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A top-half tDNA minihelix is a good substrate for the eubacterial CCA-adding enzyme

PEI-YONG SHI,[1] ALAN M. WEINER,[1,2] and NANCY MAIZELS[1,2]

[1] Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06520-8024, USA
[2] Department of Genetics, Yale University School of Medicine, New Haven, Connecticut 06520-8024, USA

ABSTRACT

The CCA-adding enzyme [ATP(CTP):tRNA nucleotidyltransferase] catalyzes the addition and regeneration of the 3′-terminal CCA sequence of tRNAs. We show that the CCA-adding enzyme will specifically add a CCA terminus to synthetic full-length tDNA and to DNA oligonucleotides corresponding to the "top half" of tRNA—the acceptor stem and TψC stem-loop of tRNA. CCA addition to the top half tDNA minihelices requires a 2′ as well as a 3′ OH at the 3′ terminus of the tDNA. Addition also depends on the length of the base paired stem, and is facilitated by, but is not dependent upon, the presence of a TψC loop. These results provide further evidence for independent functions of the top and bottom halves of tRNA, and support the hypothesis that these two structurally distinct and functionally independent domains evolved independently.

Keywords: nucleotidyltransferase; tRNA

INTRODUCTION

The CCA-adding enzyme [ATP(CTP):tRNA nucleotidyltransferase] catalyzes the addition and regeneration of the 3′-terminal CCA sequence of tRNA (Deutscher, 1982) and of certain plant viral RNA genomes (Rao et al., 1989; Giegé, 1996). CCA addition is an essential step in 3′ processing of tRNA in organisms that do not encode the terminal CCA, including eukaryotes, many archaea, and some eubacteria. The CCA-adding enzyme also repairs CCA termini depleted by exonucleolytic attack, and this is the primary function of the enzyme in eubacteria such as *Escherichia coli*, where all tRNA genes encode CCA (Zhu & Deutscher, 1987).

The CCA-adding enzyme belongs to the nucleotidyltransferase superfamily (Martin & Keller, 1996; Yue et al., 1996). This diverse superfamily includes enzymes that add nucleotides to DNA (terminal deoxynucleotidyltransferase, DNA polymerase β), RNA (poly(A) polymerase), protein (glutamine synthase adenylyltransferase), and antibiotics (kanamycin and streptomycin nucleotidyltransferases) (Holm & Sander, 1995). Like two other members of the superfamily, terminal deoxynucleotidyltransferase and poly(A) polymerase, the CCA-adding enzyme adds nucleotides in a primer-dependent but template-independent fashion. Unlike these enzymes, however, the CCA-adding enzyme displays remarkable specificity, uniquely recognizing tRNA as substrate and adding a precisely defined 3′-terminal CCA sequence.

tRNA consists of two coaxially stacked helices that appear to be independent structural and functional domains. The domain referred to as the "top half" contains the acceptor stem and the TψC stem-loop; the "bottom half" contains the D stem-loop and the anticodon stem-loop (Fig. 1). The top-half domain, sometimes referred to as a tRNA "minihelix," has been shown to provide the determinants necessary for specific charging by aminoacyl tRNA synthetases (Shi et al., 1992; Schimmel & Pouplana, 1995); for interaction with elongation factor Tu (Rudinger et al., 1994); for 5′ processing by RNase P (McClain et al., 1987); and for distinguishing elongator from initiator tRNA (Puglisi et al., 1994). However, it has not been established if top-half minihelices could function as substrates for the CCA-adding enzyme.

Because $tRNA^{Phe}$ and $tRNA^{Lys}$ analogues made of DNA have been shown to serve as substrates for aminoacylation by the cognate aminoacyl-tRNA synthetases (Khan & Roe, 1988), it seemed likely that tDNAs might similarly be substrates for the CCA-adding enzyme. Synthetic DNA oligonucleotides provide significant ad-

Reprint requests to: Nancy Maizels, Department of Molecular Biophysics and Biochemistry, Yale University, 266 Whitney Avenue, P.O. Box 208114, New Haven, Connecticut 06520-8114, USA; e-mail: nancy.maizels@yale.edu.

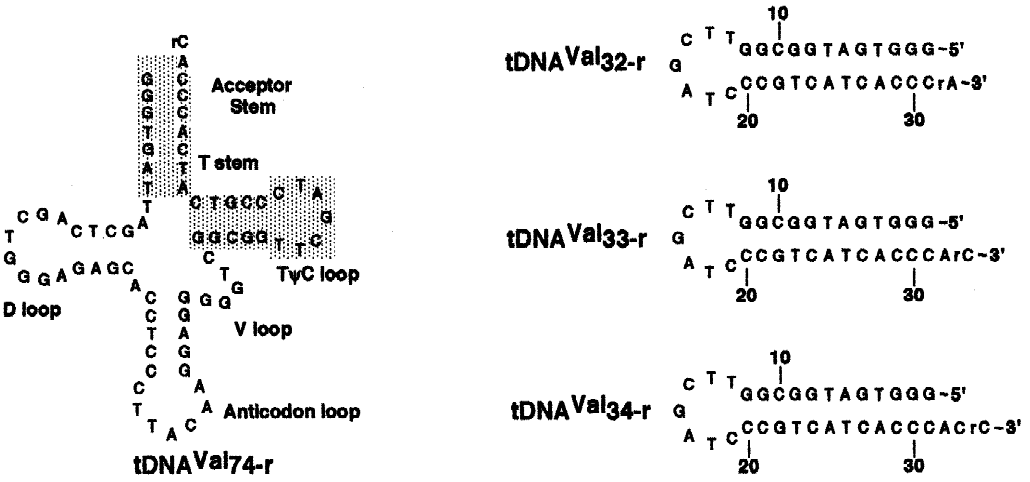

FIGURE 1. tDNAs. Secondary structure of full-length and top-half tDNA^{Val} molecules. The full-length tDNA^{Val} oligonucleotide is 74 nt in length; the top-half molecules are 32, 33, and 34 nt; all include the discriminator base and have a 3′-terminal ribonucleotide residue. The top-half oligonucleotides correspond to minihelices containing the acceptor stem and the TψC loop (shaded portion of the full-length tDNA).

vantages over RNA for many kinds of structural and functional analysis, because they are made easily and inexpensively. Here we report that the CCA-adding enzyme specifically adds CCA termini to synthetic tDNA and to tDNA minihelices corresponding to the top half domain of tRNA. Addition to tDNA minihelices is slightly more efficient than addition to full-length tDNA. Addition requires that the tDNA substrate carry a 3′-terminal ribonucleotide, and is sensitive to the length of the base paired stem, but relatively tolerant of changes in the TψC loop.

These results provide further evidence for independent functions of the top and bottom halves of tRNA. They also support the hypothesis that these two structurally independent domains evolved independently (Weiner & Maizels, 1987; Maizels & Weiner, 1993; Noller, 1993; Schimmel et al., 1993).

RESULTS

The CCA-adding enzyme specifically adds CCA to top-half tDNAs

We generated synthetic oligonucleotide substrates corresponding in sequence to the top half or the complete sequence of *E. coli* tRNA^{Val} (Fig. 1). tDNA^{Val}32-r, tDNA^{Val}33-r, and tDNA^{Val}34-r correspond to top-half minihelices 32, 33, or 34 nt in length that include the discriminator base but lack the 3′-terminal CCA, CA, or A, respectively. Oligonucleotide tDNA^{Val}74-r is 74 nt in length and corresponds to a complete tRNA molecule lacking the 3′-terminal CA. All four of these tDNA substrates carry a 3′ ribose. We also generated by in vitro transcription a full-length tRNA substrate, tRNA^{Asp}75, that carries an extra nucleotide in the D loop, and a 54-mer RNA carrying a stem-loop from the 3′ terminus of West Nile virus (Shi et al., 1996) as a control for the specificity of CCA addition. When all these molecules were analyzed by denaturing gel electrophoresis and ethidium bromide staining, it became apparent that synthesis of the full-length tDNA^{Val}74-r produced heterogeneous products, of which only about 40% were full-length molecules (Fig. 2A, lane 2). Synthesis of deoxyoligonucleotides that terminate with a 3′ ribose was performed in the 3′ to 5′ direction on a matrix containing the 3′ ribose, so the truncated molecules correspond to a series of nested 5′ deletions. The presence of premature termination products was not unanticipated, because residual secondary structure can interfere with coupling efficiency under the gentler conditions that must be used for synthesis of oligonucleotides terminating with a 3′ ribonucleotide.

Initially, we compared the ability of the *E. coli* CCA-adding enzyme to add [α-^{32}P]CTP to the full-length tDNA^{Val}74-r and top-half tDNA^{Val}33-r oligonucleotides (Fig. 2B). Addition occurred using as substrate unpurified (Fig. 2B, lane 2) or gel-purified (Fig. 2B, lane 3) tDNA^{Val}74-r, or top-half tDNA^{Val}33-r (Fig. 2B, lane 4). A single labeled product was generated regardless of whether the full-length tDNA^{Val}74-r substrate had been gel-purified, indicating that the enzyme is specific for a base paired acceptor stem. The specificity of the enzyme was further confirmed by the observation that the 3′-terminal 54-mer stem-loop of the West Nile virus (WNV) RNA genome did not function as a substrate for the enzyme (Fig. 2B, lane 5).

Addition to the tDNA^{Val}33-r top-half minihelix was 2.7-fold greater than addition to the full-length tDNA^{Val}74-r (Fig. 2B, compare lanes 3 and 4), suggesting that the top-half minihelix is a somewhat better substrate than

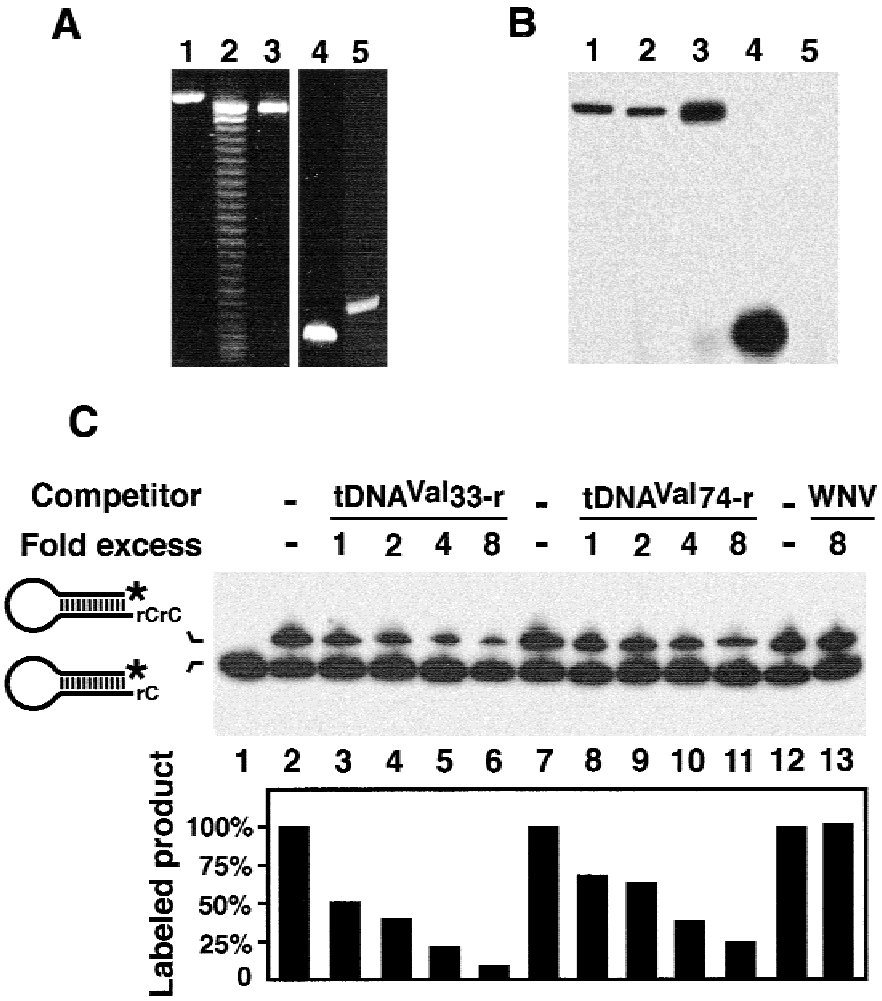

FIGURE 2. Synthetic oligonucleotide substrates for the CCA-adding enzyme. **A:** Denaturing polyacrylamide gel analysis of $tRNA^{Asp}$75-mer (lane 1); full-length $tDNA^{Val}$74-r, unpurified (lane 2) and gel purified (lane 3); top-half $tDNA^{Val}$33-r (lane 4); and the 3′-terminal 54-mer of West Nile virus genomic RNA (WNV, lane 5) (Shi et al., 1996). Oligonucleotides were visualized by ethidium bromide staining. **B:** Addition of [α-^{32}P]CTP to full-length $tDNA^{Val}$74-r (unpurified, lane 2, and purified, lane 3), top-half $tDNA^{Val}$33-r (lane 4), and WNV RNA (lane 5). End-labeled $tRNA^{Asp}$75-mer is shown as a marker (lane 1). Oligonucleotide substrate concentration was 15 μM in the reactions shown in lanes 3, 4, and 5; equal mass amounts of substrate were added to reactions shown in lanes 2 and 3. **C:** Competition assay comparing top-half $tDNA^{Val}$33-r and full-length $tDNA^{Val}$74-r. Above, addition of CTP to 5′-labeled $tDNA^{Val}$33-r was assayed in the absence (lanes, 2, 7, and 12) or presence of unlabeled $tDNA^{Val}$33-r (lanes 3–6), $tDNA^{Val}$74-r (lanes 8–11), or WNV RNA (lane 13). The molar excess of competitor is indicated above each lane. Input substrate (lane 1). Hairpin diagrams at side of the figure indicate termini of products; asterisks represent 5′ end label. Below, quantitation of labeled product in the absence or presence of competitors. The amount of labeled product in the absence of competitor was defined as 100%.

full-length tDNA. This result was confirmed by a competition experiment in which we assayed addition of cold CTP to a 5′ end-labeled $tDNA^{Val}$33-r substrate. Under these conditions, $tDNA^{Val}$33-r is extended by a single nucleotide to produce a 3′ sequence lacking only the terminal A (Fig. 2C, lanes 2, 7, 12). When unlabeled $tDNA^{Val}$33-r was included in the reactions, the amount of extended product decreased with increasing competitor concentration (Fig. 2C, lanes 3–6). The $tDNA^{Val}$74-r substrate also functioned as competitor (Fig. 2C, lanes 8–11), whereas WNV RNA had no effect on addition (Fig. 2C, lane 13). Quantitation of the fraction of substrate extended in the presence of minihelix or full-length competitor tDNAs (Fig. 2C, bottom panel) confirmed that the full-length tDNA competed less effectively than the minihelix substrate.

We conclude that both top-half tDNAs and full-length tDNAs can serve as substrates for the CCA-adding enzyme, but the top half appears to be a somewhat better substrate. Top-half tDNA may have greater flexibility than full-length tDNA, which is constrained by interactions between the top- and bottom-half domains. Because top-half tDNAs can be synthesized readily, we used these molecules as substrates in subsequent experiments.

A 2′ hydroxyl group at the 3′ terminus is critical for CCA addition

We assayed the specificity of CCA addition to top-half tDNA using a series of synthetic oligonucleotide substrates corresponding in sequence to the top half of *E. coli* $tRNA^{Val}$. Oligonucleotides were 32, 33, and 34 nt in length, and were synthesized as DNA with a single ribonucleotide at the 3′ position (Fig. 1). As shown in Figure 3, in the presence of cold CTP, the CCA-adding enzyme incorporated [α-^{32}P]ATP into all three tDNA minihelices, converting them to molecules of 35 nt in length (lanes 1–3). In contrast, only $tDNA^{Val}$32-r and $tDNA^{Val}$33-r incorporated [α-^{32}P]CTP in the presence of cold ATP (Fig. 3, lanes 4, 5); tDNAVal34-r did not (Fig. 3, lane 6). No addition was detected in assays containing [α-^{32}P]GTP or [α-^{32}P]UTP (data not shown).

Molecules of identical sequence but made entirely of DNA ($tDNA^{Val}$32-d and $tDNA^{Val}$34-d, Fig. 3, lanes 7–8) were not substrates for CCA addition. This shows that

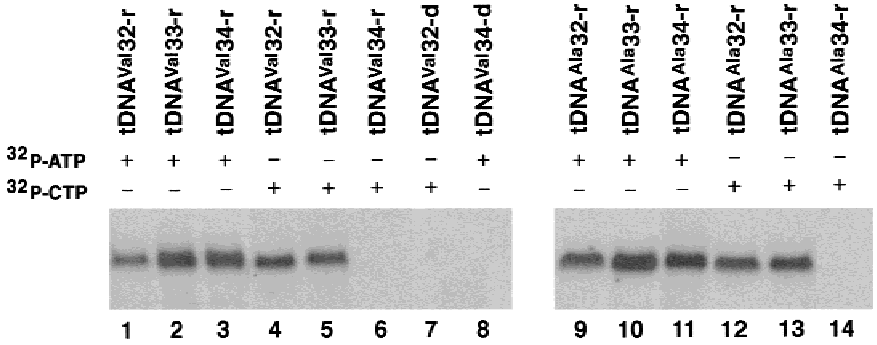

FIGURE 3. Specific addition of CCA to tDNA minihelices. CCA addition to tDNA^{Val} substrates (Fig. 1) and to tDNA^{Ala} substrates (see Materials and Methods) was assayed by denaturing 12% PAGE. Substrates carried either a 3′-terminal deoxyribonucleotide (d) or ribonucleotide (r). Assays were performed as described in Materials and Methods, except that lanes 1, 2, 9, and 10 contained 30 μM tDNA and 300 ng of *E. coli* CCA-adding enzyme.

the 2′ hydroxyl group on the 3′-terminal ribonucleotide is critical for CCA addition. To establish that tDNAs other than tDNA^{Val} could function as specific substrates, we also assayed addition to a series of tDNA minihelices corresponding in sequence to the top half of tRNA^{Ala} (Fig. 3, right). The CCA-adding enzyme incorporated [α-^{32}P]ATP into all three tDNAs in the presence of cold CTP (Fig. 3, lanes 9–11); conversely, [α-^{32}P]CTP was incorporated into tDNA^{Ala}32-r and tDNA^{Ala}33-r, but not tDNA^{Ala}34-r (Fig. 3, lanes 12–14). We conclude that top-half tDNAs with a 3′-terminal ribonucleotide can function as substrates for specific CCA addition. Addition is presumably to the 3′ hydroxyl of the substrate, because three consecutive nucleotides (CCA) can be added to substrates lacking CCA (Fig. 3, lanes 1, 4, 9, 12).

A 12-base pair (bp) stem is optimal for CCA addition

In most tRNAs, the top-half minihelix is 12 bp in length; two exceptions are *E. coli* tRNA^{His} (Keith et al., 1983) and selenocysteine tRNA, in which this stem is 13 bp long (Leinfelder et al., 1988). We assayed the effect of stem length on CCA addition using tDNA^{Val} minihelices modified by deletion or insertion of a single base pair (Fig. 4A). Three oligonucleotides were tested: tDNA^{Val}del(8,24), a deletion of residues 8 and 24 (numbered with reference to the 34-nt tDNA^{Val}34-r substrate shown in Fig. 1); tDNA^{Val}del(10,22), a deletion of residues 10 and 22; and tDNA^{Val}ins(8,26), an insertion of an A:T base pair at residues 8 and 26. The deletion mutations reduced addition about twofold compared to tDNA^{Val}32-r (Fig. 4A, lanes 1–3). The insertion mutation had a more dramatic effect, reducing CCA addition by fivefold (Fig. 4A, lane 4). These results show that the CCA-adding enzyme inspects the length of the top-half tDNA minihelix.

TψC loop facilitates but is not essential for CCA addition

The TψC loop is highly conserved, raising the question of whether it might be critical for CCA addition. We there-

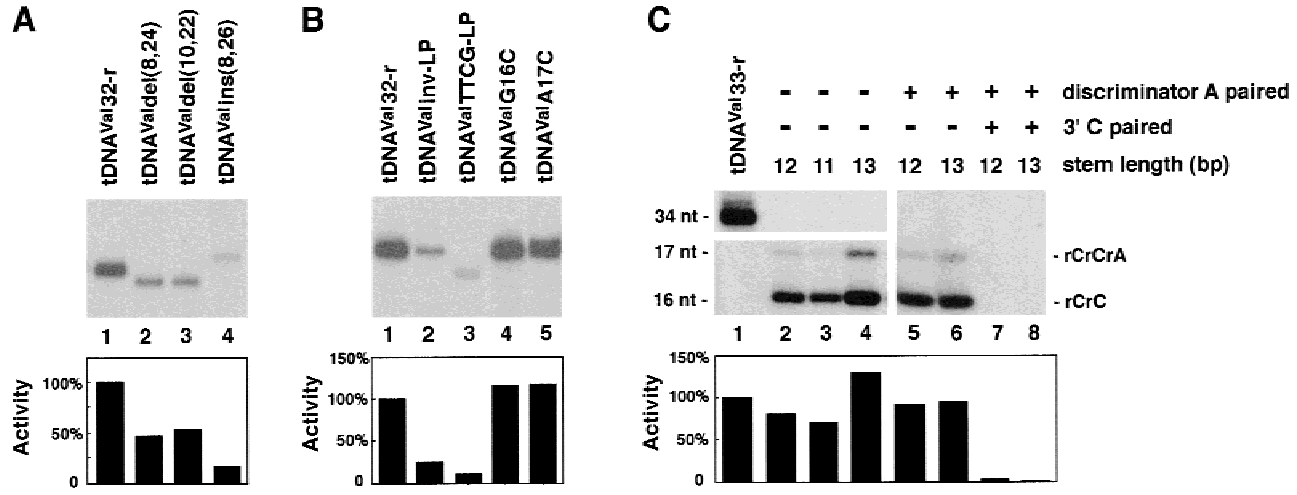

FIGURE 4. Effect of stem length, TψC loop, and discriminator base on CCA addition. Assays were performed as described in Materials and Methods unless otherwise indicated. The upper portion of each panel shows denaturing gel analysis of reaction products; and the lower portion shows addition normalized to the tDNA^{Val}32-r substrate. **A:** Addition of [α-^{32}P]CTP to tDNA^{Val}32-r (lane 1); to two deletion mutants, del(8,24), lane 2; and del(10,22), lane 3; and to the insertion mutant ins(8,26), lane 4. **B:** Addition of [α-^{32}P]CTP to tDNA^{Val}32-r, lane 1; to loop inversion mutant tDNA^{Val}inv-LP, lane 2; to tDNA^{Val}TTCG-LP, lane 3; to tDNA^{Val}G16C, lane 4; and to tDNA^{Val}A17C, lane 5. **C:** Addition of [α-^{32}P]CTP to duplex substrates. The addition reaction contained 500 μM ATP and 100 μM CTP, and products were analyzed by 20% denaturing PAGE.

fore assayed addition to several substrates carrying mutations in this loop. Inversion of the loop (by changing TTCGATC to CTAGCTT) reduced addition by approximately fourfold (Fig. 4B, compare lanes 1 and 2). Deletion of three nucleotides from the 3′ end of the loop generated a TTCG tetraloop sequence, which reduced CCA addition by 10-fold (Fig. 4B, lane 3). Although TTCG tetraloops are considerably less stable as DNA than as RNA (James & Tinoco, 1993; Allain & Varani, 1995), this dramatic decrease may reflect a structural constraint inflicted by the tetraloop on the tDNA structure. CCA addition was not measurably affected by single base changes at two conserved positions in the loop, either mutation of the highly conserved G16 to C (tDNA^{Val}G16C-r), or mutation of the universally conserved A17 to C (tDNA^{Val}A17C-r; Fig. 4B, compare lanes 4 and 5 with lane 1).

Because mutations in the TψC loop had comparatively little effect on CCA addition, we asked whether the loop might be dispensible. This possibility was tested using substrates in which two oligonucleotides were annealed to produce a base paired minihelix stem with no loop. Three different substrates were generated by annealing a single 3′ oligonucleotide 15 nt in length with three different 5′ oligonucleotides, to produce a series of substrates with minihelix stems of 11, 12, or 13 bp (see Materials and Methods). These substrates carried an unpaired discriminator base and a 3′-terminal ribonucleotide C, but lacked the terminal CA. Addition to the substrates with 11- or 12-bp stems was found to be only slightly less efficient than addition to substrates containing the intact TψC loop (30% and 20% reduced, respectively), and addition to the substrate with a 13-bp stem was slightly enhanced (30% increase) (Fig. 4C, lanes 1–4). Limiting concentrations of substrate could account for the fact that most molecules are extended by only one nucleotide, to 16 nt final length, rather than 17 nt. Li et al. (1996) found that changes in the TψC loop sequence impaired addition to tRNA substrates. We found no evidence of this in the tDNA minihelix substrates.

CCA addition requires that the 3′-terminal ribonucleotide be unpaired

The "discriminator base" of tRNA is located one nucleotide upstream of the CCA end, and this base is almost always unpaired. To ask whether pairing of the discriminator base affected CCA addition, we used minihelix stems generated by annealing two oligonucleotides to produce molecules containing either 12- or 13-bp stems in which the discriminator A was paired, but the 3′-terminal C (the first C of the CCA sequence) was unpaired. CCA addition was unaffected by pairing of the discriminator base (Fig. 4C, lanes 5, 6). This may indicate that the enzyme interacts exclusively with the tDNA backbone at this position, as might be expected for an enzyme that must accommodate any of the four nucleotides as the discriminator base.

Because addition was insensitive to pairing of the discriminator base, we further investigated the influence of base pairing within the acceptor stem by assaying addition to substrates with 12- or 13-bp stems in which the first C of the CCA sequence was base paired. These blunt minihelix termini were not substrates for CCA addition (Fig. 4C, lanes 7, 8).

Parameters of the CCA addition to tDNA minihelices

In natural tRNA substrates, the 5′ end is either phosphorylated or bears a 5′ leader sequence that will be removed by RNase P (Oh & Pace, 1994; Hardt et al., 1995; Liu & Altman, 1996; Svard et al., 1996). The synthetic tDNA oligonucleotides are synthesized with a 5′ hydroxyl group, and can be readily phosphorylated by treatment with polynucleotide kinase and ATP (e.g., Fig. 2). To determine whether 5′ phosphorylation affected CCA addition, we compared addition to synthetic tDNA^{Val}32-r, tDNA^{Val}33-r, and tDNA^{Val}34-r minihelices with 5′ hydroxyl or 5′ phosphate termini. Phosphorylation of the 5′ end had no effect on addition (Fig. 5A).

Using tDNA substrates that had been 5′ end-labeled with γ-^{32}P-ATP, we could then monitor CCA addition to tDNA minihelices at varying concentrations of nucleoside triphosphate substrate. Addition to ^{32}P-labeled tDNA^{Val}33-r was measured at concentrations of CTP ranging from 0 to 500 μM (Fig. 5B), and addition to ^{32}P-labeled tDNA^{Val}34-r was measured at concentrations of ATP ranging from 0 to 1,000 μM (Fig. 5C). The efficiency of C addition to tDNA^{Val}33-r increased with increasing concentrations of CTP up to 250 μM; at this point, the reaction saturated, and C had been added to 30% of the substrate. The efficiency of A addition to tDNA^{Val}34-r increased with increasing concentrations of ATP up to 500 μM; at this point, A had been added to more than 70% of the substrate. Had only a very small fraction of tDNA molecules proved to be potential substrates, it might have suggested that addition depended upon a rare conformer of the substrate. This is clearly not the case, because a substantial fraction of tDNA minihelix molecules are substrates for addition.

The data in Figure 5B and C allow us to estimate the K_m for nucleotide addition: K_m = 25 μM for CTP and K_m = 175 μM for ATP. These K_m values are in good agreement with those obtained with natural tRNA: K_m = 15–200 μM for CTP, and K_m = 100–200 μM for ATP (Deutscher, 1982).

Kinetic studies were performed as previously described (Li et al., 1996), measuring addition to tDNA^{Val}34-r, the 34-nt minihelix that lacked the 3′-terminal A, in the presence of saturating ATP (500 μM). The observed Michaelis constant K_m was approxi-

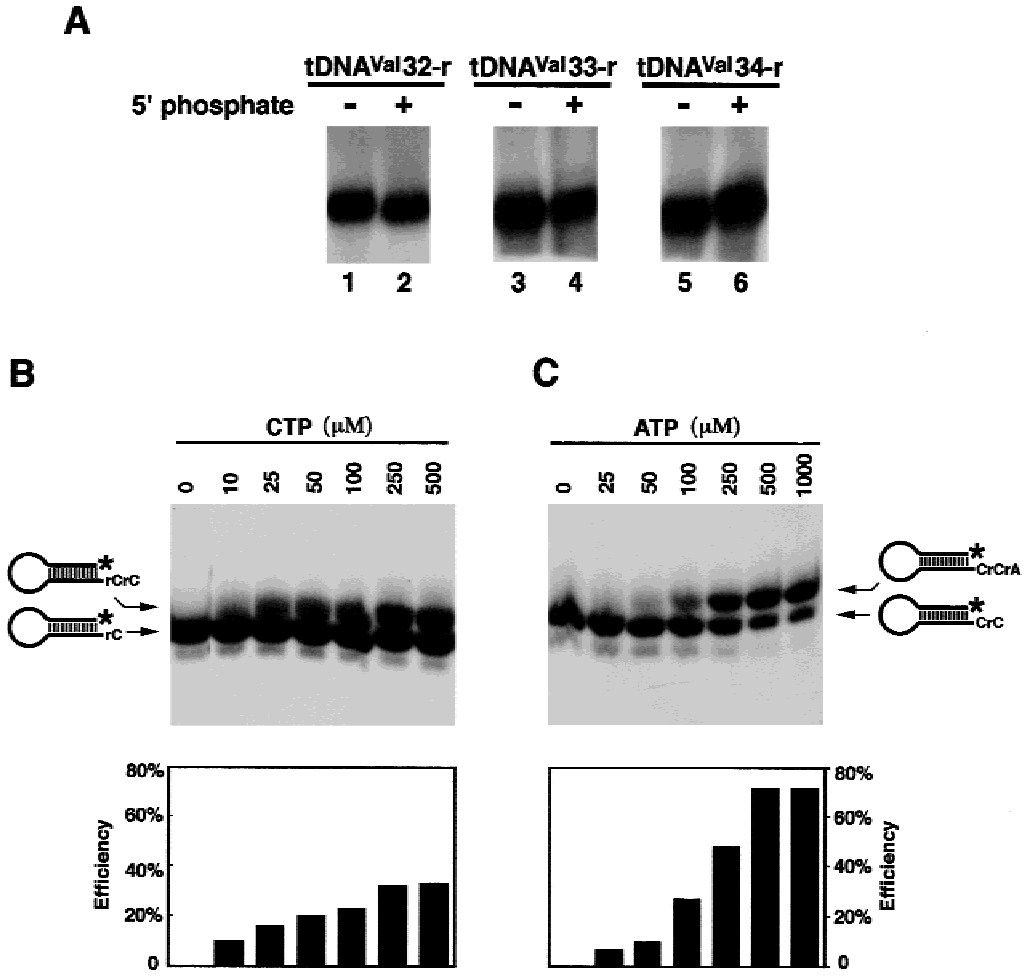

FIGURE 5. tDNA top-half is a good substrate for the CCA-adding enzyme. **A:** 5′ Phosphorylation does not affect the activity of a top-half tDNA substrate. Top-half tDNA minihelices tDNA^{Val}32-r, tDNA^{Val}33-r, and tDNA^{Val}34-r (Fig. 1) were 5′ phosphorylated with T4 polynucleotide kinase in the presence of cold ATP before CCA addition. **B:** Addition to tDNA^{Val}33-r substrates at varying concentrations of CTP. Substrates were 5′-labeled with ^{32}P. Above, denaturing gel electrophoresis of reaction products. Hairpin diagrams at either side of figure indicate termini of products; asterisks represent 5′ end label. Below, substrate efficiency expressed as the percentage of substrate converted to product. **C:** As in B, except addition to tDNA^{Val}34-r substrates was assayed at varying ATP concentrations, as indicated.

mately 35 μM, about fourfold higher than that of a natural tRNA (Deutscher, 1982). The estimated V_{max} was approximately 20 μmol/h/mg enzyme, 30-fold less than natural tRNA assayed with the rabbit liver CCA-adding enzyme (Deutscher, 1982). These results indicate that the top-half tDNA is a good substrate for CCA addition.

DISCUSSION

We found that both full-length tDNA and tDNA top-half minihelices are substrates for the *E. coli* CCA-adding enzyme. This shows that these DNA substrates possess or readily adopt a conformation that is recognized by the CCA-adding enzyme. This may not be surprising, because a ribose backbone is not always required for the activity of RNA molecules. DNA analogues with or without ribonucleotides at specific positions can form active hammerhead ribozymes (Dahm & Uhlenbeck, 1990; Yang et al., 1992), and can serve as substrates for pre-tRNA cleavage by RNase P (Perreault & Altman, 1992) or for aminoacylation by tRNA synthetases (Khan & Roe, 1988; Perreault et al., 1989; Musier-Forsyth & Schimmel, 1992). Moreover, the structures of tRNA and tDNA are globally similar (Paquette et al., 1990; Holmes & Hecht, 1993; Lim & Barton, 1993), although the absence of the 2′ hydroxyl clearly affects local structure (Paquette et al., 1990), and the more B-like conformation of tDNA disfavors GT base pairs (Lim & Barton, 1993). Duplex RNA must adopt the A form to accommodate 2′ hydroxyl groups, whereas duplex DNA is relatively flexible in structure and can exist in either A or B form (Dickerson et al., 1982). The structural flexibility of DNA may even allow the top-half tDNA

substrates to undergo a conformational switch from B to A form upon binding to the CCA-adding enzyme.

The observation that top-half tDNAs are somewhat better substrates than full-length molecules supports the notion that essential determinants for enzyme recognition reside in the top half. Addition to the tDNA minihelices depended upon the presence of a 3′-terminal ribonucleotide and its 2′ hydroxyl group. The observation that a 3′-terminal ribose is critical for CCA addition was quite unexpected, and may reflect a strong selection against CCA addition to the 3′ end of DNAs generated during replication, repair, or recombination.

All known CCA-adding enzymes belong to the nucleotidyl transferase superfamily, and share related active site signature sequences; however, the eubacterial and eukaryotic enzymes also share extensive homology outside the signature region, but are only distantly related (if at all) to the archaeal CCA-adding enzymes (Martin & Keller, 1996; Yue et al., 1996). [Thurlow et al. (1997) speculated that two adjacent unidentified open reading frames in the published *Methanococcus jannaschii* genome sequence (Bult et al., 1996) might be homologues of the *Sulfolobus shibatae* CCA-adding gene. We had also noted this anomaly, resequenced the *M. jannaschii* segment, identified the sequencing error that generated a termination codon between the MJ1111 and MJ1112 ORFs, and communicated the corrected sequence to Bult et al. (P.-Y. Shi, N. Maizels, & A.M. Weiner, unpubl.).] Studies to determine whether the substrate specificity of the archaeal class I enzymes is similar to that of the eubacterial/eukaryotic class II enzymes are in progress.

Addition of CCA to tDNA was efficient: C was added to 30% of substrate, and A to more than 70% of substrate. The observed K_m was approximately 35 μM, and the V_{max} was approximately 20 μmol/h/mg enzyme. These values for K_m and V_{max} are fourfold higher and 30-fold lower than values measured in assays of addition by rabbit liver CCA-adding enzyme to cellular tRNA substrates (Deutscher, 1982). One explanation for these different values is that regions within natural tRNAs that are missing in the top-half tDNAs improve enzyme–substrate interaction and catalysis. It is also possible that these differences reflect differences between experimental materials and protocols. The synthetic tDNAs we have assayed were homogeneous, whereas the substrate tRNAs assayed previously were prepared by periodate oxidation or snake venom phosphodiesterase digestion of total cellular tRNAs (Evans & Deutscher, 1976). Either treatment will remove CCA, but to varying extents, and the products will be heterogeneous. In addition, the earlier assays measured CCA addition as total acid-precipitable counts (Deutscher, 1982), whereas our experiments have used denaturing gel electrophoresis to resolve labeled products. There were known to be contaminating RNA molecules in cellular tRNA preparations that could be labeled by the CCA-adding enzyme, although much less efficiently than tRNA (Deutscher, 1982). Acid precipitation would not have distinguished such products from labeled tRNAs, so addition may have been somewhat overestimated in previous assays. This complicates any comparison of our data with earlier published values, but the apparent decrease in K_m and increase in V_{max} that we observe are consistent with the intuition that the tDNAs would not be fully comparable to natural tRNAs as substrates, either in binding or catalysis.

Based upon the widespread roles of top-half tRNA-like structures in replication of contemporary RNA genomes, we suggested that tRNA evolved in replication prior to the advent of templated protein synthesis, and that the top-half domain had an independent and more ancient origin than the anticodon domain (Weiner & Maizels, 1987; Maizels & Weiner, 1993). The separable interactions of the top-half and anticodon domains with the 23S and 16S rRNAs, respectively, also led Noller (1993) to suggest that these domains of tRNA evolved independently. Schimmel et al. (1993) reached similar conclusions by a very different route, based on the modular interaction of tRNA synthetases with the top and bottom halves of tRNA. Indeed, as cited above, the top half of tRNA can function as a substrate for many key enzymes of tRNA metabolism (McClain et al., 1987; Shi et al., 1992; Puglisi et al., 1994; Rudinger at el., 1994; Schimmel & Pouplana, 1995). The fact that the top half of tRNA contains sufficient determinants for specific addition by the CCA-adding enzyme, itself an ancient activity present in all three living kingdoms (Yue et al., 1996), provides additional experimental support for the hypothesis that the top-half domain of tRNA evolved independently from the bottom half.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides were synthesized by the W.M. Keck Biotechnology Resource Laboratory at Yale University using ABI 380B and 394 DNA synthesizers. All-DNA oligonucleotides were synthesized by standard procedures; DNA oligonucleotides with a single 3′-terminal ribonucleotide were synthesized on RNA columns (Perseptive Biosystems) by standard cyanoethyl DNA chemistry (ABI), and are indicated by an "r" at the end of the sequence. The full-length oligonucleotide $tDNA^{Val}$74-r, and the top-half oligonucleotides $tDNA^{Val}$34-r, $tDNA^{Val}$33-r, and $tDNA^{Val}$32-r, are shown in Figure 1. Sequences of other oligonucleotides are as follows. $tDNA^{Ala}$34-r, corresponding to the top half of RNA^{Ala}, GCCCGGACTTG GTTCGATTCCGAGTCCGGGCACCr; $tDNA^{Ala}$32-r, GCCCG GACTTGGTTCGATTCCGAGTCCGGGCAr; $tDNA^{Ala}$33-r, GCCCGGACTTGGTTCGATTCCGAGTCCGGGCACr; stem deletion mutant del(8,24), GGGTGATGCGGTTCGATCCCG TATCACCCAr; stem deletion mutant del(10,22), GGGT GATGGGGTTCGATCCCTCATCACCCAr; stem insertion mutant ins(8,26), GGGTGATAGGCGGTTCGATCCCGTCTAT

CACCCAr; loop mutant tDNA^{Val}inv-LP, GGGTGATGGCG GCTAGCTTCCGTCATCACCCAr; loop mutant tDNA^{Val}TTCG-LP, GGGTGATGGCGGTTCGCCGTCATCACCCAr; loop mutant tDNA^{Val}G16C, GGGTGATGGCGGTTCCATCCCGT CATCACCCAr; and loop mutant tDNA^{Val}A17C, GGGTGATG GCGGTTCGCTCCCGTCATCACCCAr.

Double-stranded duplex molecules were generated by annealing the oligonucleotide CCCGTCATCACCCACr to equimolar amounts of the following all-DNA oligonucleotides: to produce duplexes with an 11-, 12-, or 13-bp stem and an unpaired discriminator A, we used the 11-mer GGGTGATG GCG, 12-mer GGGTGATGGCGG, and 13-mer GGGTGATG GCGGG; to produce duplexes with 12- or 13-bp stems with discriminator A paired and the 3′-terminal C unpaired, we used the 12-mer TGGGTGATGGCG and 13-mer TGGGT GATGGCGG; to produce duplexes with 12- or 13-bp stems having both the discriminator A and 3′-terminal C paired, we used the 12-mer GTGGGTGATGGC and 13-mer GTGG GTGATGGCG.

Expression and purification of the *E. coli* CCA-adding enzyme

The *E. coli cca* gene (Cudny et al., 1986) was PCR-amplified using genomic DNA as template and two primers; CATATG AAGATTTATCTGGTCGGT corresponds to the N-terminus of the gene and carries a 5′ *Nde* I site (underlined), and GGATCC TCATTCAGGCTTTGGGCA corresponds to the complement of the C-terminus of the gene and carries a 3′ *Bam*H I site (underlined). The PCR product was cloned into the pGEM-T vector (Promega), excised by digestion with *Nde* I and *Bam*H I, and subcloned into the corresponding sites of the pET-22b(+) expression vector. By site-directed mutagenesis (Novagen), the *cca* gene stop codon (TGA) was changed to CTA to produce a construct, pET-22-CCA, in which the *cca* gene was fused with six His codons at its C-terminus. For protein expression, *E. coli* BL21(DE3) was transformed by pET-22-CCA, and transformed cells were grown in YT broth with 50 μg/mL of ampicillin and induced at an OD_{600} of 0.6 with 0.6 mM IPTG for 2 h. Overexpressed soluble protein was then purified by binding to a nickel chelate column as described by the manufacturer (Novagen). The insoluble enzyme in inclusion bodies was renatured and purified as described elsewhere (Shi et al., 1997).

Assays of CCA addition

Oligonucleotide substrates were denatured by heating to 80 °C for 3 min followed by quick cooling on ice for 10 min, then incubated at a final concentration of 15 μM for 20 min at 37 °C in a 10-μL reaction containing 100 mM glycine, pH 9.0, 10 mM $MgCl_2$, 1 mM DTT, 50 μM ATP, 50 μM CTP, 0.05 μM [α-^{32}P]ATP or [α-^{32}P]CTP (3,000 Ci/mmol, Amersham), and 50 ng of purified recombinant *E. coli* CCA-adding enzyme (unless otherwise indicated). In competition experiments, addition to substrates that had been 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase was assayed in 10-μL reactions containing 40 μM 5′-labeled tDNA^{Val}33-r, 400 μM CTP, 200 ng enzyme, and the indicated molar excess of tDNA^{Val}33-r or tDNA^{Val}74-r; other assays with end-labeled substrates were performed in 10-μL reactions containing 75 μM tDNA and 350 ng of *E. coli* CCA-adding enzyme. After addition of 8 μL of formamide/dye, samples were denatured at 95 °C for 2 min and analyzed by electrophoresis on a 12% acrylamide, 7 M urea gel in TBE. For quantitation, gel slices containing the labeled substrates or products were excised and ^{32}P levels determined by Cerenkov counting.

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