends of the tRNA, which are closest to the active site.

The CCA-adding enzyme recognizes primarily the top half tRNA minihelix

Figure 5 summarizes the data from both protection and interference footprinting analysis. The protected phosphates in the tRNA-C and tRNA-CC substrates all fall within the T-stem–loop. No protection was observed in either the D-stem–loop or the anticodon stem–loop, or within the acceptor stem. The pattern was slightly different for the product of addition, tRNA-CCA, where two acceptor stem phosphates were protected, at positions 70 and 71. Positions at which alkylation interfered with addition were restricted to the T-stem–loop and the

acceptor stem. The interference pattern was identical for the *E.coli* and *S.shibatae* enzymes.

All phosphates which are protected by the enzyme or interfere with enzyme activity are located within the top half of tRNA. These data argue that significant interactions between the enzyme and tRNA substrates are restricted to the top half of tRNA. This domain can be thought of as a minihelix consisting of the T-stem–loop coaxially stacked on the acceptor stem (Figure 5B). The importance of contacts in this region is consistent with the ability of the enzyme to add efficiently and specifically to synthetic substrates comprised of only a top half minihelix (Li *et al.*, 1997; Shi *et al.*, 1998). In this respect, the CCA-adding enzymes resemble two other enzymes, elongation factor Tu (Rudinger *et al.*, 1994) and RNase P (McClain *et al.*, 1987), which must also recognize all species of tRNA, and do so by interacting primarily with top half determinants. In contrast, aminoacyl-tRNA synthetases need only interact with a small family of tRNA isoacceptors and may, as a consequence, be free to recognize the anticodon stem–loop (bottom half) of tRNA, albeit through a separate, less conserved protein domain (Rould *et al.*, 1989, 1991; Shepard *et al.*, 1992; Cavarelli *et al.*, 1993).

We were surprised that alkylation of phosphates located on nearly opposite faces of the top half of tRNA (phosphates 54 and 55, and phosphates 69 and 70) would interfere with CCA-adding activity. Co-crystal structures of tRNAs with cognate synthetases may provide precedents for understanding these interference data. One possibility is that the CCA-adding enzyme wraps around the top half of the tRNA substrate; $tRNA^{Ser}$ is known to interact with the cognate synthetase mainly through its 3′ side of the acceptor stem, but also through the 5′ side of the T-loop and both sides of the variable arm stem (Biou *et al.*, 1993). Alternatively, the structure of the tRNA acceptor stem may be altered upon binding to the enzyme, especially near the 3′ end of tRNA where catalysis occurs. Indeed, the 3′-terminal base pair of the $tRNA^{Gln}$ acceptor stem melts upon binding to the cognate synthetase (Rould *et al.*, 1989).

In each of the three kingdoms, a single CCA-adding enzyme appears to be responsible for CCA addition to all tRNAs (Zhu and Deutscher, 1987; Aebi *et al*., 1990; Yue *et al*., 1996). The enzyme must therefore recognize structural elements common to all tRNAs. Alkylation of several phosphates in the T-loop interferes with CCA-adding activity (data summarized in Figure 5). Modification of nucleotides responsible for tertiary interactions between the D- and T-loops (Spacciapoli *et al.*, 1989) and cytidine substitutions at nucleotides 57 or 58 of the TΨCG loop (Li *et al.*, 1996) have also been shown to inhibit the CCA-adding enzyme. However, it is not yet clear whether inhibition of activity resulting from alkylation of T-loop phosphates blocks interactions between the T-loop and the enzyme directly, or inhibits enzyme activity indirectly by locally distorting the RNA backbone. Nor do we understand why alkylation of phosphates 48 and 49 in the variable loop dramatically stimulates addition to both tRNA-C and tRNA-CC (Figure 3). Stimulation could be due to a favorable local distortion of the tRNA backbone, or elimination of an unfavorable negative charge by alkylation.

CCA addition occurs without translocation

All of the phosphates that our experiments have identified as critical for binding or activity fall within the top half of the tRNA substrate (Figure 5). The fact that critical positions are identical in two different substrates, tRNA-C and tRNA-CC, argues that enzyme–substrate interactions do not change dramatically during addition of the terminal CA. The top half of the tRNA substrate therefore appears to remain nearly fixed on the surface of the enzyme during addition of the terminal CA residues, and perhaps for addition of the entire CCA sequence. Consistent with this interpretation, tRNA–enzyme complexes remain competent for CA addition even after UV cross-linking (Figure 4).

A model for CCA addition without translocation

The *E.coli* and *S.shibatae* CCA-adding enzymes both belong to the nucleotidyltransferase superfamily (Martin and Keller, 1996; Yue *et al.*, 1996), albeit to different subfamilies (Yue *et al.*, 1996). Both enzymes appear to have only a single active site with a conserved signature motif, and we have confirmed that mutations in either conserved aspartate (underlined) of the *S.shibatae* active site signature motif GSFRKGTWLRQDTDVD abolish both CTP and ATP addition (D.Yue, N.Maizels and A.M. Weiner, in preparation).

If the tRNA substrate is fixed on an enzyme with a single active site, and does not translocate or rotate, how then can polymerization of CCA occur? One possibility is that the tRNA-binding domain and the active site domain translocate relative to each other as polymerization proceeds; this would almost certainly require large movements of two protein domains relative to each other, and partially independent nucleotide-binding sites for C74, C75 and A76 (Masiakowski and Deutscher, 1980a,b). Alternatively, the distance between the tRNA-binding domain and the active site domain could be fixed, and the 3′ end of the tRNA substrate could refold to accommodate each additional nucleotide. This would almost certainly require re-use of a single nucleotide-binding site, and templating of each additional nucleotide by a collaboration between the enzyme and the tRNA substrate.

Figure 6 presents an explicit model for CCA polymerization without translocation. tRNA is bound on the enzyme surface in a fixed position relative to the single active site. Progressive refolding of the 3′-terminal tRNA sequence allows re-use of a single CTP-binding site. Additional interactions with the enzyme may stabilize refolding of the 3′ terminus in subsequent steps. The ATP-binding site is created collaboratively by the refolded CC terminus together with the CTP-binding site. Nucleotide addition ceases when the binding pocket is full. The template for CCA addition is neither pure protein, nor pure RNA, but a dynamic ribonucleoprotein (RNP) structure.

This model is simple, and does not require large macromolecular movements or multiple nucleotide-binding sites in close proximity on the surface of the enzyme. The model takes advantage of the emerging realization that short RNA sequences are capable of adopting unusual, stable structures (Doudna and Cate, 1997). Indeed, there is good evidence that CCA may be highly structured in tRNA: the 3′-terminal NCCA sequence contributes to the stability of the acceptor stem as judged by NMR (Limmer

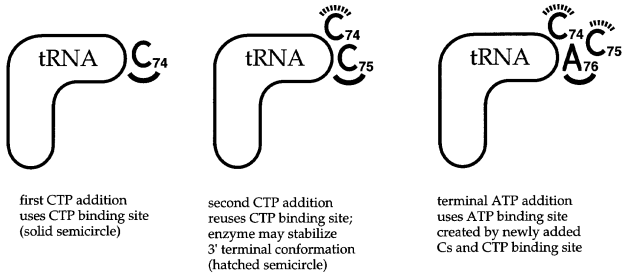

Fig. 6. A model for collaborative templating of CCA addition without translocation. tRNA (hollow boomerang) is bound by the enzyme in a fixed position relative to the single active site. As CCA is added one nucleotide at a time, the 3′-terminal tRNA sequence progressively refolds to allow re-use of a single CTP-binding site (solid semicircle). Additional interactions with the enzyme (hatched semicircles) may stabilize refolding of the 3′-terminus as polymerization proceeds. The ATP-binding site is created collaboratively by the refolded CC terminus together with the CTP-binding site. Polymerization ceases when the binding pocket is full. The template for CCA addition is neither pure protein, nor pure RNA, but a dynamic RNP structure that blurs the distinction between 'enzyme' and 'substrate'.

et al., 1993; Puglisi *et al.*, 1994), and we find that addition of C and CA to tRNA-C progressively stabilizes the conformation of the molecule during native PAGE (Figure 1).

This model also makes evolutionary sense. The *E.coli* or *S.shibatae* CCA-adding enzymes share no obvious sequence homology outside the active site signature of the nucleotidyltransferase superfamily (Martin and Keller, 1996; Yue *et al.*, 1996), yet the very same phosphates interfere with both CTP and ATP addition when alkylated (Figure 3). Such extraordinary conservation of mechanism, without comparable conservation of sequence, seems more consistent with the simple model we propose than with a complex mechanism invoking large movements of protein domains and multiple nucleotide-binding sites. Furthermore, CCA-adding enzymes and poly(A) polymerases are both members of the nucleotidyltransferase superfamily (Martin and Keller, 1996; Yue *et al.*, 1996) and appear to have intraconverted at least once in the course of evolution (Yue *et al.*, 1996). Conversion of a poly(A) polymerase into a CCA-adding enzyme would be far more difficult to envisage if large protein movements and additional nucleotide-binding sites were required. This model for CCA addition resembles that proposed for the *Euplotes* telomerase (Hammond *et al.*, 1997). As with the CCA-adding enzyme, covalent cross-linking of primer DNA to the *Euplotes* enzyme does not interfere with telomere synthesis, presumably because the internal RNA template of this RNP enzyme progressively refolds in an 'inchworm' fashion.

Collaborative templating specifies CCA addition

In conventional protein-catalyzed, template-directed RNA or DNA polymerization, selection of the incoming nucleotide represents a collaboration between protein and nucleic acid. The correct nucleotide is favored, and incorrect nucleotides discriminated against, by at least four different interactions: pairing with the template base; stacking of the newly formed base pair on the previous base pair; interactions of the incoming ribose triphosphate moiety with the enzyme; and stacking of the incoming base between the previous base pair and a critical tyrosine that forms a flat hydrophobic wall immediately ahead of the active site, as seen in *Taq* (Eom *et al*., 1996), T7 (Doublie *et al*., 1998) and *E.coli* DNA polymerase (Kiefer *et al*., 1998). Neither the protein nor the primer–template duplex possess an independent nucleotide-binding site; a collaborative effort between protein and nucleic acid rebuilds a binding site of the proper specificity before each successive nucleotide addition. Only the balance of these interactions would be changed in our model for CCA addition (Figure 6). Conventional base pairing may play no role, but unusual stacking and hydrogen-bonding interactions that can stabilize RNA turns would be exploited (for review see Doudna and Cate, 1997). The protein may interact specifically with the bases as well as with the ribose triphosphate moiety.

This model appears to be consistent with, and quite possibly may explain, much of the meticulous body of enzyme kinetics defining effective 'subsites' for binding CTP and ATP during CCA addition (Masiakowski and Deutscher, 1980a,b; Deutscher, 1982). Indeed, an enzyme-stabilized CCA structure like that shown in Figure 6 could explain why one of the CTP-binding subsites appears to overlap with or be identical to the subsite that recognizes the terminal C of tRNA-C; why the CTP- and ATP-binding sites interact with each other; why ATP stimulates CTP addition at concentrations (5 mM) greatly exceeding the K_m; why neither the CTP nor ATP subsites are absolutely specific for the respective nucleotides; and why the dinucleoside monophosphate CpC can function as an acceptor for ATP addition (Masiakowski and Deutscher, 1980a,b). It may be very difficult, and perhaps impossible, to distinguish by kinetics alone between the relative roles of protein and RNA in building each successive nucleotide-binding site of the CCA-adding enzyme. The structure of a co-crystal between tRNA and a CCA-adding enzyme would go a long way toward answering these questions.

Materials and methods

CCA addition

CCA addition reactions contained 100 mM glycine/NaOH (pH 9.0), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and the indicated amounts of enzyme and tRNA. CTP and ATP concentrations were 100 μM, except where noted. For 3′ end-labeling, 1 μM [α-^{32}P]CTP or 1 μM [α-^{32}P]ATP (both 3000 Ci/mmol, Amersham) were included in standard reactions. Reactions were incubated for 5–15 min as indicated, either at 70°C (*S.shibatae* CCA-adding enzyme) or at 37°C (*E.coli* CCA-adding enzyme). CCA-adding enzyme was assayed, and the effect of ethanol and ENU determined (Figure 1C), using 2 μg of crude wheat germ tRNA substrate (Yue *et al.*, 1996) in a standard 10 μl reaction. The reactions were analyzed by denaturing 12% PAGE. The gel was dried, autoradiographed, and the excised bands were quantitated by Cerenkov counting after 3 h rehydration.

Expression and purification of recombinant CCA-adding enzymes

Overexpression and purification of recombinant CCA-adding enzyme from *E.coli* has been described previously (Shi *et al.*, 1997). The protocol for purifying the *S.shibatae* enzyme (Yue *et al.*, 1996) was modified as follows. A single colony of *E.coli* strain BL21 freshly transformed with the expression plasmid was inoculated into LB broth containing 50 μg/ml of ampicillin and grown at 37°C to OD_{600} 0.6, at which time 0.3 mM isopropyl-β-D-galactopyranoside (IPTG) was added to induce *cca* gene expression. Cells were harvested after overnight growth, the cell pellet was resuspended in buffer I [20 mM sodium phosphate pH 7.0, 1 mM DTT, 0.5 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and the cells lysed by sonication. After a clearing spin, the crude lysate was incubated at 70°C for 10 min to denature most

E.coli proteins. The thermostable *S.shibatae* CCA-adding enzyme in the supernatant was purified on an SP Sepharose column (Pharmacia Biotech). Fractions containing CCA-adding activity were combined, dialyzed into buffer II (20 mM Tris–HCl pH 7.0, 1 mM DTT, 0.5 mM EDTA and 0.5 mM PMSF), and loaded onto a HiTrap Q column (Bio-Rad). Enzyme in the flowthrough was dialyzed into buffer I, then fractionated on a HiTrap blue column (Bio-Rad), dialyzed into buffer III (10 mM Tris–HCl pH 7.2, 1 mM DTT, 20 mM NaCl and 3 mM $MgCl_2$), concentrated using a Centricon-30 (Amicon) and stored in 50% glycerol at –20°C. Approximately 4 mg of pure enzyme was obtained from a 1 l culture.

The purified *S.shibatae* CCA-adding enzyme migrated as a single 48 kDa band upon SDS–PAGE, and the purified His_6-tagged *E.coli* enzyme as a single 56 kDa band (data not shown). Enzyme preparations were shown to be free of contaminating ribonuclease activity by assaying the integrity of 3′ end-labeled tRNA following incubation with 0.5 mg/ml enzyme for 5 h at room temperature under standard assay conditions (data not shown).

In vitro transcription and purification of tRNA substrates

The pmBsDCCA plasmid, designed to allow *in vitro* transcription of *B.subtilis* $tRNA^{Asp}$ lacking part or all of the 3′-terminal CCA sequence (Oh and Pace, 1994), was a generous gift from Dr N.R.Pace (University of California, Berkeley). pmBsDCCA digested with *Fok*I was used as template for run-off transcription of tRNA-C lacking the 3′-terminal CA. The *in vitro* transcription reaction was carried out for 3 h at 37°C in 40 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM DTT, 2 mM each NTP, 1 U/ml pyrophosphatase (New England Biolabs), 70 μg/ml T7 polymerase and 100 μg/ml plasmid template; a 100 μl transcription reaction typically yielded 30 μg of crude RNA product. tRNA-C was purified by denaturing 12% PAGE, visualized by UV shadowing, recovered by electroelution in a commerical apparatus (Schleicher and Schuell), concentrated by ethanol precipitation, and stored at –70°C. tRNA-CC lacking the 3′-terminal A was prepared by incubating 2 μg of tRNA-C with 100 ng of *S.shibatae* CCA-adding enzyme for 10 min at 70°C in a 20 μl addition reaction containing CTP but no ATP. Full-length tRNA-CCA was prepared under similar conditions in a reaction containing both CTP and ATP.

End-labeling and renaturation of tRNAs

For 5′ labeling, tRNA-C (1 μg) was dephosphorylated by treatment with calf intestinal alkaline phosphatase (Boehringer Mannheim), and labeled with [γ-^{32}P]ATP (6000 Ci/mmol, Amersham) and T4 polynucleotide kinase as described by the supplier (New England BioLabs). To prepare 3′-labeled tRNA-CC and tRNA-CCA, tRNA-C (1 μg) was incubated with either the *S.shibatae* or *E.coli* CCA-adding enzyme (50 ng) for 10 min in a 10 μl addition reaction containing either 1 μM [α-^{32}P]CTP and 5 μM CTP, or 50 μM CTP, 1 μM [α-^{32}P]ATP (both 3000 Ci/mmol, Amersham) and 5 μM ATP. Labeled tRNAs were purified by denaturing 15% PAGE, localized by autoradiography, eluted overnight at room temperature into buffer (500 mM ammonium acetate, 1 mM EDTA, and 0.1% SDS) with gentle agitation, concentrated by ethanol precipitation and stored at –20°C. Labeled tRNAs were renatured by heating to 85°C for 3 min in 5 mM HEPES (pH 7.9), brought to 50 mM NaCl and 10 mM $MgCl_2$, and slowly cooled to room temperature.

Gel mobility shift assay

CCA-adding enzyme (100–500 ng) was incubated with ~25 ng of tRNA (5×10^4 c.p.m.) in 10 μl of CCA addition buffer lacking CTP or ATP at 25°C for 15 min. The reactions were analyzed by electrophoresis on a non-denaturing 6% polyacrylamide gel (29:1 acrylamide:bisacrylamide, 0.4 mm thick) run at 220 V in 1× TBE at room temperature. The bands were quantitated by Cerenkov counting as decribed above.

Footprinting of tRNA–enzyme complexes

Alkylation of tRNA with ENU was performed as described (Romby *et al.*, 1985). Briefly, 10^5 c.p.m. of renatured, end-labeled tRNA (~50 ng) was pre-incubated with 1.6 μg of enzyme in 20 μl of 300 mM sodium cacodylate (pH 8.0), 2 mM EDTA, 20 mM $MgCl_2$, 50 mM NaCl at 37°C for 10 min; 5 μl of a saturated solution of ENU (Sigma) in 100% ethanol was then added, and incubation continued for 3 h at room temperature. The amount of enzyme was sufficient to complex all tRNA as judged by the gel mobility shift assay (Figure 1B). Reactions were terminated by addition of 5 μg of carrier tRNA and 100 μl of 300 mM sodium acetate (pH 6.0). After three consecutive ethanol precipitations to remove all traces of ENU, alkylated tRNAs were taken up in 10 μl of 100 mM Tris–HCl (pH 9.0), phosphotriester bonds cleaved by incubation at 50°C for 5 min, and cleavage products recovered by ethanol precipitation at –80°C for 1 h to minimize loss of small fragments. Ethanol precipitation served to adjust the pH and volume of the sample, and also reduced background in the lower region of the gel. Fragments were resuspended in loading dye containing 10 M urea and resolved by denaturing 15 or 20% PAGE. The specificity of alkylation and integrity of tRNA substrates were verified in control reactions which contained enzyme and substrate, but were treated with ethanol lacking ENU. ENU-generated bands were assigned by reference to a rapid RNA sequencing ladder produced by partial digestion with RNase T1, which cleaves 3′ of G residues (Knapp, 1989). The effect of enzyme binding was determined by comparing reactions in which alkylation had been carried out in the presence or absence of the CCA-adding enzyme.

Ethylnitrosourea interference experiments

To identify phosphates that are essential for CTP addition to tRNA-C, 3 μg of unlabeled tRNA-C was alkylated with ENU, and residual ENU removed by ethanol precipitation as described above. Aliquots of the alkylated tRNA-C (1 μg) were incubated with *S.shibatae* or *E.coli* CCA-adding enzyme (5, 15 or 30 ng) in 15 μl addition reactions containing 1 μM [α-^{32}P]CTP. After 5 min incubation, reactions were stopped by addition of 10 μl of loading buffer (deionized formamide containing 0.1% w/v xylene cyanol and bromphenol blue). The 3′ end-labeled tRNA-CC was purified by denaturing 15% PAGE, eluted and cleaved at sites of alkylation as described above. To map the alkylation sites on free tRNA-CC, the tRNA-C substrate was first 3′ end-labeled with [α-^{32}P]CTP, then treated with ENU, cleaved in alkali and resolved by denaturing PAGE. The same protocol was used to identify phosphates that are essential for ATP addition to tRNA-CC, except that unlabeled tRNA-CC was alkylated and selectively 3′ end-labeled with [α-^{32}P]ATP by the *E.coli* or *S.shibatae* CCA-adding enzyme prior to cleavage. Alkylation sites on free tRNA-CCA were mapped by 3′ end-labeling the tRNA-CC substrate with [α-^{32}P]ATP before ENU treatment, cleavage and PAGE.

CCA addition to UV cross-linked tRNA–enzyme complexes

For UV cross-linking, 500 ng of unlabeled tRNA-C or 25 ng of 3′ end-labeled tRNA-CC (5×10^4 c.p.m.) was incubated with 0.5 μg of *S.shibatae* CCA-adding enzyme for 5 min at room temperature in a 10 μl addition reaction with no CTP or ATP. Following 254 nm irradiation under a Stratalinker (Stratagene) for 10 min on ice, 0.5 μM [α-^{32}P]CTP and 50 μM ATP, or [α-^{32}P]ATP and 50 μM CTP was added, and incubation was continued for 10 min at 70°C. Samples were analyzed by 10% SDS–PAGE.

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References

Aebi,M., Kirchner,G., Chen,J.Y., Vijayraghavan,U., Jacobson,A., Martin,N.C. and Abelson,J. (1990) Isolation of a temperature-sensitive mutant with an altered tRNA nucleotidyltransferase and cloning of the gene encoding tRNA nucleotidyltransferase in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **265**, 16216–16220.

Biou,V., Yaremchuk,A., Tukalo,M. and Cusack,S. (1994) The 2.9 Å crystal structure of *T.thermophilus* seryl-tRNA synthetase complexed with tRNA(Ser). *Science*, **263**, 1404–1410.

Cavarelli,J., Rees,B., Ruff,M., Thierry,J.-C. and Moras,D. (1993) Yeast aspartyl-tRNA synthetase: a structural view of the aminoacylation reaction. *Nature*, **362**, 181–184.

Deutscher,M.P. (1982) tRNA nucleotidyltransferase. *The Enzymes*, **15**, 183–215.

Doudna,J.A. and Cate,J.H. (1997) RNA structure: crystal clear? *Curr. Opin. Struct. Biol.*, **7**, 310–316.

Doublié,S., Tabor,S., Long,A. M., Richardson,C. C. and Ellenberger,T. (1998) Crystal structure of bacteriophage T7 DNA polymerase complexed to a primer–template, a nucleoside triphosphate, and its processivity factor thioredoxin. *Nature*, **391**, 251–258.

Eom,S.H., Wang,J. and Steitz,T.A. (1996) Structure of *Taq* polymerase with DNA at the polymerase active site. *Nature*, **382**, 278–281.

Garret,M., Labouesse,B., Litvak,S., Romby,P., Ebel,J.P. and Giegé,R. (1983) Tertiary structure of animal $tRNA^{Trp}$ in solution and interaction of $tRNA^{Trp}$ with tryptophanyl-tRNA synthetase. *Eur. J. Biochem.*, **138**, 67–75.

Garret,M., Romby,P., Giegé,R. and Litvak,S. (1984) Interactions between avian myeloblastosis reverse transcriptase and $tRNA^{Trp}$. Mapping of complexed tRNA with chemicals and nucleases. *Nucleic Acids Res.*, **12**, 2259–2271.

Giegé,R. (1996) Interplay of tRNA-like structures from plant viral RNAs with partners of the translation and replication machineries. *Proc. Natl Acad. Sci. USA*, **93**, 12078–12081.

Hammond,P.W., Lively,T.N. and Cech,T.R. (1997) The anchor site of telomerase from *Euplotes aediculatus* revealed by photo-crosslinking to single- and double-stranded DNA primers. *Mol. Cell. Biol.*, **17**, 296–308.

Hegg,L.A. and Thurlow,D.L. (1990) Cytidines in tRNAs that are required intact by ATP/CTP:tRNA nucleotidyltransferases from *Escherichia coli* and *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **18**, 5975–5979.

Holm,L. and Sander,C. (1995) DNA polymerase β belongs to an ancient nucleotidyltransferase superfamily. *Trends Biochem. Sci.*, **20**, 345–347.

Kiefer,J.R., Mao,C., Braman,J.C. and Beese,L.S. (1998) Visualizing DNA replication in a catalytically active *Bacillus* DNA polymerase crystal. *Nature*, **391**, 304–307.

Knapp,G. (1989) Enzymatic approaches to probing of RNA secondary and tertiary structure. *Methods Enzymol.*, **180**, 192–212.

Li,Z., Gillis,K.A., Hegg,L.A., Zhang,J. and Thurlow,D.L. (1996) Effects of nucleotide substitutions within the T-loop of precursor tRNAs on interaction with ATP/CTP:tRNA nucleotidyltransferases from *Escherichia coli* and yeast. *Biochem. J.*, **314**, 49–53.

Li,Z., Sun,Y. and Thurlow,D.L. (1997) RNA minihelices as model substrates for ATP/CTP:tRNA nucleotidyltransferase. *Biochem. J.*, **327**, 847–851.

Limmer,S., Hofman,H.-P., Ott,G. and Sprinzl,M. (1993) The 3′-terminal end (NCCA) of tRNA determines the structure and stability of the aminoacyl acceptor stem. *Proc. Natl Acad. Sci. USA*, **90**, 6199–6202.

Margison,G.P. and O'Connor,P.J.O. (1978) Nucleic acid modification by *N*-nitroso compounds. In Grover,P.L. (ed.), *Chemical Carcinogens and DNA*. CRC Press Inc., Boca Raton, FL, Vol. 1, pp. 112–151.

Martin,G, and Keller,W. (1996) Mutational analysis of mammalian poly(A) polymerase identifies a region for primer binding and catalytic domain, homologous to the family X polymerases, and to other nucleotidyltransferases. *EMBO J.*, **15**, 2593–2603.

Masiakowski,P. and Deutscher,M.P. (1980a) Dissection of the active site of rabbit liver tRNA nucleotidyltransferase: specificity and properties of the tRNA and acceptor subsites determined with model acceptor substrates. *J. Biol. Chem.*, **255**, 11233–11239.

Masiakowski,P. and Deutscher,M.P. (1980b) Dissection of the active site of rabbit liver tRNA nucleotidyltransferase: specificity and properties of subsites for donor nucleoside triphosphates. *J. Biol. Chem.*, **255**, 11240–11246.

McClain,W.H., Guerrier-Takada,C. and Altman,S. (1987) Model substrates for an RNA enzyme. *Science*, **238**, 527–530.

Oh,B.-K. and Pace,N.R. (1994) Interaction of the 3′-end of tRNA with ribonuclease P RNA. *Nucleic Acids Res.*, **22**, 4087–4094.

Puglisi,E.V., Puglisi,J.D., Williamson,J.R. and RajBhandary,U.L. (1994) NMR analysis of tRNA acceptor stem microhelices: discriminator base change affects tRNA conformation at the 3′ end. *Proc. Natl Acad. Sci. USA*, **91**, 11467–11471.

Rao,A.L., Dreher,T.W., Marsh,L.E. and Hall,T.C. (1989) Telomeric function of the tRNA-like structure of brome mosaic virus RNA. *Proc. Natl Acad. Sci. USA*, **86**, 5335–5339.

Riehl,N., Giegé,R, Ebel,J.P. and Ehresmann,B. (1983) Effect of elongation factor Tu on the conformation of phenylalanyl-$tRNA^{Phe}$. *FEBS Lett.*, **154**, 42–46.

Romby,P., Moras,D., Bergdoll,M., Dumas,P., Vlassov,V.V., Westhof,E., Ebel,J.P. and Giegé,R. (1985) Yeast $tRNA^{Asp}$ tertiary structure in solution and areas of interaction of the tRNA with aspartyl-tRNA synthetase. A comparative study of the yeast phenylalanine system by phosphate alkylation experiments with ethylnitrosourea. *J. Mol. Biol.*, **184**, 455–471.

Rould,M.A., Perona,J.J., Söll,D. and Steitz,T.A. (1989) Structure of *E.coli* glutaminyl-tRNA synthetase complexed with tRNA(Gln) and ATP at 2.8 Å resolution. *Science*, **246**, 1135–1142.

Rould,M.A., Perona,J.J. and Steitz,T.A. (1991) Structural basis of anticodon loop recognition by glutaminyl-tRNA synthetase. *Nature*, **352**, 213–218.

Rudinger,J., Blechschmidt,B., Ribeiro,S. and Sprinzl,M. (1994) Minimalist aminoacylated RNAs as efficient substrates for elongation factor Tu. *Biochemistry*, **33**, 5682–5688.

Shepard,A., Shiba,K. and Schimmel,P. (1992) RNA binding determinant in some class I tRNA synthetases identified by alignment-guided mutagenesis. *Proc. Natl Acad. Sci. USA*, **89**, 9964–9968.

Shi,P.-Y., Maizels,N. and Weiner,A.M. (1997) Recovery of soluble, active recombinant protein from inclusion bodies. *BioTechniques*, **23**, 1036–1038.

Shi,P.-Y., Weiner,A.M. and Maizels,N. (1998) A top-half tDNA minihelix is a good substrate for the eubacterial CCA-adding enzyme. *RNA*, **4**, 276–284.

Spacciapoli,P., Doviken,L., Mulero, J.J. and Thurlow,D.L. (1989) Recognition of tRNA by the enzyme ATP/CTP:tRNA nucleotidyltransferase. Interference by nucleotides modified with diethyl pyrocarbonate or hydrazine. *J. Biol. Chem.*, **264**, 3799–3805.

Sprinzl,M. and Cramer,F. (1979) The -C-C-A end of tRNA and its role in protein biosynthesis. *Prog. Nucleic Acid Res. Mol. Biol.*, **22**, 1–69.

Vlassov,V.V., Giegé,R. and Ebel,J.P. (1981) Tertiary structure of tRNAs in solution monitored by phosphodiester modification with ethylnitrosourea. *Eur. J. Biochem.*, **119**, 51–59.

Vlassov,V.V., Kern,D., Romby,P., Giegé,R. and Ebel,J.P. (1983) Interaction of $tRNA^{Phe}$ and $tRNA^{Val}$ with aminoacyl-tRNA synthetases. A chemical modification study. *Eur. J. Biochem.*, **132**, 537–544.

Weiner,A.M. and Maizels,N. (1987) tRNA-like structures tag the 3′ ends of genomic RNA molecules for replication: implications for the origin of protein synthesis. *Proc. Natl Acad. Sci. USA*, **84**, 7383–7387.

Yue,D., Maizels,N. and Weiner,A.M. (1996) CCA-adding enzymes and poly(A) polymerases are all members of the same nucleotidyltransferase superfamily: characterization of the CCA-adding enzyme from the archaeal hyperthermophile *Sulfolobus shibatae*. *RNA*, **2**, 895–908.

Zhu,L. and Deutscher,M.P. (1987) tRNA nucleotidyltransferase is not essential for *Escherichia coli* viability. *EMBO J.*, **6**, 2473–2477.

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