The 3′-terminal CCA sequence of tRNA is faithfully constructed and repaired by the CCA-adding enzyme (ATP(CTP):tRNA nucleotidyltransferase) using CTP and ATP as substrates but no nucleic acid template. Until recently, all CCA-adding enzymes from all three kingdoms appeared to be composed of a single kind of polypeptide with dual specificity for adding both CTP and ATP; however, we recently found that in *Aquifex aeolicus*, which lies near the deepest root of the euubacterial 16S rRNA-based phylogenetic tree, CCA addition represents a collaboration between closely related CC-adding and A-adding enzymes (Tomita, K. and Weiner, A. M. (2001) *Science* 294, 1334–1336). Here we show that in *Synechocystis* sp. and *Deinococcus radiodurans*, as in *A. aeolicus*, CCA is added by homologous CC- and A-adding enzymes. We also find that the euubacterial CCA-, CC-, and A-adding enzymes, as well as the related euubacterial poly(A) polymerases, each fall into phylogenetically distinct groups derived from a common ancestor. Intriguingly, the *Thermatoga maritima* CCA-adding enzyme groups with the A-adding enzymes, suggesting that these distinct tRNA nucleotidyltransferase activities can intraconvert over evolutionary time.

The 3′-terminal CCA sequence (positions 74–76 in the standard cloverleaf representation) is universally present in the tRNAs of all organisms (1) and is important for many aspects of gene expression. The CCA terminus is required for the aminoacylation of tRNA (2, 3) and for translation on the ribosome where the CCA sequences of the aminoacyl- and peptidyl-tRNA pair with the large ribosomal RNA near the peptidyltransferase center (4–6). In euubacteria, the CCA sequence is required for the efficient maturation of the 5′-end of tRNA by RNase P (7, 8). In eukaryotes, the CCA sequence serves as an antinucleotidase block 3′-exonuclease activity (9), and it is essential for the export of mature tRNA from the nucleus to the cytoplasm (10, 11).

The CCA-adding enzyme (ATP(CTP):tRNA nucleotidyltransferase) builds and repairs the 3′-terminal CCA sequence of all tRNAs (12). CCA-adding activity has been identified in all three kingdoms, suggesting conservation of function and perhaps structure throughout evolution (13). CCA-adding activity is essential in some eubacteria as well as in all archaea and eukaryotes where some or all tRNA genes do not encode CCA (14). Yet, even in organisms such as *Escherichia coli* where all tRNA genes do encode CCA, CCA-adding activity confers a substantial selective advantage, probably by repairing tRNAs that have been subject to errant nucleolytic attack (15).

The CCA-adding enzyme belongs to the nucleotidyltransferase (NTR) family, a large protein superfamily that encompasses template-dependent DNA polymerases (DNA polymerase β) and template-independent RNA and DNA polymerases (poly(A) polymerase, terminal deoxynucleotidyltransferase, and CCA-adding enzymes) as well as metabolic regulators (GlnB uridylyltransferase, glutamine synthase adenyllyltransferase) and antibiotic resistance factors (kanamycin nucleotidyltransferase and streptomycin adenyllyltransferase) (13, 16, 17). The CCA-adding enzyme is unique among these NTRs because, unlike other the other DNA and RNA polymerases in the superfamily, it does not use a nucleic acid template, yet it faithfully constructs a defined nucleotide sequence by the addition of mononucleotides. Several models have been proposed to explain how the CCA addition could be templated by protein alone or by a tRNA/protein complex (18–23), but the detailed mechanism of the CCA addition remains unknown.

Until recently, all CCA-adding enzymes characterized from all three kingdoms were composed of a single kind of polypeptide with dual specificity for the 3′-terminal addition of CTP and ATP to tRNA primers. However, we found that CCA-adding activity in *Aquifex aeolicus* reflects a collaboration between two closely related nucleotidyltransferases (24). The smaller polypeptide (Aa.S) is a CC-adding enzyme that adds CTP at positions 74 and 75 of a tRNA primer. The larger polypeptide (Aa.L) is an A-adding enzyme that adds a single ATP at position 76 of a tRNA primer. Remarkably, the *A. aeolicus* Aa.S and Aa.L polypeptides do not appear to bind to each other or to work in concert. We also found that *Thermatoga maritima* CCA-adding enzyme encodes a polypeptide that is homologous to AA.1 over its entire length, yet it has CCA- instead of A-adding activity; moreover, the homologous N-terminal halves of the *T. maritima* CCA-adding enzyme and the AA.1 A-adding enzyme are dispensable for NTR activity.

Here we show that the addition of CCA to tRNA is the joint responsibility of homologous CC- and A-adding enzymes in two other euubacteria, *Synechocystis* sp. and *Deinococcus radiodurans*. 

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‡ Present address: Dept. of Integrated Bioscience, Graduate School of Frontier Sciences, University of Tokyo, Bldg. FSB-401, 5-1-5, Kashiwa-noha, Chiba, Chiba Prefecture 277-8562, Japan.

§ To whom correspondence should be addressed. Tel.: 206-543-1768; Fax: 206-685-9231; E-mail: amweiner@u.washington.edu.

1 The abbreviations used are: NTR, nucleotidyltransferase; Aa.S, shorter *Aquifex aeolicus* CC-adding polypeptide; Aa.L, longer *A. aeolicus* A-adding polypeptide; Sy.S, shorter *Synechocystis* sp. CC-adding polypeptide; Sy.L, longer *Synechocystis* sp. A-adding polypeptide; Tm, *Thermatoga maritima* CCA-adding enzyme; DR.1, *Deinococcus radiodurans* CC-adding polypeptide; DR.2, *D. radiodurans* A-adding polypeptide.
durans, just as it is in A. aeolicus. A phylogenetic analysis of eubacterial CCA-, CC-, and A-adding enzymes, as well as of poly(A) polymerases, places the CC- and A-adding enzymes in distinct groups from CCA-adding enzymes and poly(A) polymerases. We discuss several different evolutionary scenarios that could explain these data.

**MATERIALS AND METHODS**

**Cloning of Nucleotidyltransferase Homologs from Synechocystis sp. and Deinococcus radiodurans**—As shown in Fig. 1A, Synechocystis sp. encodes two nucleotidyltransferase homologs, designated Sy.S and Sy.L for short and long polypeptides, with accession numbers NP_294915 and NP_444147 respectively (25). The coding regions were amplified from Synechocystis sp. PCC 6803 genomic DNA (generous gift from S. Wolin of Yale University) using PCR primer pairs Sy.SCF (5′-GCCCTGCAAGATGTTTCTGAGCACCAGCTG-3′) and Sy.SBR (5′-CCCCCTCGAGAATAGCTTTTGGTAACCGGATA-3′) for Sy.S and Sy.LCF (5′-GGGCTATGGGCGAGGCTTGTTAGCGGATTGTG-3′) and Sy.LCR (5′-CCCCCTCGAGAATAGCTTTTGGTAACCGGATA-3′) for the C-terminal half of Sy.L (residues 399–942). To allow cloning of the coding region between the NdelI and XhoI sites of the pET22b (+) expression vector (Novagen), the Sy.SF and Sy.LCF primers contained an NdelI site (underlined) overlapping the start codon, and the Sy.SR and Sy.LCR primers contained a XhoI site (underlined and italicized) overlapping the natural stop codon, which was changed to a serine TCG codon. The resulting plasmids expressing Sy.S and Sy.LC were pETSy.S and pETSy.LC, respectively. Note that Sy.LC contains about 50% larger N-terminal residues than the region of Sy.S corresponding with Aa.LC and Tm.C (Fig. 1 and Ref. 24); without these N-terminal residues, Sy.LC was insoluble whether it had an N-terminal or a C-terminal hexahistidine tag.

As shown in Fig. 1A, Deinococcus radiodurans encodes two NTR homologs, designated DR.1 and DR.2, with accession numbers NP_294707 and NP_294915 (26). The coding regions were amplified from genomic DNA (generous gift from K. Kaneko of the Kazusa DNA Research Institute) using PCR primer pairs DR.1F (5′-ACGAGTGCTCGCGCCAGGCAGGTCTGG-3′) and DR.1R (5′-CCCCCTCGAGAAATGCTTTTGGTAACCGGATA-3′) for Sy.S and Sy.LCF (5′-GGGCTATGGGCGAGGCTTGTTAGCGGATTGTG-3′) and Sy.LCR (5′-CCCCCTCGAGAATAGCTTTTGGTAACCGGATA-3′) for the C-terminal half of Sy.L (residues 399–942). To allow cloning of the coding region between the NdelI and XhoI sites of the pET22b (+) expression vector (Novagen), the Sy.SF and Sy.LCF primers contained an NdelI site (underlined) overlapping the start codon, and the Sy.SR and Sy.LCR primers contained a XhoI site (underlined and italicized) overlapping the natural stop codon, which was changed to a serine TCG codon. The resulting plasmids expressing Sy.S and Sy.LC were pETSy.S and pETSy.LC, respectively. Note that Sy.LC contains about 50% larger N-terminal residues than the region of Sy.S corresponding with Aa.LC and Tm.C (Fig. 1 and Ref. 24); without these N-terminal residues, Sy.LC was insoluble whether it had an N-terminal or a C-terminal hexahistidine tag.

**Expression and Purification of Recombinant Proteins**—The expression vectors were transformed into E. coli strain BL21(DE3) carrying a plasmid encoding the minor tRNA

SDS-PAGE. A. aeolicus A-adding and CC-adding enzymes were prepared as described (24).

**tRNA Substrates Lacking CCA, CA, and A—**tRNA transcripts lacking CA and A (tRNA-DC and tRNA-DCC, where D is a discriminator nucleotide at position 73) were prepared by in vitro transcription of the pmBSCCA plasmid (a kind gift of N. R. Pace) (8) linearized with FokI and Bsil, respectively, as described (27). For tRNA lacking CCA (tRNA-D), the plasmid was mutated using the QuikChange mutagenesis kit (Stratagene) as described (24) and digested with FokI prior to in vitro transcription. Transcripts were purified by denaturing polyacrylamide gel electrophoresis with one nucleotide resolution, excised after UV shadowing, and then eluted, ethanol precipitated, with cold 70% ethanol, and dried. Uniformly labeled tRNAs were prepared identically to [32P]UTP (Amersham Pharmacia, 3000 Ci/mmol), 25 μM UTP, and 500 μM CTP, ATP, and GTP. The desired products were located by autoradiography and then excised, eluted, and concentrated as for unlabeled products.

**CCA-adding Assay in Vitro**—CCA-adding assays were carried out in 50 mM glycine-NaOH (pH 8.5), 10 mM MgCl2, 25 mM KCl, 2 mM dithiothreitol, 2 μM tRNA, 15 mM enzyme, 1 mM ATP, 1 mM CTP, and 150 μM [α-32P]UTP or [α-32P]CTP (300 Ci/mmol). After incubation for 20 min at 37 °C, reactions were stopped by adding an equal volume of stop solution (9 M urea, 0.02% xylene cyanol, and 0.02% bromphenol blue) and resolved by 12% polyacrylamide gel electrophoresis in the presence of 7 M urea. The A. aeolicus CCA-adding enzyme (Aa.S) and A-adding enzyme (Aa.LC) were assayed identically, but at 60 °C.

**Phylogenetic Analysis**—The following eubacterial CCA-adding, A-adding, and CC-adding enzymes and poly(A) polymerase were used for the phylogenetic analysis: the eubacteria CCA-adding enzymes of E. coli (28), Haemophilus influenzae (29), Bacillus subtilis (30), Mycobacterium leprae (31), Mycobacterium tuberculosis (32), and T. maritima (33); the A-adding enzymes from A. aeolicus (34), Synechocystis sp. (25), and D. radiodurans (26); the CC-adding enzymes from A. aeolicus (34), Synechocystis sp. (25), and D. radiodurans (26); and the poly(A) polymerases from E. coli (28) and H. influenza (29). The protein sequences were aligned by Clustal W (35), the alignment of the conserved 25 kDa core of the class II nucleotidyltransferase family (15) was edited manually, and insertions were omitted. Phylogenetic trees were constructed by the neighbor-joining method (36) based on a distance matrix with 1000 bootstrap trials.

**RESULTS AND DISCUSSION**

**Two NTR Homologs in Synechocystis sp. and D. radiodurans**—We recently found that the addition of CCA to tRNA in A. aeolicus reflects a collaborative effort between two different but closely related polypeptides, one that adds CC at positions 74 and 75 and another that adds A at position 76 (24). A BLAST search (37) using the A. aeolicus CC-adding and A-adding enzyme sequences revealed that Synechocystis sp. (25) encodes two NTRs designated Sy.S and Sy.L (accession numbers NP_442458 and NP_444147 respectively). Sy.S is homologous to the A. aeolicus CC-adding enzyme (Aa.S), and Sy.L is homologous to the A. aeolicus A-adding enzyme (Aa.L). Sy.L also possesses the same N-terminal 44-kDa extension of unknown function seen in the A. aeolicus A-adding enzyme (Aa.L) and T. maritima CCA-adding enzyme (Tm.C) (Fig. 1).

A BLAST search using the A. aeolicus CC-adding polypeptide (Aa.S) revealed that the genome of D. radiodurans (26) encodes an Aa.S homolog designated DR.1 (accession number NP_294915). Amino acid identity between DR.1 and Aa.S is high over the entire length of the proteins. A BLAST search for homologs of the A. aeolicus A-adding enzyme (Aa.L) did not reveal any full-length homologs in D. radiodurans, but rather a smaller homolog, designated DR.2, corresponding to the C-terminal half of Aa.L (Aa.LC) (accession number NP_294707). Thus, three eubacteria (Synechocystis sp., A. aeolicus, and T. maritima) have NTR homologs with homologous N-terminal extensions of unknown function (Sy.L, Aa.L, and Tm), but D. radiodurans does not.

These data indicate that the Synechocystis Sy.S and Deinococcus DR.1 polypeptides are most closely related to the A. aeolicus CC-adding enzyme, and the Synechocystis Sy.L and Deinococcus DR.2 polypeptides most closely related to the A. aeolicus
A-adding enzyme. The implication is that CCA-adding activity in Synechocystis sp. and Deinococcus radiodurans, as in A. aeolicus, may be the joint responsibility of two distinct but related polypeptides, although the N-terminal extension found in the Aa.L and Tm homologs is absent from DR.2.

CCA-adding Activity Can Be Reconstituted by the Synechocystis Sy.S CC-adding (Sy.S) and A-adding (Sy.LC) Polypeptides—Synechocystis Sy.S and the C-terminal half of Sy.L (Sy.LC) amino acid residues 399–942, where Thr-399 is changed to Met) were expressed in E. coli as hexahistidine-tagged proteins (Fig. 1A). The recombinant polypeptides were assayed in the presence of both ATP and CTP using tRNA substrates lacking CCA, CA, or A (tRNA-D, tRNA-DC, and tRNA-DCC, respectively, where D is a discriminator base at position 73). Sy.S adds one or more CMPs to tRNA-D and tRNA-DC, but not to tRNA-DCC, and does not add AMP to any tRNA substrate (Fig. 1B, lanes 1–3). DR.2 adds AMP to tRNA-DCC, but not to tRNA-D or tRNA-DC, and does not add CMP to any tRNA substrate (Fig. 3B, lanes 1–3). DR.2 adds AMP to tRNA-DCC, but not to tRNA-D or tRNA-DC, and does not add CMP to any tRNA substrate (Fig. 3B, lanes 4–6). Thus DR.1 and DR.2 are likely to be CC-adding and A-adding enzymes, respectively. Moreover, as expected, CCA adding activity could be reconstituted when DR.1 and DR.2 were combined (Fig. 3B, lanes 7–9). The D. radiodurans DR.2 polypeptide lacks the N-terminal extension of unknown function found in the homologous Aa.L, Sy.L, and Tm polypeptides (Fig. 1). When the assays were performed using uniformly labeled tRNA substrates in the presence of all of four nucleotides, DR.1 added one nucleotide to tRNA-D and two nucleotides to tRNA-DCC, DR.2 added only one nucleotide to tRNA-DCC, and together DR.1 and DR.2 added three nucleotides to tRNA-D (Fig. 3C). DR.1 incorporates only CMP into tRNA lacking CCA and CA, whereas DR.2 incorporates only AMP into tRNA lacking 3’-terminal A (Fig. 3D). These are the same results obtained for the A. aeolicus Aa.S and Aa.LC polypeptides (24) and the Synechocystis Sy.S and Sy.LC polypeptides (Fig. 2, B–D). We conclude that the D. radiodurans CCA-adding activity is also divided between two closely related polypeptides of different activity and specificity as in A. aeolicus and Synechocystis sp.

A Phylogeny of Eubacterial CCA-, CC-, and A-Adding Enzymes and Poly(A) Polymerases—Having found that CCA-adding activity in Synechocystis sp. and D. radiodurans represents a collaboration between two distinct but related polypeptides (Figs. 2 and 3) just as in A. aeolicus (24), we wanted to understand the phylogenetic relationship between eubacterial CCA-, CC-, and A-adding enzymes, as well as the related eubacterial polyclA polymerases. All known CCA-adding enzymes belong to the nucleotidyltransferase superfamily, which can be divided into Class I and Class II enzymes (13). The archaeal CCA-adding enzymes (Class I) share a highly homologous 45-kDa core, and the eubacterial and eukaryotic CCA-adding enzymes (Class II) share a highly homologous 25-kDa core; however, Class I and Class II enzymes exhibit little obvious homology with each other outside of the immediate vicinity of the nucleotidyltransferase active site signature (17). The prokaryotic polyclA polymerases belong to Class II and share the 25-kDa core of the CCA-adding enzymes (13); the eukaryotic polyclA polymerases belong to Class I (15) and exhibit very modest homology with the archaeal CCA-adding enzymes over roughly 25 kDa of the 45-kDa core.

To avoid needless complication, we compared only Class II enzymes. Because it is not yet possible to distinguish Class II CCA-adding enzymes from polyclA polymerases based solely on sequence analysis (38), we included only those enzymes whose enzymatic activities had been experimentally demonstrated or could be confidently assumed. These are the H. influenzae CCA-adding enzyme and polyclA polymerase, both of which are almost identical to their experimentally characterized E. coli counterparts (21, 23, 39), the B. subtilis and M. leprae CCA-adding enzymes (38), and the CCA-adding enzyme from M. tuberculosis, which is almost identical to M. leprae enzyme (31). A multiple alignment of the 25-kDa core regions of these Class II enzymes, including the active site signature, is shown in Fig. 4.

As shown in Fig. 5, eubacterial nucleotidyltransferases are divided into four main groups (Groups 1–4) and two subgroups (Group 4a and 4b). The Gram-negative eubacterial E. coli and H. influenzae CCA-adding enzymes (EC-CCA and HI-CCA) form a distinct group (Group 2) from the corresponding polyclA polymerases and Gram-positive eubacterial CCA-adding enzymes (Group 4). The Gram-positive B. subtilis, M. leprae and

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2H.-D. Cho, C. Verlinde, and A. M. Weiner, manuscript in preparation.
FIG. 2. CCA-adding activity is divided between two different polypeptides in *Synechocystis* sp. *A*, the purified recombinant histidine-tagged *Synechocystis* Sy.S and Sy.LC polypeptides were resolved by 10% SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. Sy.LC contains C-terminal residues 399–942 of Sy.L. *B*, Sy.S adds CMPs to tRNA-D and tRNA-DC. Sy.LC adds AMP into tRNA-DCC. CCA-adding activity is reconstituted in the presence of both Sy.S and Sy.LC. C, Sy.S is specific for CMP incorporation, and Sy.LC is specific for AMP incorporation. Assays were performed in the presence of all four ribonucleotide triphosphates, one of which was labeled. D, as controls, *A. aeolicus* CC- and A-adding enzymes were assayed as described for panel C, except at 60°C. Asterisks indicate α[32P]labeled nucleotides.

**FIG. 3.** CCA-adding activity is divided between two different polypeptides in *Deinococcus* radiodurans. *A*, the purified recombinant histidine-tagged *Deinococcus* DR.1 and DR.2 polypeptides were resolved by 10% SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue. *B*, DR.1 adds CMP to tRNA-D and tRNA-DC. DR.2 adds AMP into tRNA-DCC. CCA-adding activity is reconstituted in the presence of both DR.1 and DR.2. *C*, DR.1 adds two nucleotides to tRNA-D and one nucleotide to tRNA-DCC. DR.2 adds one nucleotide to tRNA-DCC. The addition of ribonucleotide triphosphates (1 mM) to uniformly [32P]labeled tRNA (2 μM) was assayed as described for Fig. 2C. D, DR.1 is specific for CMP incorporation into tRNA-D and tRNA-DC. DR.2 is specific for AMP incorporation. Asterisks indicate α[32P]labeled nucleotides.

*M. tuberculosis* CCA-adding enzymes (Group 4b; BS-CCA, ML-CCA and MT-CCA) are more closely related to the Gram-negative poly(A) polymerases (Group 4a; EC-PA and HI-PA) than to the CCA-adding enzymes from Gram-negative *E. coli* and *H. influenzae* (Group 2). Among Group 4 enzymes, the *B. subtilis* CCA-adding enzyme is most closely related to Gram-negative poly(A) polymerases (Group 4a), consistent with a recent biochemical study showing that the *B. subtilis* nucleoti-
FIG. 4. Multiple alignment of the 25-kDa core region that includes the active site signature (DXD, underlined) from eubacterial NTRs. The CCA-adding enzymes are E. coli (EC-CCA, NP_417528), H. influenza (HI-CCA, NP_439748), B. subtilis (BS-CCA, NP_390126), M. leprae (ML-CCA, NP_302720), Mycobacterium tuberculosis (MT-CCA, NP_2184241) and T. maritima (TM-CCA, NP_2494195). The A-adding enzymes are A. aeolicus (AA-A, NP_213288), Synechocystis (SY-A, NP_441479), and D. radiodurans (DR-A, NP_294707). The CC-adding enzymes are A. aeolicus (AA-CC, NP_214480), Synechocystis (SY-CC, NP_442458), and D. radiodurans (DR-CC, NP_294915). The poly(A) polymerases are E. coli (EC-PA, NP_414685) and H. influenza (HI-PA, NP_438236). Residues conserved among all NTRs are shown as white letters on a black background; regions shaded in gray were excluded from the analysis shown in Fig. 5.
Fig. 5. Phylogeny of eubacterial CCA-, CC-, and A-adding enzymes and poly(A) polymerases. CC-adding enzymes and A-adding enzymes group separately from CCA-adding enzymes and poly(A) polymerases. This unrooted tree is based on the sequence alignment shown in Fig. 4, using the neighbor-joining method with 1000 bootstrap scores (out of 1000) are indicated. Abbreviations as in Fig. 4.

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