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22. *Drosophila* polytene chromosome spreads were prepared from salivary glands of third-instar larvae and processed as described (*11*). Fluorescent double-labeling of proteins was carried out as described (*11*) using rat N1 (*4*) at 1:20 dilution and affinity-purified rabbit C2 or S1, each at 1:150 dilution. Image files of labeled chromosomes were acquired with an Olympus microscope equipped with a digital camera.
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24. Selection of mutant embryos was performed using GFP balancer chromosomes. RNA in situ hybridization of whole-mount embryos using digoxigenin-labeled *Ubx* and *lacZ* probes was performed as described (*4, 11*).
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Collaboration Between CC- and A-Adding Enzymes to Build and Repair the 3′-Terminal CCA of tRNA in *Aquifex aeolicus*

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The universal 3′-terminal CCA sequence of all transfer RNAs (tRNAs) is repaired, and sometimes constructed de novo, by the CCA-adding enzyme [ATP(CTP):tRNA nucleotidyltransferase]. This RNA polymerase has no nucleic acid template, yet faithfully builds the CCA sequence one nucleotide at a time using cytidine triphosphate (CTP) and adenosine triphosphate (ATP) as substrates. All previously characterized CCA-adding enzymes from all three kingdoms are single polypeptides with CCA-adding activity. Here, we demonstrate through biochemical and genetic approaches that CCA addition in *Aquifex aeolicus* requires collaboration between two related polypeptides, one that adds CC and another that adds A.

The 3′-terminal CCA sequence (positions 74, 75, and 76) found on all mature tRNAs is required for tRNA aminoacylation (*1*) and for peptide bond formation by the ribosome (*2–4*). CCA-adding enzymes (*5*) have been identified in each of the three kingdoms (*6*) and are essential in organisms where not every tRNA gene encodes CCA (*7*). The CCA-adding enzyme is unique among nucleotidyltransferases (*6, 8, 9*) because it adds an ordered nucleotide sequence to a specific primer without using a nucleic acid template; moreover, the enzyme is sensitive to register, and can faithfully rebuild tRNAs with incomplete 3′ ends (tRNAD, tRNADC, and tRNADCC, where D is the discriminator base). Several models have been proposed to explain these properties (*10–14*), but the molecular details remain unknown. Compounding the mystery, all CCA-adding enzymes characterized to date consist of a single polypeptide with dual specificity for adenosine monophosphate and cytosine monophosphate incorporation.

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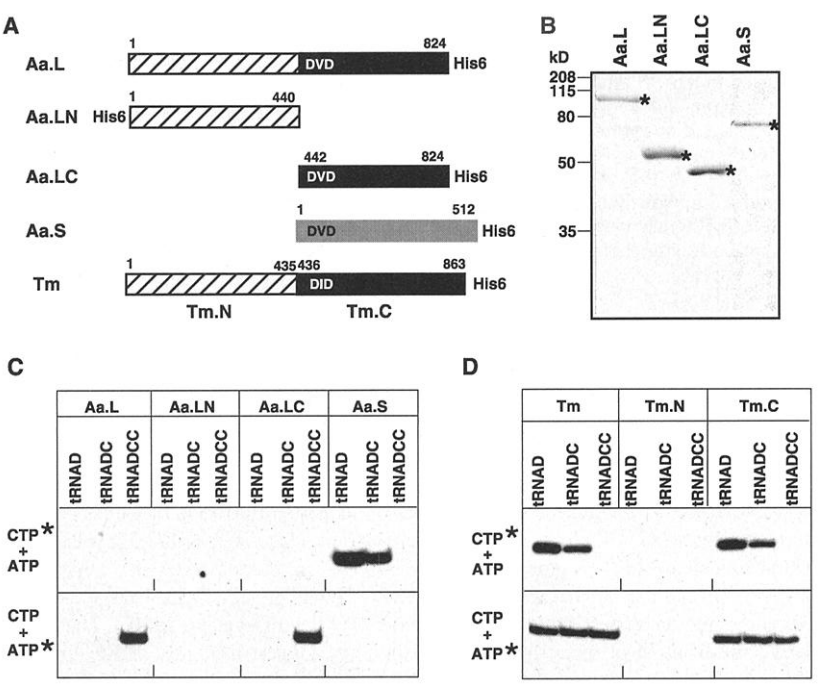

Fig. 1. C- and A-addition to tRNA are carried out by distinct, but related polypeptides in *A. aeolicus*. (**A**) *Aquifex aeolicus* nucleotidyltransferase homologs and the domains used in this study. Aa.L, the large polypeptide; Aa.LN, NH_2-terminal region of Aa.L; Aa.LC, COOH-terminal region of Aa.L; and Aa.S, the small polypeptide. The *T. maritima* nucleotidyltransferase is shown for comparison. DVD and DID indicate the active site signature found in all nucleotidyltransferase family members (*8*); His6 is the hexahistidine tag; striped boxes denote NH_2-terminal similarity, black boxes strong COOH-terminal similarity, and the gray box weaker COOH-terminal similarity. (**B**) Hexahistidine-tagged proteins were resolved by 12% SDS–polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. (**C**) Aa.L and Aa.LC have A-adding activity and Aa.S has C-adding activity. (**D**) The *T. maritima* nucleotidyltransferase is a CCA-adding enzyme and activity resides within the COOH-terminal region.

tRNA nucleotidyltransferase homologs can be identified in almost every new genomic sequence, but we were surprised to find two potential CCA-adding enzyme homologs in the genome of *Aquifex aeolicus* (NC_000918), a eubacterium where some tRNA genes do not encode CCA. The shorter homolog of 512 residues (Aa.S, GenBank AAC07883) is comparable in size to most archaeal and eubacterial CCA-adding enzymes, whereas the longer homolog of 824 residues (Aa.L, GenBank AAC06692) possesses an NH_2-terminal extension of unknown function (Fig. 1A). Homologs of the Aa.L polypeptide are readily identified in the *Thermotoga maritima*, *Synechocystis sp.*, and *Deinococcus radiodurans* genomes (GenBank AAD35797, BAA18195, and AE001950, respectively). *Synechocystis spp.* and *D. radiodurans* also contain homologs of the short Aa.S polypeptide (GenBank BBA10528 and AE001968, respectively), but *T. maritima* does not. Initially, we found that the recombinant *T. maritima* homolog of the long Aa.L polypeptide has full CCA-adding activity, and that this activity resides entirely within the COOH-terminal region (residues 436 through 863) (Fig. 1, A and D). We therefore expected that Aa.L would also have full CCA-adding activity; however, Aa.L adds neither CTP nor ATP to tRNA lacking CCA or CA, but does add ATP (and not CTP) to tRNA lacking a terminal A (Fig. 1C) (*15, 16*). The A-adding activity of Aa.L resides entirely within the COOH-terminal region, and C-adding activity is not observed when the NH_2-terminal extension is deleted (Fig. 1C; Aa.LC, residues 442 through 824). We next investigated whether the smaller *A. aeolicus* homolog might have full CCA-adding activity; however, recombinant Aa.S adds only CTP (and not ATP) to tRNA lacking CCA and CA, and does not add CTP or ATP to tRNA lacking a terminal A (Fig. 1C; Aa.S). CCA-adding activity could be reconstituted by mixing the two *A. aeolicus* polypeptides in vitro (Fig. 2A) where Aa.L (or Aa.LC) adds a single A at position 76 only, and Aa.S adds C at positions 75 and 74 only (Fig. 2, B and C). These two peptides do not interact with each other, and no ternary complex was formed in the presence of tRNA (*17*), suggesting that CCA addition by these peptides occurs by a dissociative rather than processive mechanism.

We expressed the two proteins in *Escherichia coli* strain CA244*cca*$^-$(*lacZ, trp, relA, spoT, cca::cam*) carrying a disrupted CCA-adding gene (*18*), and then analyzed the 3′-terminal status of tRNAs in vitro (*19*). Expression of the A-adding enzyme Aa.LC reduced the level of tRNAs lacking A, and also tRNAs lacking CA or CA, whereas expression of the CC-adding enzyme Aa.S increased the level of tRNAs lacking A, CA, and CCA (Fig. 3, A and B). We interpret the latter result to mean that the CC-adding enzyme rescues tRNAs lacking CA or CCA from complete degradation by 3′ exonucleases, but endogenous polyadenylate [poly(A)] polymerase I or a similar activity (*20*) cannot repair tRNAs lacking A as rapidly as the CC-adding enzyme generates them. When both Aa.LC and Aa.S are expressed, the level of defective tRNAs decreases, as expected (Fig. 3, A and B; Aa.LC/S and Aa.S/LC). Next, we coexpressed Aa.LC and Aa.S individually, or both Aa.LC and Aa.S, in CA244*cca*$^-$ along with the conditional amber suppressor tRNASup^{3+}(CGG_{OH}) with 3′-terminal CGG at positions 74 to 76 (*21*). Only when the 3′-terminal GG sequence is resected, and CA is added, will suppression of the *lac*Z amber mutation be observed (*22*). Consistent with reconstitution, coexpression of the A- and CC-adding enzymes (Aa.LC and Aa.S) with tRNASup^{3+}-(CGG_{OH}) suppressed the amber mutation (Fig. 3D; pMAa.LC/S and pMAa.S/LC), whereas expression of tRNASup^{3+}(CGG_{OH}) alone did not suppress (Fig. 3C). However, slight but significant suppression was observed when the CC-adding enzyme Aa.S alone, but not the A-adding enzyme Aa.LC alone, was coexpressed with tRNASup^{3+}(CGG_{OH}) (Fig. 3D; pMAa.S and pMAa.LC). This must reflect a low level of 3′-terminal A addition by endogenous poly(A) polymerase I or a similar activity (*20*), because a variant tRNASup^{3+} gene with 3′-terminal CCG exhibits suppressor activity in the absence of both the A- and CC-adding enzymes (Fig. 3C). We conclude that the A- and CC-adding enzymes collaborate to reconstitute CCA-adding activity in vivo as well as in vitro (Figs. 1 and 2) and that CCA-adding activity in *A. aeolicus* is the joint responsibility of two distinct but related polypeptides with different specificity and activity.

All previously characterized CCA-adding enzymes are composed of a single kind of polypeptide with dual specificity for CTP and ATP addition. In fact, even the *T. maritima* enzyme, which is highly homologous to both the NH_2- and COOH-terminal regions of the *A. aeolicus* A-adding polypeptide, has full CCA-adding activity. *Thermotoga maritima* and *A. aeolicus* are among the most deeply rooted, slowly evolving eubacteria (*23, 24*); thus, separate CC- and A-adding activities may be the more primitive state, and presence of dual-specificity polypeptides capable of full CCA-adding activity is a derived state. The *T. maritima* CCA-adding enzyme is more closely related to the A-adding enzyme of *A. aeolicus* than to the CC-adding enzyme, suggesting that the *T. maritima* CCA enzyme may have arisen from a primitive A-adding enzyme. The division of CCA-adding activity into two different polypeptides is not unique to *A. aeolicus*, but

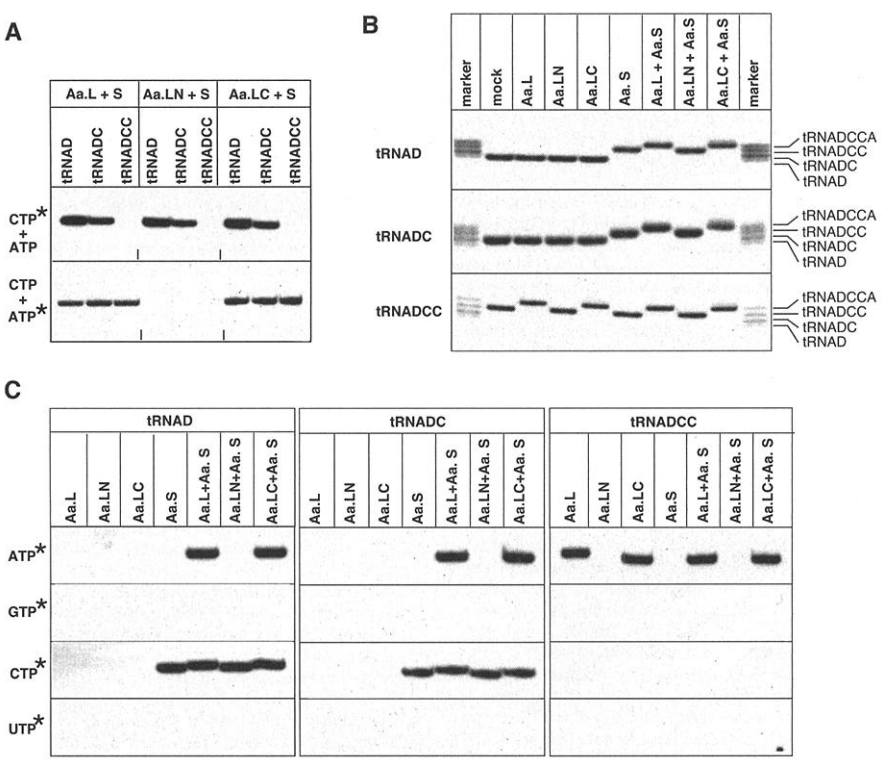

Fig. 2. Reconstitution of CCA-adding activity in vitro by two distinct *A. aeolicus* polypeptides. (**A**) Nucleotide specificity of Aa.L and Aa.S. The polypeptides were mixed pairwise and assayed as in Fig. 1C. (**B**) Sequence specificity of Aa.L and Aa.S. Uniformly [α-^{32}P]UTP-labeled tRNA lacking CCA, CA, or A (2 μM) was incubated with the polypeptides singly or in combination (12.5 nM each protein) in the presence of all of four nucleoside triphosphates (NTPs) (1 mM each), and products were resolved by 12% polyacrylamide gel electrophoresis. (**C**) Nucleotide and sequence specificity of Aa.L and Aa.LC. Assays contained 2 μM nonradiolabeled tRNA, 1 mM all four unlabeled NTPs, and one [α-^{32}P]-labeled NTP (indicated by an asterisk) (100 nM, 3000 Ci/mmol). Aa.L and Aa.LC add only ATP to tRNA and Aa.S adds only CTP.

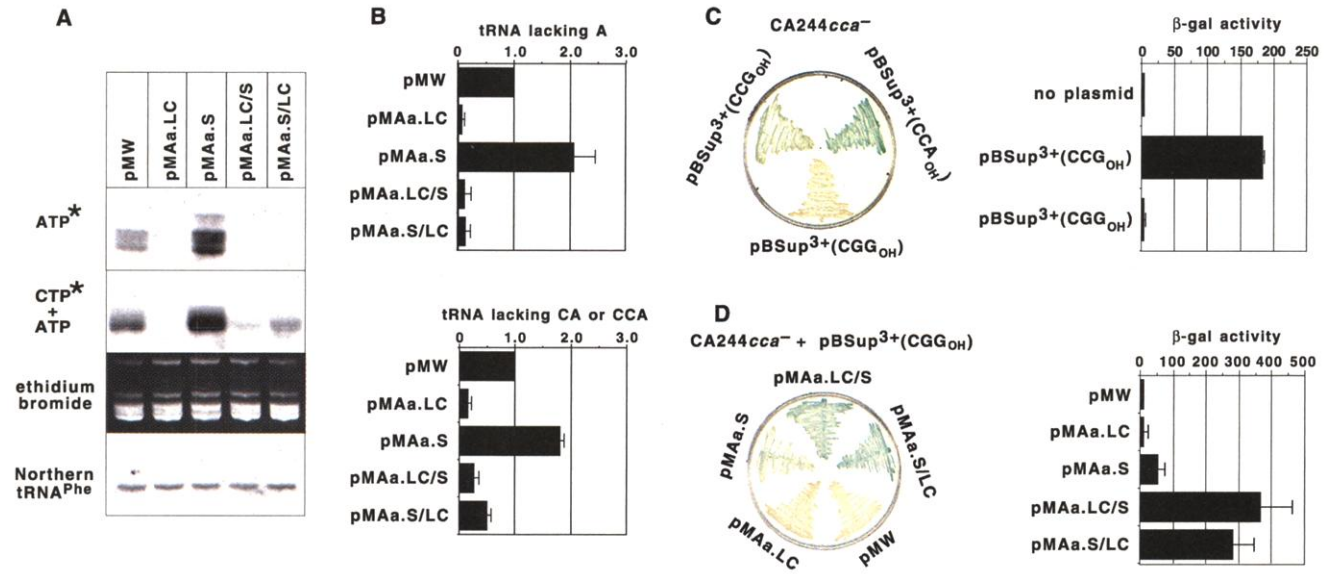

Fig. 3. The *A. aeolicus* Aa.LC and Aa.S polypeptides collaborate to repair the 3′ end of tRNA in vivo. (**A**) A biochemical assay for CCA-addition in vivo. The 3′-terminal status of bulk tRNA in vivo was analyzed (*19*). Upper two panels show assays for 3′-terminal ATP and CTP incorporation, where the asterisk indicates the labeled NTP. The lower two panels show an identical gel stained with ethidium bromide, and subjected to Northern blotting for $tRNA^{Phe}$, as a control for equal loading. (**B**) The 3′-terminal status of tRNAs in vivo. (Top) Relative levels of tRNA lacking 3′-terminal A; (bottom) relative levels of tRNA lacking CA or CCA. The tRNA concentrations were normalized relative to $tRNA^{Phe}$; data were normalized relative to tRNA prepared from the *cca*$^-$ strain carrying an empty plasmid (pMW); and error bars indicate the SD. (**C**) An amber suppression assay for CCA addition in vivo. (Left) $tRNASup^{3+}(CCA_{OH})$ and $tRNASup^{3+}(CCG_{OH})$ suppress, but $tRNASup^{3+}(CGG_{OH})$ does not, as visualized by growth of CA244*cca*$^-$ on Luria broth (LB) plates containing 1 mM IPTG, 50 μg/ml X-gal, 20 μg/ml tetracycline, and 34 μg/ml chloramphenicol. (Right) β-galactosidase activity assayed as described (*22*); error bars represent the SD. (**D**) Coexpression of *A. aeolicus* Aa.LC and Aa.S allows amber suppression by $tRNASup^{3+}(CGG_{OH})$. The expression constructs pMAa.LC/S and pMAa.S/LC carry the Aa.LC and Aa.S coding regions in reverse order; other details as in panel (C) except for addition of 100 μg/ml ampicillin to the LB plate.

is also found in *D. radiodurans* and *Synechocystis* (*17, 25*). Strong sequence homology between ribonucleotidyltransferases of different function—poly(A) polymerases, CCA-adding enzymes, and now CC- and A-adding enzymes—also imposes significant new constraints on possible mechanisms for CCA addition (*10–14*) by a single active site (*26*) in the absence of a nucleic acid template.

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16. Transfer RNA substrates lacking a terminal CA or A (tRNADC and tRNADCC) were prepared by in vitro transcription of linearized plasmid pmBSDCCA (*27*). The pmBSDCCA plasmid was mutagenized, using the Quickchange kit (Stratagene), to generate a template for in vitro transcription of tRNA lacking CCA (tRNAD). Standard assays for CTP or ATP incorporation were carried out in buffer containing 50 mM glycine-NaOH (pH 8.5), 10 mM $MgCl_2$, 25 mM KCl, 2 mM dithiothreitol, 1 mM ATP (and/or 500 μM CTP) and 100 nM [α-^{32}P]ATP (3000 Ci/mmol) or [α-^{32}P]CTP (3000 Ci/mmol), 2 μM tRNA substrate, and 12.5 nM recombinant protein at 60°C for 15 min, and products were resolved by denaturing 12% polyacrylamide gel electrophoresis. Others (*13, 14*) have reported that the *E. coli* CCA-adding enzyme has poly(C) polymerase activity in the presence of CTP alone, although it faithfully adds CCA in the presence of both ATP and CTP. Also several CCA-adding enzymes will use uridine triphosphate (UTP) in place of CTP, but CTP competitively inhibits UTP incorporation (*28*). We were therefore careful to assay the Tm, Aa.L, and Aa.S polypeptides in the presence of both ATP and CTP (Fig. 1) or all four nucleotides (Fig. 2) to maintain the physiological conditions that ensure faithful CCA addition.
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22. For assays of β-galactosidase activity, a single colony coexpressing the suppressor $tRNASup^{3+}$ and an *A. aeolicus* nucleotidyltransferase (Aa.LC, Aa.S, or both Aa.LC and Aa.S) was inoculated into YT medium and grown to saturation in the presence of ampicillin, tetracycline, chloramphenicol, and 1 mM IPTG at 37°C.
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