An inhibitory C-terminal region dictates the specificity of A-adding enzymes

Sandy Tretbar^a, Anne Neuenfeldt^{a,b}, Heike Betat^a, and Mario Mörl^{a,1}

^aInstitute for Biochemistry, University of Leipzig, Brüderstrasse 34, 04103 Leipzig, Germany; and ^bUnité Propre de Recherche 9002 de Centre National de la Recherche Scientifique, Institut de Biologie Moléculaire et Cellulaire, 15 rue René Descartes, 67084 Strasbourg Cedex, France

Edited by Dieter Söll, Yale University, New Haven, CT, and approved October 28, 2011 (received for review October 4, 2011)

For efficient aminoacylation, tRNAs carry the conserved 3'-terminal sequence C-C-A, which is synthesized by highly specific tRNA nucleotidyltransferases (CCA-adding enzymes). In several prokaryotes, this function is accomplished by separate enzymes for CC- and A-addition. As A-adding enzymes carry an N-terminal catalytic core identical to that of CCA-adding enzymes, it is unclear why their activity is restricted. Here, it is shown that C-terminal deletion variants of A-adding enzymes acquire full and precise CCA-incorporating activity. The deleted region seems to be responsible for tRNA primer selection, restricting the enzyme's specificity to tRNAs ending with CC. The data suggest that A-adding enzymes carry an intrinsic CCA-adding activity that can be reactivated by the introduction of deletions in the C-terminal domain. Furthermore, a unique subtype of CCA-adding enzymes could be identified that evolved out of A-adding enzymes, suggesting that mutations and deletions in nucleotidyltransferases can lead to altered and even more complex activities, as a simple A-incorporation is converted into sequence-specific addition of C and A residues. Such activity-modifying events may have had an important role in the evolution of tRNA nucleotidyltransferases.

The tRNA nucleotidyltransferases are specialized polymerases that add the sequence CCA to the 3'-end of tRNAs, corresponding to positions 74–76 (numbering according to ref. 1). These enzymes do not depend on a nucleic acid template, yet they synthesize the sequence at high accuracy. To achieve this fidelity, CCA-adding enzymes use two different strategies dividing them into two separate groups (2–4). In Archaea, the enzymes (categorized as class I) use a combination of amino acid residues in the nucleotide binding pocket and phosphate groups of the bound tRNA primer to form specific hydrogen bonds to the base moiety of the incoming NTP (5–9). Eukaryotic and bacterial enzymes (class II) form an amino acid-based template that specifically interacts with CTP and ATP, without any involvement of the tRNA primer backbone. To discriminate between the nucleotides, the templating amino acids are pushed into different rotameric orientations in the binding pocket, leading to mutually exclusive recognition of either CTP or ATP (10).

Whereas such CCA-adding enzymes are found in most organisms, several bacteria like *Aquifex aeolicus*, *Synechocystis species*, *Deinococcus radiodurans*, *Bacillus halodurans*, *Geobacter sulfurreducens*, and *Thermus thermophilus* carry two closely related class II enzymes with partial activities (11–15). Here, one enzyme adds two C residues to the tRNA (CC-adding enzyme), and the second nucleotidyltransferase incorporates the terminal A (A-adding enzyme). In the N-terminally located catalytic core, both types of enzymes share conserved motifs with CCA-adding enzymes (10). Motifs A and B are involved in metal ion binding and ribose recognition, respectively. Motif D carries the amino acid template consisting of three conserved residues (EDxxR), forming hydrogen bonds with CTP and ATP. The domain-connecting motif C as well as motif E, involved in interactions with the tRNA 3'-end, are also present. A typical feature has been identified only for CC-adding enzymes (13). These proteins have lost a flexible loop region—located between motifs A and B—that functions in CCA-adding enzymes as a lever to switch the specificity of the

amino acid template from CTP toward ATP (16, 17). Furthermore, a pair of basic and acidic residues located immediately upstream of the loop, is also deleted. This basic/acidic (B/A) element positions the tRNA C₇₄C₇₅-end for addition of the terminal A (6, 13). Hence, this deletion represents the molecular basis of why these enzymes incorporate only C residues but cannot add the complete CCA end. A-adding enzymes, on the other hand, carry the full set of catalytic core motifs as well as flexible loop and B/A motif (Fig. 1). Thus, it is not understood why these enzymes fail to synthesize a complete CCA end.

In contrast to the N terminus, the C terminus shows no sequence conservation, although it seems to be involved in RNA substrate selection (6, 18–20). Here, it is shown that this region contributes to the catalytic restriction of A-adding enzymes. C-terminal deletion variants as well as chimeras consisting of the N-terminal catalytic core of an A-adding enzyme and the C-terminal region of a CC-adding enzyme exhibit a highly accurate CCA-adding activity. Phylogenetic data indicate that similar conversions of A-adding enzymes into CCA-adding enzymes occurred in evolution, leading to a unique subclass IIa of CCA-adding enzymes that are more related to A-adding enzymes than to their conventional class II counterparts. It seems that nucleotidyltransferases have a surprising catalytic flexibility responsible for frequent interconversion of enzymatic activities.

Results

C-terminal Deletions and Replacements Convert A-Adding Enzymes into CCA-Adding Enzymes. To explore which elements lead to the restricted nucleotide incorporation of A-adding enzymes, CC- and A-adding enzymes of *D. radiodurans* (15) were investigated in vitro. As the N-terminal part of the A-adding enzyme carries the full set of catalytic core motifs for CCA-addition, the C terminus was replaced with the corresponding part of the CC-adding enzyme, resulting in chimera A/CC1 (Fig. 2A). This construct carries an N terminus of the A-adding enzyme ending with the last position of motif E (positions 1–213) and a C terminus of the CC-adding enzyme (residues 182–434). In a second chimera A/CC2, the fusion position was shifted for 74 residues downstream, leading to an extended N terminus (positions 1–287) and an accordingly reduced C terminus (positions 262–434). As no crystal structures for *Deinococcus* enzymes are available, the fusion positions were chosen based on boundaries of predicted α -helical elements, using the PSIPRED algorithm (21, 22).

Recombinant proteins were incubated in the presence of NTPs and a radioactively labeled tRNA transcript lacking the CCA terminus (tRNA₇₃). Reaction products were separated on a denaturing polyacrylamide gel and visualized by autoradiography (Fig. 2B). While exhibiting an A-adding activity comparable to

Author contributions: H.B. and M.M. designed research; S.T. and A.N. performed research; S.T., A.N., H.B., and M.M. analyzed data; and S.T. and M.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹To whom correspondence should be addressed. E-mail: moerl@uni-leipzig.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1116117108/-DCSupplemental.

Fig. 1. Alignment of catalytic core elements of experimentally verified class II CCA- and A-adding enzymes. Invariant positions are indicated in red. Carboxylates involved in metal ion binding are labeled by red dots. Both CCA- and A-adding enzymes carry the full set of conserved motifs A to E as well as the B/A element and the flexible loop. Hence, the composition of the catalytic core cannot be used to distinguish A- from CCA-adding enzymes, and the molecular basis for the restricted activity of A-adding enzymes must be located outside of these elements. *B.st.*, *Bacillus stearothermophilus*; *E.c.*, *Escherichia coli*; *H.s.*, *Homo sapiens*; *B.su.*, *Bacillus subtilis*; *B.hal.*, *Bacillus halodurans*; *A.ae.*, *Aquifex aeolicus*; *D.rad.*, *Deinococcus radiodurans*; *Syn.*, *Synechocystis species*; *T.th.*, *Thermus thermophilus*.

that of the parental enzyme (Fig. S1), both chimeras also catalyze the addition of two C residues. Other nucleotides were not accepted, indicating that the acquired activity is not the result of a general loss of specificity. Furthermore, in the presence of all four NTPs, the addition of three residues could be observed. To identify the nature of the incorporated nucleotides, reaction products of A/CC1 were isolated and sequenced, showing a highly accurate CCA-synthesis (Table S1). Hence, the replacement of the C-terminal part of the A-adding enzyme led to an additional CC-incorporating activity in the chimera, similar to the specific incorporation of CTP and ATP observed for bona fide CCA-adding enzymes.

To investigate whether the removal of the original C terminus or the presence of the corresponding part of a CC-adding enzyme is responsible for C-incorporation, a deletion variant *D. rad* A-Δ287, representing residues 1–287 of the A-adding enzyme, was constructed (Fig. 2A). Again, this truncated enzyme showed efficient incorporation of two C residues as well as accurate CCA-addition (Table S1), whereas UTP and GTP were not incorporated (Fig. 2B). These data indicate that the activity of the chimera is not an effect of the presence of a C-terminal region derived from a CC-adding enzyme. Instead, the N-terminal part of the A-adding enzyme, carrying the catalytic cleft and the tRNA 3'-end binding region, is able to synthesize a complete CCA end. A similar construct from the A-adding enzyme of *B. halodurans* (11) was generated, consisting of residues 1–302 (*B. hal* A-Δ302) (Fig. 2A). According to structure predictions, position 302 corresponds to the deletion site in the *D. radiodurans* A-adding enzyme. This variant also exhibited CCA-adding activity (Fig. 2B).

Fig. 2. Enzyme chimeras and deletion variants synthesize complete CCA-termini. (A) Schematic representation of *D. rad* A- and CC-adding enzymes (WT) and the corresponding constructs, including a deletion variant of the *B. hal* A-adding enzyme. A-adding enzymes are indicated in red, CC-adding enzymes in blue. Elements of the catalytic core are labeled in orange (A–E, conserved motifs; L, flexible loop element). In chimera A/CC1, the A-adding enzyme part ends at position 213, immediately downstream of motif E. The fused C terminus contains residues 182–434 of the CC-adding enzyme. In chimera A/CC2, the fusion position was shifted for 74 residues to the C terminus. Deletion variant *D. rad* A-Δ287 corresponds to the N-terminal part of chimera A/CC2. Deletion variant *B. hal* A-Δ302 represents the analogous N terminus of the *B. halodurans* A-adding enzyme. (B) Nucleotide incorporation activities of chimeras and deletion variants. In the presence of all four NTPs, each variant incorporated three nucleotides, representing complete CCA ends. When individual nucleotides were offered, only CTP residues were efficiently incorporated. *B. hal* A-Δ302, however, showed also some incorporation of individual U or A residues, respectively. Mock, incubation of tRNA substrate without enzyme; control, reaction catalyzed by *E. coli* CCA-adding enzyme.

(Fig. 3). Reaction products were separated on a denaturing polyacrylamide gel and the radioactive signals were visualized. The efficiency of the A-addition to tRNA- $C_{74}C_{75}$ by the WT A-adding enzyme was not affected, regardless how much competitor was added (Fig. 3A, *Left*). This observation is consistent with the enzyme's substrate requirement for tRNA with two terminal C residues. In contrast, A-addition catalyzed by *D. rad* A-Δ287 was strongly inhibited by increasing amounts of tRNA $_{73}$ (Fig. 3A, *Right*). In a quantitative analysis, intensities of radioactive signals were determined using a phosphor imager software (Image-Quant) (Fig. 3B). Reaction products without competitor were set to 100% and relative efficiencies of A-addition in the presence of increasing amounts of competitor were calculated. Whereas the A-Δ287 variant shows a strong reduction down to 30% at 20-fold excess of the competitor, the WT A-adding enzyme remains almost unaffected. Here, the highest competitor concentration led only to a slight reduction of about 80% of the reaction (because of the limited solubility of tRNA, it was not possible to use higher concentrations). The presented data (Fig. 3B) allow deducing apparent IC_{50} values to quantify the binding behavior of these enzymes. For the truncated enzyme, tRNA $_{73}$ has an IC_{50} value of about 1.25 μ M. For the full-length enzyme, an IC_{50} could only be extrapolated. This calculation results in an apparent IC_{50} of 21.34 μ M, indicating that the WT enzyme recognizes the competitor tRNA $_{73}$ with a 17-fold reduced affinity compared to the deletion variant. Thus, the truncated enzyme was capable of interacting with both tRNA- $C_{74}C_{75}$ as well as tRNA $_{73}$, whereas the full-length enzyme showed an efficient interaction only with tRNA- $C_{74}C_{75}$.

As only ATP was offered, one can exclude that C-incorporation occurred as a competing reaction in the case of the deletion variant, which would also lead to product reduction. Hence, the observed inhibition is not an effect on catalysis, but is solely based on tRNA recognition.

A Small C-terminal Extension Shifts the Activity from CCA- Back to A-Incorporation. Obviously, the C-terminal part that was deleted in *D. rad* A-Δ287 is responsible for the restricted A-incorporating activity of the WT enzyme. To identify elements involved in this restriction, the deletion site was shifted 33 residues downstream (Fig. 4A), as structure prediction programs indicated α -helical elements ending at this position. Hence, such deletion positions are likely not to interfere with correct protein folding, leading to the production of a soluble and active protein. Surprisingly, this new variant A-Δ320 showed the same activities as the full-length WT enzyme. On a radioactively labeled tRNA $_{73}$, no nucleotide incorporation was observed, whereas a CC-adding enzyme as a positive control added two C residues (Fig. 4B, *Top*).

Fig. 3. Competition studies with *D. rad* A-adding enzyme and *D. rad* A-Δ287 deletion variant. (A) Enzymes were incubated with radioactively labeled tRNA- $C_{74}C_{75}$ and increasing amounts of unlabeled tRNA $_{73}$ as competitor. Whereas the WT A-adding enzyme is not affected by the competitor and readily adds the terminal A (*Left*), the deletion variant shows an increasing inhibition, correlating with the amount of added competitor (*Right*). Competition effects are indicated by black and white arrows. (B) Graphical representation of competition effects, resulting from three independent experiments. Relative amounts of A-addition were plotted, starting with the reaction without competitor as 100%. The WT A-adding enzyme (grey) shows only a slight reduction in A-addition at high competitor concentrations. The activity of A-Δ287 (black), however, is severely affected, showing a final reduction to about 30%.

The unlabeled second tRNA substrate ending with $C_{74}C_{75}$, however, was accepted by A-Δ320 as well as the WT A-adding enzyme (Fig. 4B, *Bottom*). Radioactively labeled ATP was incorporated, leading to a detectable signal for a single nucleotide addition. Similar results were obtained with the *B. halodurans* A-adding enzyme. Whereas the *B. hal* A-Δ302 deletion construct incorporated complete CCA-termini (Fig. 2B), a C-terminal elongation (*B. hal* A-Δ363) showed restricted A-adding activity (Fig. S2). These results indicate that a small region in the C terminus of A-adding enzymes (33 residues in the case of *D. radiodurans*, 61 in *B. halodurans*) is responsible for the limited nucleotide incorporation.

Some A-Adding Enzymes have a Weak Intrinsic C-Adding Activity. The finding that the catalytic cores of two A-adding enzymes are able

Fig. 4. Deletion variants *D. rad* A-Δ287 and A-Δ320 have different activities. (A) Schematic representation of *D. rad* A-Δ287 and *D. rad* A-Δ320. Both enzymes carry the complete set of motifs of the N-terminal catalytic core (orange boxes). The C terminus of A-Δ320 is 33 residues longer than that of A-Δ287. (B) This extension has a dramatic effect on the catalytic activity of the enzyme. The A-Δ320 variant is no longer able to add C residues to tRNA $_{73}$ (*Top*, showing C-addition on a labeled tRNA primer), but readily incorporates the terminal A in tRNA- $C_{74}C_{75}$. (*Bottom*; here, labeled A is added to an unlabeled tRNA). This activity is indistinguishable from that of the WT A-adding enzyme. The CC-adding enzyme was used as a control for C-incorporation. The radioactive label is represented by a red asterisk.

to synthesize complete CCA ends led us to speculate that some of the WT A-adding enzymes might express a weak intrinsic CCA-adding activity as well. To test this possibility, several enzymes were evaluated in an assay with increased sensitivity, using both ^{32}P -labeled tRNA₇₃ as well as α - ^{32}P -CTP (in addition to unlabeled NTPs). Whereas the *B. halodurans* and *A. aeolicus* en-

zymes could not add C residues to the tRNA substrate, enzymes of *D. radiodurans* and *T. thermophilus* showed a weak but detectable C incorporation (Fig. 5A). This activity was further corroborated by sequence analysis of the reaction product of the *Deinococcus* enzyme, where the addition of C residues could be detected. The data suggest that A-adding enzymes are much closer relatives of bona fide CCA-adding enzymes than previously thought.

Such a close relation is supported by the observation that the tRNA nucleotidyltransferase of *Thermotoga maritima* is a true CCA-adding enzyme (Fig. 5A) even though it contains sequence motifs typical for A-adding enzymes (14, 17, 24). As these facts are not in agreement with the hitherto discussed evolutionary distribution of class II tRNA nucleotidyltransferases (3, 13, 15), a rooted phylogenetic tree was calculated, containing further putative CCA-adding enzymes of the phylum *Thermotogae*, in addition to experimentally verified bacterial tRNA nucleotidyltransferases (Fig. 5B). Although the tree supports the close evolutionary relation of CCA- and CC-adding enzymes, the *Thermotogae* CCA-adding enzymes are more closely related to A-adding enzymes than to conventional class II CCA-adding enzymes. Furthermore, a screen of 1,590 bacterial genomes revealed that an increasing number of other phyla like *Proteobacteria*, *Acidobacteria*, *Firmicutes*, and *Deferribacteres* also express CCA-adding enzymes that apparently originate from A-adding enzymes (Table S2). Hence, class II tRNA nucleotidyltransferases are evolutionary more heterogeneous than previously assumed and can be subdivided into an additional subclass IIa of CCA-adding enzymes (Fig. 5B).

Discussion

The fact that A-adding enzyme variants with C-terminal deletions or replacements catalyze CCA-addition suggests that the native C terminus has an inhibiting effect on the WT enzyme's range of nucleotide incorporation. As this region is not part of the catalytic cleft, the active site cannot be directly affected. Furthermore, the C-terminal region does not carry an RNA binding motif and shows no sequence conservation (2, 18), which is in agreement with a low substrate binding affinity, as no k_D values could be determined (23). Rather, the conservation of the C terminus seems to be a structural one, as this part forms body and tail domains of the seahorse-like enzyme shape (6, 10, 25, 26). Nevertheless, our competition study indicates that the C terminus is involved in discriminating among tRNA substrates based on the presence of positions C₇₄C₇₅. This idea is strongly supported by the cocrystal structure of the related A-adding enzyme of *A. aeolicus* with a tRNA-C₇₄C₇₅ substrate (6) (Fig. 6A, Left). The catalytic core in the neck domain directly interacts with the incoming 3'-terminal positions 73–75 of the tRNA primer. A₇₃ and C₇₄ are recognized by two amino acid residues of motif E, whereas R191 interacts with the phosphate group of C₇₄ and N194 binds to the ribose O2' position of A₇₃ (6). C₇₅ is recognized by R79, being part of the B/A motif (6, 13). In the C-terminal region, positions S281, S284, R287, and H321 are in close contact to the tRNA acceptor stem. However, as these positions are not conserved, this contact does not represent a specific tRNA interaction. This observation is supported by our analysis of a quadruple mutant, where these residues were replaced by alanine. This variant showed an activity identical to that of the WT A-adding enzyme, without any detectable C-addition. Thus, the contribution of the C terminus to the nucleotide addition is not mediated via specific binding of the tRNA acceptor stem. Instead, it seems to form a physical constraint that holds the tRNA acceptor stem rigidly in place and does not allow any movement of the tRNA (and the 3'-end in the catalytic core) relative to the enzyme (Fig. 6B, Left).

When a deletion equivalent to *D. rad* A- Δ 287 and *B. hal* A- Δ 302 (showing complete CCA-addition) is superimposed on the structure of the *A. aeolicus* A-adding enzyme, it becomes apparent that these C-terminal barrier elements are no longer present,

Fig. 5. Intrinsic C-incorporation by WT A-adding enzymes and phylogenetic tree of class II tRNA nucleotidyltransferases. (A) Nucleotide incorporation of A-adding enzymes in tRNA₇₃ in the presence of all four NTPs. Whereas A-adding enzymes of *B. halodurans* and *A. aeolicus* do not add any nucleotide to the tRNA, the enzymes of *D. radiodurans* and *T. thermophilus* incorporate three residues, leading to a weak but detectable reaction product. Sequence analysis of the product labeled with an asterisk showed that the *D. rad* A-adding enzyme indeed incorporated C residues into the tRNA primer, demonstrating that some A-adding enzymes have a weak intrinsic C-adding activity. (B) Phylogenetic tree of class II CCA-, CC-, and A-adding enzymes. The CCA-adding enzyme of *H. sapiens* was used as an outgroup. Green boxes represent CCA-adding enzymes, blue boxes CC-adding enzymes, and the red box indicates A-adding enzymes. CC-adding enzymes descend from CCA-adding enzymes and group together in an individual branch. A-adding enzymes form an independent branch, indicating an early separation from CC- and CCA-adding enzymes. As only a single tRNA nucleotidyltransferase is encoded in *Thermotogae* genomes, it is highly likely that these proteins represent CCA-adding enzymes, as it was shown for *T. maritima*. The *Thermotogae* enzymes are not grouping together with other CCA-adding enzymes, but form a separate branch that evolved out of A-adding enzymes, leading to a subclass IIa of CCA-adding enzymes. This observation is supported by the fact that some A-adding enzymes have an intrinsic C-incorporating activity and might rather easily evolve into CCA-adding enzymes (question mark). With the exception of the *Thermotogae* branch, all proteins represent experimentally verified enzymes. FN, *Fervidobacterium nodosum*; TA, *Thermosiphon africanus*; TME, *Thermosiphon melanesiensis*; TM, *Thermotoga maritima*; TL, *Thermotoga lettingiae*; KO, *Kosmotoga olearia*; AAE, *Aquifex aeolicus*; SSPEC, *Synechocystis spec*; BH, *Bacillus halodurans*; DR, *Deinococcus radiodurans*; TTH, *Thermus thermophilus*; WIGGL, *Wigglesworthia glossinidia*; EC, *Escherichia coli*; BST, *Bacillus stearothermophilus*; BSU, *Bacillus subtilis*; HS, *Homo sapiens*.

Fig. 6. A possible function for the C-terminal domain as a guide rail for the tRNA substrate. (A) Contact sites between the *A. aeolicus* A-adding enzyme and an RNA primer, based on the cocrystal structure of the enzyme in complex with a tRNA acceptor stem ending with CC (6). (Left) The single stranded 3'-end of tRNA-C₇₄C₇₅ (green) is in close contact with conserved elements (green) of the catalytic cleft (orange). The C-terminal domain (blue) forms a barrier that holds the tRNA rigidly in place, constraining the primer orientation for A-addition only. (Right) In a C-terminally truncated enzyme corresponding to *D. rad* Δ287 or *B. hal* A-Δ302, the barrier element is removed, and the physical constraints for the tRNA orientation are relaxed. (B) Hypothetical mechanism leading to different substrate specificities in WT and deletion forms of A-adding enzymes. (Upper Left) A tRNA (gray) ending with C₇₄C₇₅ can enter the catalytic cleft of the WT A-adding enzyme, where the 3'-end is bound (green). The barrier element (blue) forces the tRNA into an orientation where only A-addition is possible. (Lower Left) If a tRNA lacking C₇₄C₇₅ approaches the enzyme, the C terminus inhibits an alternative positioning of the tRNA, interfering with CCA-addition. (Upper and Lower Right) In a C-terminally truncated enzyme, both tRNA-C₇₄C₇₅ as well as tRNA₇₃ (lacking C₇₄C₇₅) can adopt alternative orientations. In this wiggling, the tRNA₇₃ primer can adopt a position that allows CCA-addition.

giving the tRNA more room to adopt alternative orientations (Fig. 6A, Right). In the elongated variants *D. rad* A-Δ320 and *B. hal* A-Δ363, this region (and the physical constraint) is restored (Fig. S24). Consequently, these enzymes retained the A-adding activity of the full-length enzyme and lost the ability to incorporate C₇₄ and C₇₅. As the competition experiments indicate that tRNA₇₃ is not recognized by the full-length enzymes, it seems that the 3'-end without C₇₄C₇₅ is too short to reach the catalytic core when the C-terminal barrier is present and prevents the tRNA from proper accommodation. In the truncated forms, however, tRNA₇₃ gains some flexibility to advance with its 3'-end into the catalytic core. This wiggle room seems to allow the tRNA primer to adopt a different geometry compatible with CCA-addition (Fig. 6B, Right). Furthermore, cocrystal structures show that CCA- and A-adding enzymes share an identical amino acid template and interact with incoming nucleotides in identical ways (6,

10, 26). Hence, the active site of A-adding enzymes is in principle fully equipped for CTP recognition.

The wiggling of the tRNA primer might also account for the observed misincorporation of U residues by the *B. hal* A-Δ302 version. Obviously, the changed geometry of the bound acceptor stem in the catalytic core reduces the fidelity of nucleotide addition. In the presence of all four nucleotides, misincorporation is abolished, as the correctly added positions lead to a proper orientation of the primer 3'-end. This scenario applies also to a backup mechanism described for class II nucleotidyltransferases, as enzyme variants with a mutated nucleotide binding pocket still show accurate addition of CCA ends (27).

The decreased catalytic efficiency of some of the deletion variants may be a further consequence of the absence of the C-terminal barrier, as the wiggling of the tRNA probably also leads to temporary primer orientations incompatible with nucleotide addition. Moreover, we do not expect an enzyme variant missing 23% of its sequence to exhibit a reaction rate comparable to that of the WT enzyme. Rather, and more important, the deletion of the C-terminal part leads to a loss of specificity in terms of tRNA substrates, but to a surprising increase in the complexity of the reaction, allowing the incorporation of two different nucleotides in a sequence-specific order.

However, as some of the WT A-adding enzymes show a weak intrinsic CCA-adding activity, it seems that the C-terminal region does not have an absolute discriminative power to reject tRNA substrates without C₇₄C₇₅. As this enzyme region is not conserved at the sequence level (2, 18), it is conceivable that the C-termini of individual A-adding enzymes differ in the efficiency of the constraint.

Furthermore, one can speculate whether it is possible that in these A-adding enzymes, the unoccupied binding pocket for the C₇₄C₇₅ positions of the primer 3'-end can also function as an alternative templating region for nucleotide addition, if the protein interacts with a tRNA₇₃ substrate. The primer CC-binding site would interact with CTP and dictate a CC-addition on such a tRNA substrate, before the regular NTP binding site would mediate the conventional terminal A-addition. To clarify such a possible contribution of the primer binding site and the question whether this element has a fixed orientation—as one would expect for a tRNA-C₇₄C₇₅ substrate determinant—or is reorganized after each nucleotide addition, further cocrystal structures of A-adding enzymes with different tRNA substrates are required.

With respect to evolution, the C-terminal part of class II nucleotidyltransferases is a highly fascinating but poorly understood element. Besides affecting the catalytic activity of A-adding enzymes, it is also an activity determinant in CCA-adding enzymes and poly(A) polymerases. It was shown that the C-terminal part of the *Escherichia coli* CCA-adding enzyme reprograms the N-terminal catalytic core of a poly(A) polymerase, leading to the synthesis of CCA ends (19). Furthermore, a reciprocal chimera consisting of the catalytic core of the CCA-adding enzyme and the C terminus of the poly(A) polymerase added poly(CCA) tails to tRNAs. Hence, it seems that class II nucleotidyltransferases use the C-terminal domain to modulate substrate interaction and polymerization reaction in terms of specificity and processivity. This idea is in agreement with the fact that the subclass IIa of CCA-adding enzymes is more closely related to A-adding enzymes than to the canonical class II CCA-adding enzymes (Fig. 5). These proteins carry active site motifs and a flexible loop identical to that of A-adding enzymes (6, 24). The sequence of the C-terminal region, however, is very different. It is likely that replacements similar to the experimental ones led to the appearance of this additional group of CCA-adding enzymes.

In conclusion, it seems that the low conservation of this enzyme region reflects an evolutionary strategy to change the specific activity by an increasing diversification of RNA recognition properties. Additional structural data are required to shed more

light into this fascinating evolutionary mode to generate different polymerization specificities.

Materials and Methods

Construction of Recombinant Clones. Nucleotidyltransferase genes of *D. radiodurans*, *B. halodurans*, *T. Thermophilus*, *A. aeolicus* (short version), and *T. maritima* (short version) were amplified and cloned into pET-30 Ek/LIC (Novagen) or pTrcHis vectors (Invitrogen) (13, 14, 26). Enzyme chimeras were constructed according to Betat et al. (19). Point mutations and stop codons (TAA; for enzyme deletion variants) were introduced into the coding sequence of the wild-type gene using the QuickChange Site-Directed Mutagenesis Kit (Stratagene).

Protein Expression and Purification. Enzymes were overexpressed with an N-terminal His-tag in *E. coli* JM109 (DE3) cca:cam or *E. coli* CA244 cca:cam, lacking an endogenous CCA-adding enzyme. Transformed cells were grown in 200–500 mL LB at 37 °C with 34 µg/mL chloramphenicol and either 30 µg/mL kanamycin or 100 µg/mL ampicillin. Expression was induced at OD₆₀₀ of 0.6 by the addition of IPTG to a final concentration of 1 mM for 3–5 h at 37 °C or overnight at room temperature. Purification of the recombinant enzymes was done according to Lizano et al. (28).

Preparation of tRNA Substrates. Yeast tRNA^{Phe} lacking the CCA terminus (tRNA₇₃) or ending with a partial CCA end (tRNA-C₇₄C₇₅) were prepared

as described in refs. 29 and 30. For internally labeled tRNA, transcription was carried out in the presence of α -³²P-ATP.

Enzyme Activity Assays. Standard activity assays were carried out according to Neuenfeldt et al. (13).

For the competition study, nonsaturating incubation times and enzyme concentrations were determined (30 mM Hepes/KOH pH 7.6, 6 mM MgCl₂, 30 mM KCl, and 200 µM DTT for 15–120 min with 10–200 ng enzyme). Incorporation of ATP was monitored using 5 pmol radioactively labeled tRNA-C₇₄C₇₅ in the presence of ATP (1 mM) under increasing amounts of competitor tRNA (5–100 pmol).

Sequence Analysis of Reaction Products. The 3'-ends of isolated reaction products were analyzed as described in ref. 19.

Computational and Phylogenetic Analysis. BLAST analyses were carried out using NCBI genomic BLAST (31). Protein sequences starting with motif A were aligned using Clustal X (32). Phylogenetic and evolutionary analyses were conducted using the PHYLIP 3.69 package (33) and MEGA 4.0 (34).

ACKNOWLEDGMENTS. We thank S. Thiele and J. Kofent for assistance in the construction of enzyme variants. Thanks to H. C. Smith, H. Dickinson, M. Grunow, S. Vörtler, and R. K. Hartmann for helpful discussions as well as J. Kelso and K. Prüfer for the support with phylogenetic analysis. This work was supported by Deutsche Forschungsgemeinschaft Grant Mo 634/2-4.

1. Sprinzl M, Horn C, Brown M, Ioudovitch A, Steinberg S (1998) Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res* 26:148–153.
2. Yue D, Maizels N, Weiner AM (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.
3. Betat H, Rammelt C, Mörl M (2010) tRNA nucleotidyltransferases: Ancient catalysts with an unusual mechanism of polymerization. *Cell Mol Life Sci* 67:1447–1463.
4. Vörtler S, Mörl M (2010) tRNA-nucleotidyltransferases: Highly unusual RNA polymerases with vital functions. *FEBS Lett* 584:297–302.
5. Xiong Y, Li F, Wang J, Weiner AM, Steitz TA (2003) Crystal structures of an Archaeal class I CCA-adding enzyme and its nucleotide complexes. *Mol Cell Biochem* 251:1165–1172.
6. Tomita K, et al. (2004) Structural basis for template-independent RNA polymerization. *Nature* 430:700–704.
7. Xiong Y, Steitz TA (2004) Mechanism of transfer RNA maturation by CCA-adding enzyme without using an oligonucleotide template. *Nature* 430:640–645.
8. Toh Y, et al. (2008) Molecular basis for maintenance of fidelity during the CCA-adding reaction by a CCA-adding enzyme. *EMBO J* 27:1944–1952.
9. Pan B, Xiong Y, Steitz TA (2010) How the CCA-adding enzyme selects adenine over cytosine at position 76 of tRNA. *Science* 330:937–940.
10. Li F, et al. (2002) Crystal structures of the *Bacillus stearothermophilus* CCA-adding enzyme and its complexes with ATP or CTP. *Cell* 111:815–824.
11. Bralley P, Chang SA, Jones GH (2005) A phylogeny of bacterial RNA nucleotidyltransferases: *Bacillus halodurans* contains two tRNA nucleotidyltransferases. *J Bacteriol* 187:5927–5936.
12. Bralley P, Cozad M, Jones GH (2009) *Geobacter sulfurreducens* contains separate C- and A-adding tRNA nucleotidyltransferases and a poly(A) polymerase. *J Bacteriol* 191:109–114.
13. Neuenfeldt A, Just A, Betat H, Mörl M (2008) Evolution of tRNA nucleotidyltransferases: A small deletion generated CC-adding enzymes. *Proc Natl Acad Sci USA* 105:7953–7958.
14. Tomita K, Weiner AM (2001) Collaboration between CC- and A-adding enzymes to build and repair the 3'-terminal CCA of tRNA in *Aquifex aeolicus*. *Science* 294:1334–1336.
15. Tomita K, Weiner AM (2002) Closely related CC- and A-adding enzymes collaborate to construct and repair the 3'-terminal CCA of tRNA in *Synechocystis species* and *Deinococcus radiodurans*. *J Biol Chem* 277:48192–48198.
16. Just A, et al. (2008) A comparative analysis of two conserved motifs in bacterial poly(A) polymerase and CCA-adding enzyme. *Nucleic Acids Res* 36:5212–5220.
17. Hoffmeier A, et al. (2010) Unusual evolution of a catalytic core element in CCA-adding enzymes. *Nucleic Acids Res* 38:4436–4447.
18. Martin G, Keller W (2007) RNA-specific ribonucleotidyl transferases. *RNA* 13:1834–1849.
19. Betat H, Rammelt C, Martin G, Mörl M (2004) Exchange of regions between bacterial poly(A) polymerase and CCA adding enzyme generates altered specificities. *Molecular cell* 15:389–398.
20. Raynal LC, Carpousis AJ (1999) Poly(A) polymerase I of *Escherichia coli*: Characterization of the catalytic domain, an RNA binding site and regions for the interaction with proteins involved in mRNA degradation. *Mol Microbiol* 32:765–775.
21. Bryson K, et al. (2005) Protein structure prediction servers at University College London. *Nucleic Acids Res* 33:W36–38.
22. Jones DT (1999) Protein secondary structure prediction based on position-specific scoring matrices. *J Mol Biol* 292:195–202.
23. Shi PY, Maizels N, Weiner AM (1998) CCA addition by tRNA nucleotidyltransferase: Polymerization without translocation? *EMBO J* 17:3197–3206.
24. Martin G, Keller W (2004) Sequence motifs that distinguish ATP(CTP) : tRNA nucleotidyl transferases from eubacterial poly(A) polymerases. *RNA* 10:899–906.
25. Augustin MA, et al. (2003) Crystal structure of the human CCA-adding enzyme: Insights into template-independent polymerization. *J Mol Biol* 328:985–994.
26. Toh Y, et al. (2009) Mechanism for the definition of elongation and termination by the class II CCA-adding enzyme. *EMBO J* 28:3353–3365.
27. Lizano E, Scheibe M, Rammelt C, Betat H, Mörl M (2008) A comparative analysis of CCA-adding enzymes from human and *E. coli*: Differences in CCA addition and tRNA 3'-end repair. *Biochimie* 90:762–772.
28. Lizano E, Schuster J, Müller M, Kelso J, Mörl M (2007) A splice variant of the human CCA-adding enzyme with modified activity. *J Mol Biol* 366:1258–1265.
29. Mörl M, Lizano E, Willkomm DK, Hartmann RK (2005) Production of RNAs with homogeneous 5' and 3' ends. *Handbook of RNA Biochemistry*, eds RK Hartmann, A Bindereif, A Schön, and E Westhof (Wiley-VCH, Weinheim, Germany), Vol 1, pp 22–35.
30. Schürer H, Lang K, Schuster J, Mörl M (2002) A universal method to produce in vitro transcripts with homogeneous 3' ends. *Nucleic Acids Res* 30:e56.
31. Cummings L, et al. (2002) Genomic BLAST: Custom-defined virtual databases for complete and unfinished genomes. *FEMS Microbiol Lett* 216:133–138.
32. Larkin MA, et al. (2007) Clustal W and Clustal X, Version 2. *Bioinformatics*, pp:2947–2948.
33. Felsenstein J (2005) PHYLIP (Phylogeny Inference Package), Version 3.6. (Department of Genome Sciences, University of Washington, Seattle), Distributed by the author.
34. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24:1596–1599.