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Identity elements for the aminoacylation of metazoan mitochondrial tRNA^{Arg} have been widely conserved throughout evolution and ensure the fidelity of the AGR codon reassignment

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Eumetazoan mitochondrial tRNAs possess structures (identity elements) that require the specific recognition by their cognate nuclear-encoded aminoacyl-tRNA synthetases. The AGA (arginine) codon of the standard genetic code has been reassigned to serine/glycine/termination in eumetazoan organelles and is translated in some organisms by a mitochondrially encoded tRNA$^{Ser}_{UCU}$. One mechanism to prevent mistranslation of the AGA codon as arginine would require a set of tRNA identity elements distinct from those possessed by the cytoplasmic tRNAArg in which the major identity elements permit the arginylation of all 5 encoded isoacceptors. We have performed comparative in vitro aminoacylation using an insect mitochondrial arginyl-tRNA synthetase and tRNAArgUCG structural variants. The established identity elements are sufficient to maintain the fidelity of tRNASerUCU reassignment. tRNAs having a UCU anticodon cannot be arginylated but can be converted to arginine acceptance by identity element transplantation. We have examined the evolutionary distribution and functionality of these tRNA elements within metazoan taxa. We conclude that the identity elements that have evolved for the recognition of mitochondrial tRNAArgUCG by the nuclear encoded mitochondrial arginyl-tRNA synthetases of eumetazoans have been extensively, but not universally conserved, throughout this clade. They ensure that the AGR codon reassignment in eumetazoan mitochondria is not compromised by misaminoacylation. In contrast, in other metazoans, such as Porifera, whose mitochondrial translation is dictated by the universal genetic code, recognition of the 2 encoded tRNAArgUCG/UCU isoacceptors is achieved through structural features that resemble those employed by the yeast cytoplasmic system.

Introduction

Metazoan mitochondrial genomes have become a valuable source of information regarding phylogenetic markers[1] and the evolution of the mitochondrial genetic code has been extensively analyzed.[2,3] Mitochondrial translation is mediated by a family of nuclear encoded aminoacyl-tRNA synthetases that catalyze the attachment of an amino acid to the cognate tRNA. Eumetazoan mitochondrially encoded tRNAs possess structures – identity elements[4,5] – that are recognized by their cognate enzyme yet are frequently rejected by corresponding cytoplasmic or bacterial aminoacyl-tRNA synthetases.[6-8] On the other hand, mitochondrial aminoacyl-tRNA synthetases may accept heterologous tRNA substrates such as those of *E. coli*[7-11] or their cytoplasmic counterparts.[12]

Identity elements that govern tRNA charging have been studied for the cytoplasmic aminoacyl-tRNA synthetases for all amino acid specificities[13] and in a number of species – although the claim that they "have been deciphered in eubacteria and most eukarya"[14] is, in view of the extent of evolutionary diversity, somewhat exagerated. Nevertheless, it is generally accepted that in view of the conserved canonical structure retained by the cytoplasmic tRNAs, only modest evolutionary divergence in the nature of identity elements has occurred.[15] In contrast, elements defining accurate eumetazoan mitochondrial aminoacylation have been established systematically only for serine,[16] tyrosine,[17] aspartate,[6] alanine[18] and leucine.[19] They appear to follow different, relaxed, identity rules reflecting the atypical, variable and often bizare tRNA secondary structures.

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Of particular interest are tRNAs that participate in codon reassignment. $tRNA^{Ser}$ has been reassigned to read AGR codons in invertebrate mitochondria[20] that encode either $tRNA^{Ser}_{GCU}$ or $tRNA^{Ser}_{UCU}$. It has been found that the anticodon is not involved in the recognition by the corresponding mitochondrial seryl-tRNA synthetase.[16] This supports the argument that participation of the anticodon in recognition would impede a codon reassignment.[21] However, AGA corresponds to arginine in the standard genetic code and the anticodon of $tRNA^{Arg}_{UCU}$ forms an integral part of the recognition mechanism for the cytoplasmic arginyl-tRNA synthetase.[22] For an efficient codon reassignment one would expect that arginylation of $tRNA^{Ser}_{UCU}$ by the mitochondrial arginyl-tRNA synthetase, which would lead to arginine misincorporation, is avoided by using an alternative set of identity rules. These might be expected to be shared by organisms that are related evolutionarily through the same codon reassignment. No extensive investigation of the evolutionary variation in mitochondrial tRNA identity elements has been performed to date.

We have recently characterized the mitochondrial arginyl-tRNA synthetase from a Coleopteran insect (*Caryedes brasiliensis*).[23] To investigate which structural features are required to aminoacylate specifically the mitochondrially encoded $tRNA^{Arg}_{UCG}$ isoacceptor a systematic in vitro base replacement and transplantation study was undertaken. A comparative examination of the activity of such tRNA variants established the features required for recognition. An analysis of the aminoacylation properties of the corresponding $tRNA^{Arg}$ from representatives of 7 metazoan phyla showed that, despite large variations in the secondary structures, these identity elements have been largely conserved evolutionarily in eumetazoans that share the AGR codon reassignment.

Results

Throughout these studies, full length tRNAs were prepared by in vitro transcription, substituting A1 or U1 by G1 to facilitate T7 RNA polymerase mediated transcription. The *C. brasiliensis* mitochondrial $tRNA^{Arg}_{UCG}$ (Accession No. KF419299) has a typical Coleopteran primary structure composed of 68 bases (after 3′ –CCA addition) and a GC content of 26.2%. The absence of modified bases in the transcript substrates may influence charging levels,[24] Modified bases such as m^1A9 play a role in stabilizing the tertiary structure[25] and Ψ contributes to base pair stability.[26-28] In the case of *Aedes albopictus* (the only insect $tRNA^{Arg}$ that has been sequenced), just 4 modified bases have been detected: m^1A9, Ψ28, Ψ32, Ψ68.[29] Despite reservations concerning kinetic studies of such transcripts, by using identical component concentrations for all aminoacylations, one can make a direct comparison of the charging efficiency through time-course measurements.[18] Using the derived equation $v_x/v_y=(k^x_{cat}/K^x_M)/k^y_{cat}/K^y_M)$[30] (where x and y refer, in this case, to the cognate and the variant tRNAs, respectively), the initial rate v reflects the catalytic efficiency, irrespective of whether k_{cat} or *K*m are effected.

Early work on mammalian systems indicated that cytoplasmic arginyl-tRNA synthetase could not recognize mitochondrial $tRNA^{Arg}$.[31,32] Subsequently, using purified bovine mitochondrial tRNAs a charging unilaterality was observed by which eubacterial aminoacyl-tRNA synthetases were unable to charge eumetazoan mitochondrial tRNAs, whereas mitochondrial aminoacyl-tRNA synthetases efficiently accepted bacterial tRNAs.[6,8] A reported exception was that *E.coli* arginyl-tRNA synthetase could weakly aminoacylate bovine mitochondrial tRNA. In view of the potential background of *E.coli* aminoacyl-tRNA synthetases in our recombinant *C. brasiliensis* mitochondrial arginyl-tRNA synthetase preparation, we established previously that the purified *E.coli* arginyl-tRNA synthetase is unable to recognize *C. brasiliensis* mitochondrial $tRNA^{Arg}_{UCG}$ [23] (or any of the tRNAs described in this work – data not shown). The functionality of the *C. brasiliensis* mitochondrial $tRNA^{Arg}_{UCG}$ transcript was confirmed by its aminoacylation by the recombinant insect mitochondrial enzyme (**Fig. 1**).

Influence of anticodon manipulation

Replacement of C35 (**Fig. 1**) [(numbering throughout refers to the conventional tRNA numbering system[33]] by G, creating a proline-inserting anticodon, abolished arginylation activity entirely. Position 36 permits reading of both arginine codon boxes in cytoplasmic $tRNA^{Arg}$ by accepting G or U and is, as such, a degenerate identity element in all 3 kingdoms of life.[34-36] In insect mitochon-

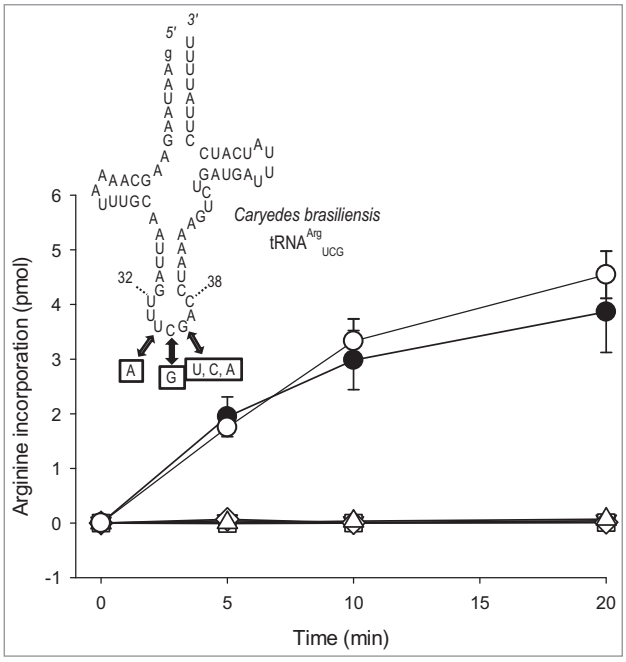

Figure 1. Effect of anticodon base replacements on the activity of *C. brasiliensis* mitochondrial $tRNA^{Arg}$. DNA templates harboring individual substitutions were transcribed and tested for arginine acceptance using *C. brasiliensis* mitochondrial arginyl-tRNA synthetase. Base replacements, are boxed, with arrows marking their positions. The lower case g1 denotes a replacement of A1 to permit T7 RNA polymerase mediated in vitro transcription. The structure is shown without the 3′-terminal –CCA sequence and the numbering corresponds to the tRNA convention. *C. brasiliensis* $tRNA^{Arg}_{UCG}$ (Wild type) (●);C35G (▽);G36C (□);G36A (◇); G36U (△);U34A (◯).

dria, this position is restricted to G, as is evident from the loss of activity when C36, A36 or U36 are inserted (**Fig. 1**). Thus, the insect mitochondrial enzyme discriminates against tRNAs bearing the UCU arginine anticodon of the universal genetic code.

Having evaluated the common cytoplasmic major identity elements (C35, G36) and since the discriminator base (position 73) contributes modest specificity only in the case of *E.coli*[13] and archaea,[35] it appeared conceivable that the anticodon alone might be the sole structural determinant for arginylation. Transplantation of the arginine UCG anticodon into the structurally dissimilar *Drosophila melanogaster* mitochondrial $tRNA^{Asp}_{GUC}$ (**Fig. 2A**) (Accession No. J01404) led to a very low L-arginine acceptance. However, transplantation of the complete $tRNA^{Arg}$ anticodon arm conferred full arginine identity to this $tRNA^{Asp}$.

The Leipzig tRNA database (http://mttrna.bioinf.uni-leipzig.de/) of mitochondrial tRNA genes provided 1332 unique eumetazoan mitochondrial $tRNA^{Arg}$ gene sequences. Alignment of these showed a high level of conservation in the anticodon arm region (**Fig. 2B**) (with considerably greater variation throughout the remaining tRNA). In order to localize the features responsible for the identity conversion, the $tRNA^{Arg}$ gene sequences of the 109 Coleopteran species [(compiled from the MetAmiga database of metazoan mitochondrial genomes (http://amiga.cbmeg.unicamp.br/) and Leipzig tRNA databases (http://mttrna.bioinf.uni-leipzig.de/)] (not shown) was examined more closely. It is evident that the anticodon loop is, with the exception of position 37 (G/A), 100% conserved within this subset. Of all the other, non-$tRNA^{Arg}$, Coleopteran mitochondrial tRNA genes (more than 400 from 19 species in the mitochondrial tRNA Leipzig database) only 22 share the anticodon loop sequence TTTNN(A/G)C. Of these, the anticodon loop sequence of Coleopteran $tRNA^{Ala}$ is variable but that of the spotted asparagus beetle (*Crioceris duodecimpunctata*[37]) (Accession No. NC_003372 REGION: 5862..5925) possesses the $tRNA^{Arg}$-like TTTNNAC motif (**Fig. 2A**). The corresponding transcript, with the native alanine anticodon bases (UGC) could not be arginylated (data not shown) but when this region was converted to the arginine (UCG) anticodon, the arginylation activity with the *Caryedes* mitochondrial arginyl-tRNA synthetase was greater than that of the cognate $tRNA^{Arg}$ (**Fig. 2a**). Evidently, although the presence of C35, G36 are critical factors, additional anticodon loop elements are required to complete the major identity element for this tRNA.

Influence of the anticodon environment

Focusing further on the anticodon loop, it was of interest to determine in more detail what influence the highly conserved

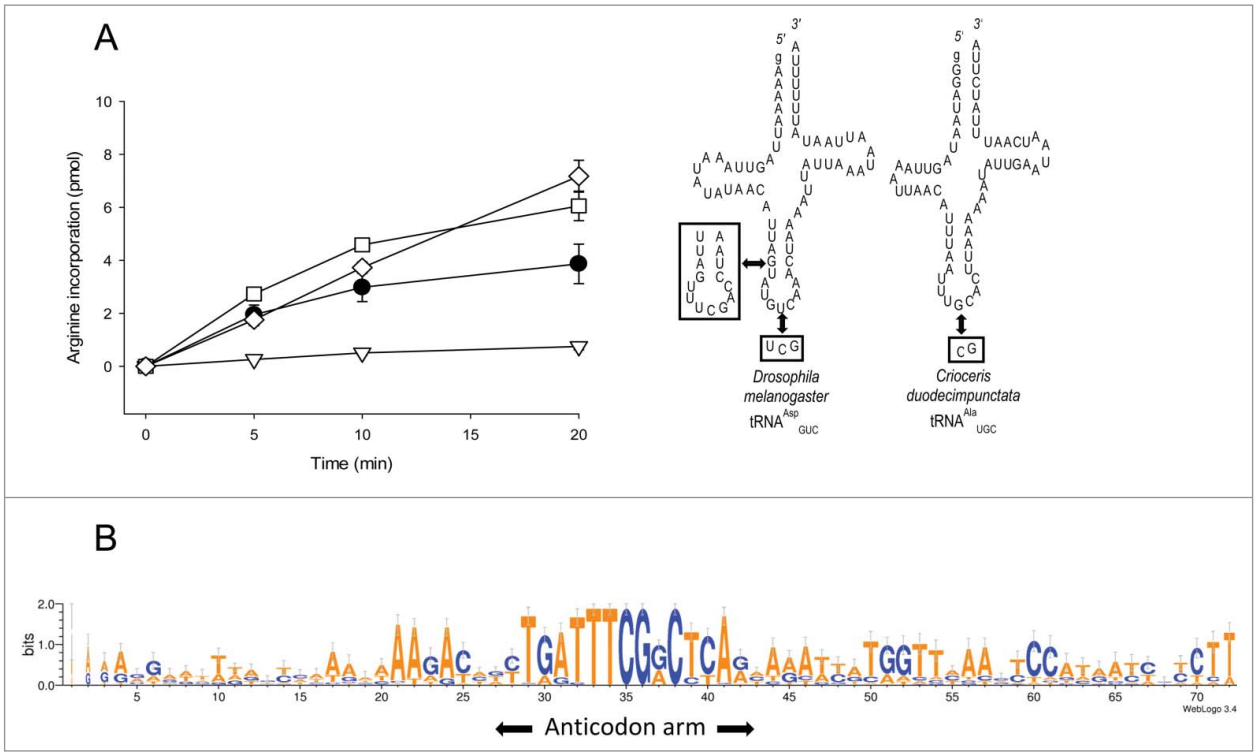

Figure 2. A. Anticodon transplantation into $tRNA^{Asp}$ and $tRNA^{Ala}$. DNA templates harboring the replacements (boxed) were transcribed and tested for arginine acceptance using *C. brasiliensis* mitochondrial arginyl-tRNA synthetase. *C. brasiliensis* mitochondrial $tRNA^{Arg}_{UCG}$ (●) compared to *D. melanogaster* $tRNA^{Asp}_{GUC \rightarrow UCG}$ (▽); *D. melanogaster* $tRNA^{Asp}_{UCG}$ / *C. brasiliensis* $tRNA^{Arg}_{UCG}$ anticodon arm (□); *C. duodecimpunctata* $tRNA^{Ala}_{UGC \rightarrow UCG}$ (◇). (**B**). Alignment of eumetazoan mitochondrial $tRNA^{Arg}$ gene sequences. The Leipzig tRNA database (http://mttrna.bioinf.uni-leipzig.de/) of mitochondrial tRNA genes provided 1332 unique eumetazoan mitochondrial $tRNA^{Arg}$ gene sequences which were aligned in CLUSTAL OMEGA (http://www.ebi.ac.uk/Tools/msa/clustalo/) and bases 1–72 of the alignment depicted in WebLogo3 (http://weblogo.threeplusone.com/create.cgi). The position of the anticodon arm within the alignment is indicated.

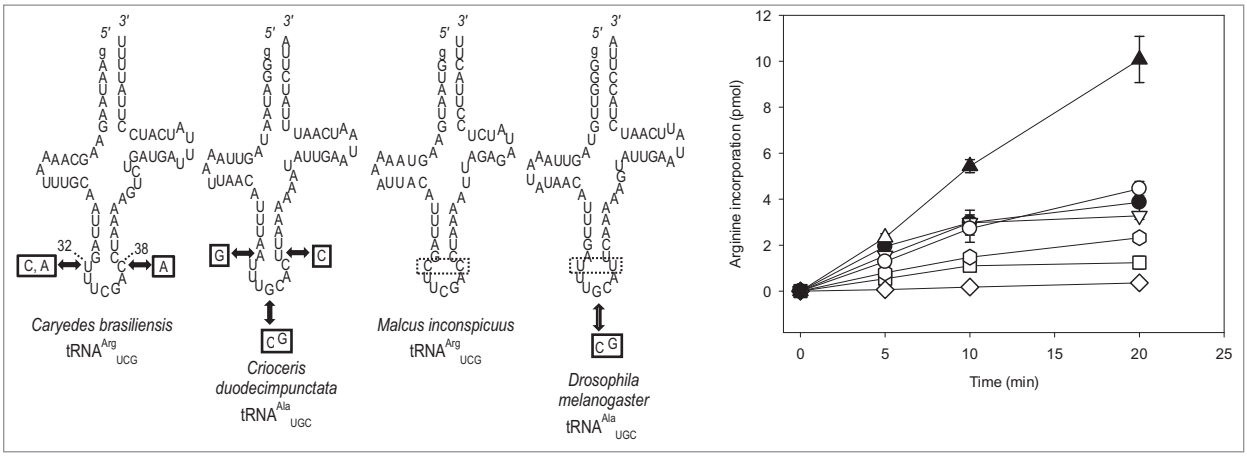

Figure 3. Influence of the anticodon environment on tRNA^{Arg} recognition. DNA templates possessing the replacements indicated in boxes were transcribed and tested for arginine acceptance using *C. brasiliensis* mitochondrial arginyl-tRNA synthetase. *C. brasiliensis* mitochondrial tRNA$^{Arg}_{UCG}$ (●) is compared to *C. brasiliensis* tRNA$^{Arg}_{UCG}$ A37G (▽); *C. brasiliensis* tRNA$^{Arg}_{UCG}$ A32:A38 (□); *C. brasiliensis* tRNA$^{Arg}_{UCG}$ C32:A38 (◇); *C. duodecimpunctata* tRNA$^{Ala}_{UGC}$ → $_{UCG}$ G31:C39 (▲), *D. melanogaster* tRNA$^{Ala}_{UGC}$ → $_{UCG}$ (□); *M. inconspicuus* tRNA$^{Arg}_{UCG}$ (○). Alternative position 32:38 component bases are framed.

nucleotides enclosing the anticodon triplet have on the tRNA identity. As would be anticipated, the nature of the conserved purine at position 37 had no impact on the activity (**Fig. 3**). In contrast, positions 32:38 proved to have essential functional roles. On the one hand, the rare natural C32:C38 combination, as in *Malcus inconspicuus* mitochondrial tRNA$^{Arg}_{UCG}$ (Accession No. NC_012458) was charged by the *Caryedes* mitochondrial arginyl-tRNA synthetase to the same level as the cognate tRNA (**Fig. 3**). A C32:A38 combination within the framework of *Caryedes* tRNA$^{Arg}_{UCG}$ led to an almost complete loss in activity (**Fig. 3**). Not quite as drastically, a change to A32:A38 resulted in a substantially reduced activity. A similar reduction was evident when using *Drosophila* tRNA^{Ala} bearing a UCG anticodon together with the native U32:U38 (**Fig. 3**) and thereby being distinct from the *C. duodecimpunctata* tRNA$^{Ala}_{UCG}$ (U32:C38) that had been shown to be an active substrate (**Fig. 2**). The arginylation of the latter construct could be further improved by introducing a G31:C39 base pair at the base of the anticodon stem, in analogy to the native *Caryedes* tRNA$^{Arg}_{UCG}$ structure (**Fig. 3**). The introduction of this base pair into the existing 5 base pair anticodon stem (compared to the 4 base paired *Caryedes* stem) may explain the higher activity of this construct. Stabilization through G31:C39 with concomitant improved aminoacylation has previously been observed for human mitochondrial tRNA^{Leu19} and tRNA^{Asp}.[6]

Codon reassignment of tRNA$^{Ser}_{UCU}$ is maintained by restrictive identity elements

An inspection of eumetazoan mitochondrial tRNA$^{Ser}_{TCT}$ gene sequences in the Leipzig database revealed all 77 available datasets were characterized by a conserved CTTCTAA anticodon loop (**Fig. 4A**) that included the negative determinants for arginylation, U36 and C32:A38. To show that these were indeed responsible for sustaining the codon reassignment of tRNA$^{Ser}_{UCU}$, appropriate base replacements in a beetle serine isoacceptor were performed (**Fig. 4B**). The anticodon swap UCU to UCG proved to be ineffective in conferring arginylation activity. However, this replacement in combination with U32:C38 yielded a transcript with an arginylation close to that of the cognate tRNA^{Arg}.

Evolutionary distribution

With a knowledge of the identity elements required by the Coleopteran mitochondrial arginyl-tRNA synthetase, one may consider their evolutionary functional relationship to other metazoan species. We have, therefore, investigated the distribution, structure and activity of mitochondrially encoded arginine isoaccepting tRNAs over a wide range of animal taxa.

In view of the highly variable overall secondary structure exhibited by tRNAs of this sub-kingdom (exemplified in **Fig. 5A**), we tested mitochondrial tRNA arginylation by the insect mitochondrial arginyl-tRNA synthetase of an additional 11 representative species from a further 6 phyla (**Fig. 5B**). As judged by the initial rate of aminoacylation under identical conditions, despite highly dissimilar structures, most showed significant activity. Within the Eumetazoans, human tRNA^{Arg}, representing Chordata, stood out in its greater activity compared to the cognate beetle tRNA. The others of this clade were generally slightly lower in their activity. Despite the disparate use of the ACG anticodon by the Cestoda class of Platyhelminthes, this isoacceptor was an adequate substrate for the insect enzyme. In contrast, the same isoacceptor from the Chromadorea branch of Nematodes was a poor (*Haemonchus contortus*) to negligible (*Caenorhabditis elegans*) substrate. As shown above (**Fig. 1**), A34 could fully replace U34 in the recognition process in the context of the insect tRNA structure. Since, despite evidence to the contrary,[38] it is considered that in tRNAs A34 is generally deaminated to inosine,[39] a potential involvement of inosine in tRNA recognition required further investigation. Reverse transcription of total *C. elegans* RNA with a primer specific for tRNAs, followed by PCR with a tRNA$^{Arg}_{ACG}$-specific primer and sequencing, gave no indication for the presence of an inosine-

derived G34[40] (**Fig. 5B**, inset). Hence, mitochondrial *C. elegans* $tRNA^{Arg}_{ACG}$ is not post-transcriptionally deaminated and the decreased activity must be related to a loss in optimal macromolecular recognition, such as through the sub-optimal U32:U38 tRNA structure or the absent T-arm that is a feature in all 11 Chromadorean $tRNA^{Arg}_{ACG}$'s in the Leipzig database.

In addition to Eumetazoa, the metazoan kingdom includes the Porifera and Placozoa phyla. In contrast to Eumetazoans, members of both these phyla (with the exception of Hexactinellida) use translation Table 4 (see http://www.ncbi.nlm.nih.gov/Taxonomy/Utils/wprintgc.cgi for mentions of Tables 4 and 5) (UCU = Arg) for decoding the mitochondrial genome. Placozoan $tRNA^{Arg}_{UCG}$, represented by *Trichoplax adhaerens*, is a good substrate for insect mitochondrial arginyl-tRNA synthetase achieving twice the charging level compared to the cognate tRNA (**Fig. 5B**); the additional mitochondrially encoded UCU isoacceptor is not expected to be a substrate (see discrimination against U36, above). The Porifera are subdivided into 3 classes. Only a single Calcarean $tRNA^{Arg}_{UCU}$ is known[41] and this was not investigated. From the large class of Demospongiae several mitochondrial genomes have been described as encoding both UCG and UCU isoacceptors. The position 32:38 bases are distinct from the optimally active U32:C38. Nevertheless, the $tRNA^{Arg}_{UCG}$ from *Iotrochota birotulata* proved to be recognized by the insect mitochondrial arginyl-tRNA synthetase (**Fig. 5B**). The $tRNA^{Arg}_{UCU}$ transcript, on the other hand, having the U36 negative determinant for the insect enzyme was inactive. The few examples of mitochondria from the borderline class of Hexactinellida encode solely the $tRNA^{Arg}_{UCG}$ isoacceptor but bear the unfavorable C32:A38 anticodon loop feature. The corresponding tRNA from *Aphrocallistes vastus* was completely devoid of arginylation activity when tested with the insect enzyme.

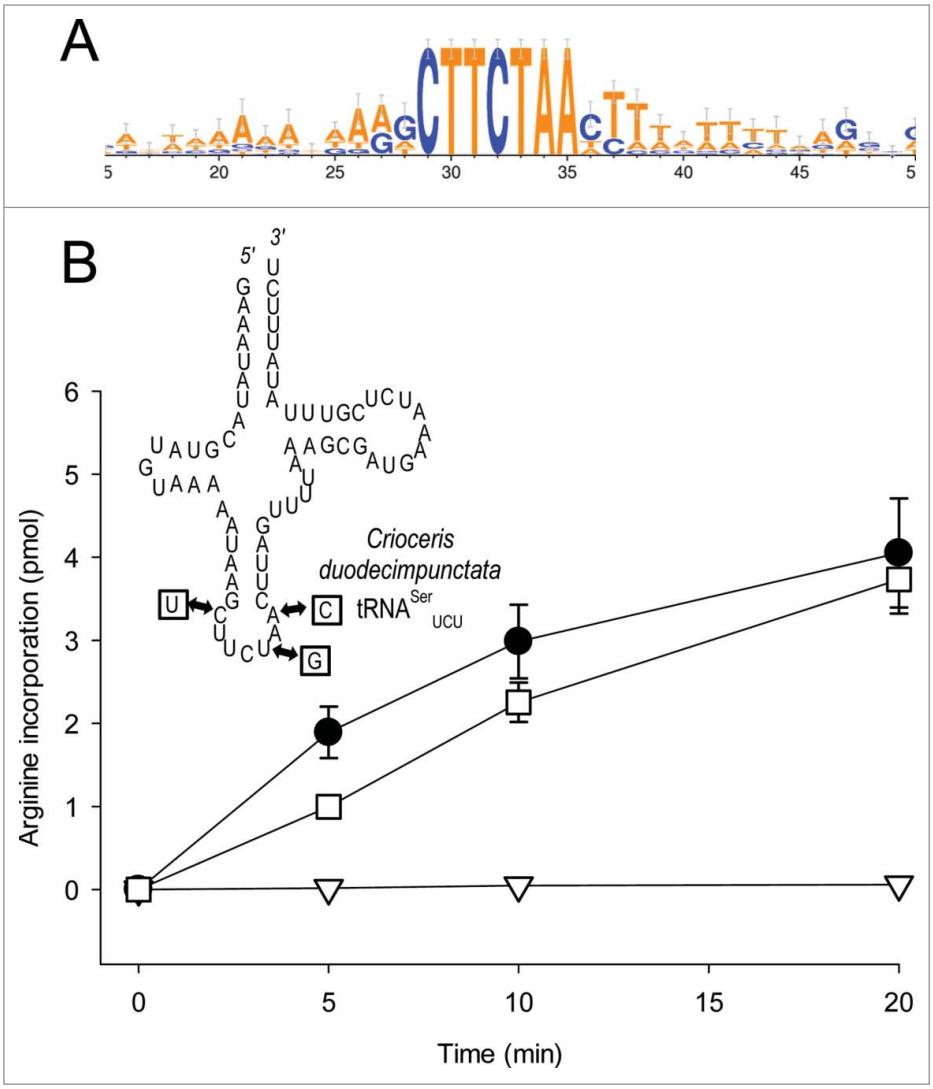

Figure 4. Restrictive identity elements maintain $tRNA^{Ser}_{UCU}$ codon reassignment. (**A**). Alignment of eumetazoan mitochondrial $tRNA^{Ser}_{UCU}$ gene sequences. The Leipzig tRNA database (http://mttrna.bioinf.uni-leipzig.de/) of mitochondrial tRNA genes provided 77 unique eumetazoan mitochondrial $tRNA^{Ser}_{UCU}$ gene sequences which were aligned in CLUSTAL OMEGA (http://www.ebi.ac.uk/Tools/msa/clustalo/) and the central 35 bases of the alignment depicted in WebLogo3 (http://weblogo.threeplusone.com/create.cgi). (**B**). Identity conversion of $tRNA^{Ser}_{UCU}$ to arginine acceptance. DNA templates possessing the replacements indicated in boxes were transcribed and tested for arginine acceptance using *C. brasiliensis* mitochondrial arginyl-tRNA synthetase. *C. brasiliensis* mitochondrial $tRNA^{Arg}_{UCG}$ (●) is compared to *C. duodecimpunctata* $tRNA^{Ser}_{UCU>UCG}$ (▽) and *C. duodecimpunctata* $tRNA^{Ser}_{UCU>UCG}$ U32:C38 (□).

Heterologous arginylation with yeast cytoplasmic arginyl-tRNA synthetase

Prokaryote $tRNA^{Arg}$ can be charged by yeast cytoplasmic arginyl-tRNA synthetase whereas the yeast $tRNA^{Arg}$ lacking the major A20 identity element is not arginylated by the *E. coli* enzyme.[42] The absence of a canonical A20 in the insect mitochondrial $tRNA^{Arg}$ might be seen as one factor preventing aminoacylation by the *E.coli* enzyme. On the other hand, the mitochondrial tRNA possesses all the elements that have been described as being essential (or permissible) for acceptance by the yeast arginyl-tRNA synthetase.[36]

Since the yeast cytoplasmic arginyl-tRNA synthetase can tolerate some variability in the D-loop,[36] we considered the insect mitochondrial $tRNA^{Arg}$ to be a potential substrate for heterologous aminoacylation. However, the wild type insect tRNA was not detectably arginylated by a yeast extract active in the

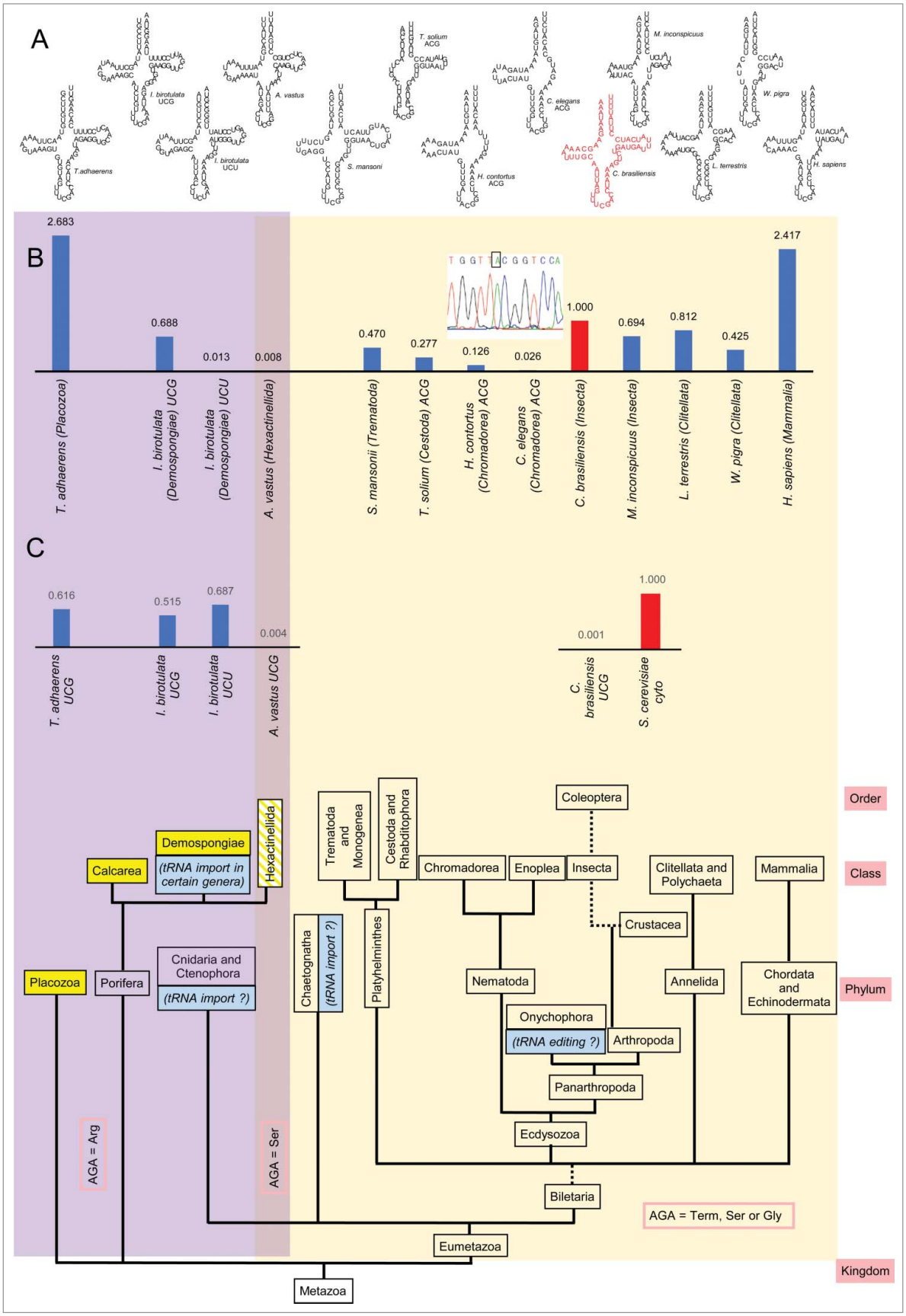

Figure 5. For figure legend, See page 1319.

aminoacylation of yeast $tRNA^{Arg}_{UCU}$ (**Fig. 5C**). On the other hand, not only were both *T. adhaerens* and *I. birotulata* UCG isoacceptors substrates for the yeast enzyme, but the sponge UCU isoacceptor also proved to be efficiently arginylated (**Fig. 5C**). The Hexactinellida $tRNA^{Arg}_{UCG}$ remained inactive.

Discussion

Cytoplasmic arginyl-tRNA synthetases recognize all nuclear encoded $tRNA^{Arg}$ isoacceptors [34,36] (and unpublished results for plants). The mitochondrial reassignment of the AGA codon from Arg to Ser (invertebrates), Gly (tunicates) or termination (vertebrates, trematodes and echinoderms)[20] relies not only on the serylation or glycinylation of mitochondrially encoded $tRNA^{Ser}_{UCU}$[16] or $tRNA^{Gly}_{UCU}$ but also on avoiding the recognition of the UCU-bearing tRNA by the mitochondrial arginyl-tRNA synthetase.

We have focused our attention on the tRNA recognition by the *C. brasiliensis* mitochondrial arginyl-tRNA synthetase.[23] We have investigated the identity elements that are contained within the structure of the tRNA by systematic base replacements and by transplanting sequence fragments from the cognate $tRNA^{Arg}$ to unrelated mitochondrial tRNAs.

The anticodon base G36 is an absolute requirement for arginylation and, in contrast to the cytoplasmic tRNA counterpart, cannot be replaced by U36 or any other base. A major identity element for the recognition of the *C. brasiliensis* mitochondrial $tRNA^{Arg}$ by its cognate synthetase resides, as is the case for cytoplasmic $tRNA^{Arg}$ from several organisms,[43] is the central anticodon position, C35. Replacement by G35 in the cognate tRNA eliminated acceptor activity. However, the anticodon triplet as a whole, while eliciting a very slight arginylation, was not adequate for efficient recognition when transplanted into *Drosophila* $tRNA^{Asp}$. The presence of the remaining bases of the highly conserved anticodon loop, however, conferred arginine acceptance to both mitochondrial $tRNA^{Asp}$ and $tRNA^{Ala}$. The identity elements are independent of the sequence of the distal acceptor stem. The major identity element A20, that has been described for the *E.coli,*[44] mammalian cytoplasmic[45] and plant arginyl-tRNA synthetases,[43] has been lost in insect mitochondria during the evolution of highly variable and, in some species, rudimentary D-stem/loops. The activity of the *C. duodecimpunctata* $tRNA^{Ala}$ whose anticodon had been replaced by UCG supports the conclusion that the discriminator base U_{73} ($tRNA^{Arg}$) or A_{73} ($tRNA^{Ala}$) does not play a role in recognition. Indeed, position 73 in 567 insect mitochondrial $tRNA^{Arg}$ gene sequences is usually A or T but can be G or C (in 22 of the annotations). Thus, the complete set of identity elements is restricted to the anticodon loop. The nature of the bases found at positions 32 and 38 plays a major role. Maximal activity was achieved with U32:C38 that is prevalent in insects, and with the less common C32:C38. Some alternative combinations were adequate, if less active, substrates. C32:A38 on the other hand, proved to be a strong negative determinant for arginylation in the context of the insect mitochondrial tRNA framework. U32 is modified to Ψ32 in insects[29] although the lack of modification does not prevent transcripts from being recognized by the arginyl-tRNA synthetase. The nature of these elements ensures that the reassigned $tRNA^{Ser}_{UCU}$ is not arginylated since U36 prevents recognition by the arginyl-tRNA synthetase. Additionally, even in the case of a U36G mutational event, all 77 eumetazoan $tRNA^{Ser}_{UCU}$ sequences in the database (http://mttrna.bioinf.uni-leipzig.de/) possess the C32:A38 pairing that, as we have shown, virtually abolishes arginylation. Replacement of both negative determinants by G36 and U32:C38 in $tRNA^{Ser}_{UCU}$ conferred arginylation activity to this tRNA.

The recognition elements of $tRNA^{Arg}$ that we have defined may be restricted to Coleoptera since the anticodon loop sequence is absolutely conserved within 109 members of this taxonomic order. However, an examination of metazoan tRNA sequences available in the databases showed that the identity elements are frequently retained in members of several phyla. Within the Metazoa, the most extensive clade is that of the Eumetazoa (**Fig. 5**). Here, the Ecdysozoa group includes the phyla Arthropoda, Onychophora (velvet worms) and Nematoda. There is little data regarding Onychophoran mitochondria. The four available organelle genomes reveal a nominal loss of 9 tRNA genes, including that of $tRNA^{Arg}$ [46] although a 31 bp fragment representing the 5' half including the anticodon loop was annotated as such (HM600781). Subsequently, extensive RNA editing was detected that conferred a potential eumetazoan $tRNA^{Arg}$ structure to this gene product.[47]

In the smaller class within the Nematoda phylum, 14 Enoplea mitochondrial sequences encode $tRNA^{Arg}_{UCG}$ with the Coleopteran anticodon-loop sequence. The larger Chromadorea class is represented by 69 sequences of which 67 have ACG, 1 UCG (*Mecistocirrus digitatus*), 1 GCG (*Strongyloides stercoralis*)

Figure 5 (**See previous page**). Phylogenetic metazoan relationships (not to scale). The nature of the codon reassignment is framed in orange. Phyla maintaining the universal genetic code are shaded in purple, those using reassigned arginine codons are shaded in beige. Organisms possessing $tRNA^{Arg}$ resembling the canonical cytoplasmic secondary structure are highlighted in yellow and those thought to be involved in extensive mitochondrial tRNA import or editing, in blue. Broken lines denote that the taxonomic lineage has been abbreviated, for clarity. (**A**). Structures of metazoan mitochondrial $tRNA^{Arg}$ isoacceptors (without the –CCA end) from 7 phyla that were tested as substrates for the insect arginyl-tRNA synthetase. The cognate tRNA is given in red. Unless otherwise annotated, UCG isoacceptors are shown. Sequences were obtained from the Leipzig database (http://mttrna.bioinf.uni-leipzig.de/). The anticodon loop sequence of the Enoplea class of Nematoda is identical to that of Insecta and was not tested. (**B**). Arginylation activity of tRNAs shown in (**A**). Initial rates (pmol/min) relative to the cognate tRNA (red) are given. Inset: DNA sequence corresponding to the reverse transcribed anticodon stem region of *C. elegans* $tRNA^{Arg}_{ACG}$ confirming the absence of A-to-I editing of the wobble base at position 34 (boxed). (**C**). Heterologous aminoacylation of metazoan mitochondrial tRNAs by yeast cytoplasmic arginyl-tRNA synthetase. The mitochondrially encoded Placozoan $tRNA^{Arg}_{UCU}$ was not tested. Initial rates (pmol/min) relative to the cognate yeast cytoplasmic tRNA (red) are given.

anticodons. The ACG anticodon is frequently associated with decoding using the deaminated inosine wobble position,[48] Within this class, *Caenorhabditis elegans* is a representative of the order Rhabditida. We have examined *C. elegans* mitochondrial $tRNA^{Arg}_{ACG}$ by reverse transcription followed by PCR and sequencing. As in the case of cytoplasmic $tRNA^{Arg}_{ACG}$ in plants[38] and in insects (unpublished results), the first position of the anticodon is not deaminated and that, therefore, translation of all arginine codons by this isoacceptor must rely on non-standard wobble interactions. The presence of A34 in the cognate tRNA did not influence the aminoacylation activity by the insect arginyl-tRNA synthetase so that the loss of arginine acceptance by the *C. elegans* $tRNA^{Arg}_{ACG}$ may be indicative of a modulated recognition mechanism for these isoacceptors.

More examples of an ACG anticodon are found within the Cestoda and Rhabdoptophora classes of Platyhelminthes, whereas in the same phylum the members of the Monogenea and Trematoda classes feature the mitochondrial UCG anticodon (with the exception of *Benedemia seriolae* with ACG). In the case of *T.solium*, that possesses the favorable U32:C38, activity was reduced to about a third compared with the cognate system suggesting that recognition may also be dominated by alternative elements.

Of 1125 Echinodermata and Chordata mitochondrial $tRNA^{Arg}$ in the Leipzig database, 21 species from 16 genera have bases at positions 32:38 that are distinct from the majority U32:C38. Four of these have been annotated as $tRNA^{Arg}$ despite having atypical anticodons [*Phocoena phocoena*; UCA, Acc.No. NC_005280.1, *Cervus elaphus*; UUG, NC_007704.1 (although 2 other *Cervus* species possess standard UCG anticodons), *Cyclemys atripons* (vertebrate); CCG, NC_010970 and *Odontobutis platycephala* (vertebrate); CCG, NC_010199.1]. Overall, more than 98% of chordate and echinodermate $tRNA^{Arg}$ sequences possess the identity elements experimentally established in the coleopteran case. The likelihood that members of this phylum share identity elements with insects is supported by the high level of arginylation of human mitochondrial $tRNA^{Arg}$ by the insect enzyme. Similarly, the small sample of available Annelida $tRNA^{Arg}_{UCG}$ sequences, with highly diverse overall structures (as evident for the 2 Clitellata examples examined here) shows a trend toward a coleopteran anticodon loop structure and, consequently, comparable identity elements.

Within the Biletaria, the exotic Chaetognath (arrow worms) phylum is enigmatic since the mitochondria of the members fail to encode any tRNAs,[49] and an import of nuclear encoded mitochondrial tRNAs (to meet the demands of codon reassignment) would be required. Cnidarians, that largely import both cytoplasmic tRNAs and their cognate synthetases to the mitochondria,[50] have retained the conventional arginine codons in the mitochondrial genome.

In sponges (Porifera) (that are classified within the Metazoa but represent a phylum distinct from Eumetazoa), 34 of the 35 mitochondrial genome sequences in the MetAmiga database code for both $tRNA^{Arg}_{UCG}$ and $tRNA^{Arg}_{UCU}$. They display a conventional structure and length with conserved U8, G18, G19, A21, U33, and A38 throughout, and the canonical GUUCRA T-loop. Decoding is according to translation Table 4, allowing UCU anticodons for arginine translation. The exceptional sponge within the MetAmiga database, *Aphrocallistes vastus,* whose $tRNA^{Arg}$ transcript is inactive with both the insect and yeast arginyl-tRNA synthetase, is one of 3 examples from the Hexactinellida class. These, whose affinity to Bilaterians has been noted previously,[51] use translation Table 5 where UCU serves as a serine decoder. *Aphrocallistes vastus* and the partial mitochondrial sequence of *Iphiteon panacea* (Accession No. EF537576) and *Sympagella nux* (EF537577) are annotated with $tRNA^{Arg}_{UCG}$, whereas the additional tRNA(UCU) found in *Aphrocallistes* is probably required for serine decoding[52] (despite being annotated as $tRNA^{Arg}$ in the database (NC_010769). Their borderline existence between the other sponge classes and Biletaria is also evident from the divergent secondary structure within the class. Thus, whereas the *Iphiteon* tRNA resembles the eumetazoan mitochondrial structure with an A-rich abbreviated D-loop, *Aphrocallistes* tRNA has features reminiscent of canonical cytoplasmic tRNAs, including the T-loop GUUCRA sequence, that are found in Demospongiae. All other sponges in the database belong to Demospongiae that use translation Table 4, and thus utilize a $tRNA^{Arg}_{UCU}$ isoacceptor. Here, only the UCG isoacceptor is a substrate for the insect mitochondrial synthetase, whereas both isoacceptors are charged by the yeast cytoplasmic enzyme. However, neither was a substrate for a higher eukaryotic (plant) cytoplasmic arginyl-tRNA synthetase (data not shown) that requires an A20 identity element.[43] Parenthetically, evidence has been presented that the yeast cytoplasmic arginyl-tRNA synthetase has mitochondrial origins.[53,54] Finally, in a third class of sponges, the Calcarea, the CGN arginine codon has been reassigned to glycine, retaining $tRNA^{Arg}_{UCU}$ as the sole arginine decoder.[41] Identity elements for all sponges are anticipated to be different from the Coleopteran consensus, requiring the recognition of both U36 and G36 in the 2 alternative isoacceptors and of divergent 32:38 positions. The challenge of recognizing all arginine isoacceptors by a single arginyl-tRNA synthetase has been met by all cytoplasmic arginyl-tRNA synthetases so that a direct import of the cytoplasmic form into Demospongian mitochondria would be one conceivable alternative to re-inventing UCG/U anticodon recognition by a specialized mitochondrial enzyme. An examination of the incomplete sole Poriferan genome in the database reveals evidence for the existence of 2 similar but distinct nuclear encoded arginyl-tRNA synthetases in *Amphimedon queenslandica*. Although only partial sequences that include the arginyl-tRNA synthetase signature sequence are available, it is interesting to note that both have N-terminal sequence motifs characteristic of the cytoplasmic form (in particular the GDYQCNNAM-like feature) (**Table S1**). Furthermore, mitochondrial targeting (by both MitoProt[55] and CELLO[56]) is predicted for one of the gene products. We would speculate that, as for some plant aminoacyl-tRNA synthetases[57] a duplicated form of the cytoplasmic enzyme has acquired a targeting sequence for import into the Demospongiae mitochondria. The canonical nature of the tRNA structures encoded by these organelles[58] permits their interaction with the conventional, but imported cytoplasmic enzyme that can master redundant anticodon recognition. This would also be in accordance with the observation that members of certain genera within

the Demospongiae lack the mitochondrial coding capacity for numerous tRNAs,[59,60] including those for $tRNA^{Arg}$ (Clade G1). Having also lost the genes coding for mitochondrial aminoacyl-tRNA synthetases, the inevitable import of cytoplasmic tRNAs would then also require the intra-organellar availability of their cognate cytoplasmic enzymes.

The Placozoan phylum within the metazoan kingdom is represented in the database by 6 sequences from 2 genera. These display a conventional cloverleaf structure having U8, G18, G19, and the T-loop GUUCRA sequence. Five of the 6 available species encode both UCG and UCU isoacceptors with the UCG anticodons enclosed by the Coleoptera-like U32:C38 anticodon loop sequence, whereas the UCU anticodon loops possess C32:A38. As in the Demospongiae a single arginyl-tRNA synthetase with an extended recognition capability, such as the cytoplasmic counterpart, is likely to arginylate both decoders.

We conclude that the identity elements that have evolved for the recognition of mitochondrial $tRNA^{Arg}_{UCG}$ by the nuclear encoded mitochondrial arginyl-tRNA synthetases of eumetazoans have been conserved, with minor modulations, and possibly alternative elements in the Chromadorea class of Nematodes. They ensure that the AGR codon reassignment in eumetazoan mitochondria is not compromised by misaminoacylation. In contrast, in other metazoans whose mitochondrial translation is governed by the universal genetic code, $tRNA^{Arg}$ recognition is achieved through structural features that resemble those employed by the yeast cytoplasmic system. The exceptional class of Hexactinellida whose $tRNA^{Arg}$ failed to be recognized by all tested enzymes (prokaryotic, eukaryotic cytoplasmic, coleopteran mitochondrial or yeast cytoplasmic) requires further investigation.

Materials and Methods

Primers for transcription were products of Sigma-Aldrich (Supplementary **Table 2**). A protocol for the preparation of a yeast extract containing arginyl-tRNA synthetase activity was kindly provided by Dr. R. Giegé (Strasbourg).[61] Reverse transcription analysis of tRNA was performed as described.[38] Total *C. elegans* RNA was kindly provided by Dr. E. Schulze (Freiburg).

For efficient in vitro transcription of the mitochondrial $tRNA^{Arg}_{UCG}$ genes, position A1 or U1 was replaced by G.[62] In vitro transcription was performed with double stranded tDNA obtained by a Klenow fill-in-reaction using Exo-Minus Klenow DNA Polymerase (Epicenter) and an appropriate full-length oligonucleotide template bearing a T7 promoter extension. One nmol of single stranded template was incubated in a 1 ml reaction volume containing 10 mM Tris-HCl pH7.5, 5 mM $MgCl_2$, 2.5 mM dNTPs, 0.75 M betaine, and 100 pmol 3'-terminal-specific 20-mer primer, for 5 min at 95°C. After cooling in ice for 5 min, 2.5 μl 1 M DTT and 50 U Klenow DNA polymerase were added. Incubation for 1 h at 37°C was followed by ethanol precipitation. A 0.5 ml in vitro transcription contained T7 RNA polymerase buffer (4% PEG8000, 40 mM Tris-HCl, pH 8.0, 12 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine-HCl, 0.002% Triton X-100), 5 mM NTP, 20 mM GMP, 0.1 U of inorganic pyrophosphatase, 0.7 nmol template DNA and 52 nM T7 RNA polymerase prepared from the recombinant pAR1219 expression plasmid.[63] The reaction mixture was incubated for 4 hours at 42°C, and purified over a NAP-5 gel filtration column (GE Healthcare). DNase digestion was performed in a 0.5-ml reaction with DNase buffer and 1 U DNase (Fermentas) for 30 min at 37°C. The reaction mixture was phenol extracted and ethanol precipitated. Confirmation that the homogeneity of the tRNA transcript was greater than 90%, was obtained by electrophoresis on 10% denaturing polyacrylamide gels. The tRNA was refolded by heating the solution for 5 min to 70°C, followed by slow cooling in the presence of 25 mM Tris-HCl, pH 7.5 and 5 mM $MgCl_2$. tRNA variants were prepared analogously, using primers given in Supplementary **Table 2**.

Aminoacylation with [^{14}C]-labeled L-arginine was carried out at 30°C in 50 μl reactions in the presence of 50 mM Hepes, pH7.5, 10 mM $MgCl_2$, 4 mM ATP, 17 μM [^{14}C]-L-arginine (300 mCi/mmol; Perkin Elmer) and 20 μg tRNA transcript. The reaction was initiated by the addition of recombinant *C. brasiliensis* mitochondrial arginyl-tRNA synthetase [23] to a final concentration of 6 nM. At the given time intervals, 10 μl aliquots were removed and spotted onto 5% TCA-pre-treated Whatman 3 MM discs, washed twice for 10 min with 5% TCA and once with ethanol before air drying and quantification by liquid scintillation counting. Results are those of at least duplicate determinations and were analyzed in SIGMAPLOT12. In contrast to the human mitochondrial $tRNA^{Leu}$, whose more conventional structure permits the replacement of the 3'-terminal A by a radioactive AMP,[64] the insect $tRNA^{Arg}$ was not a substrate for *E. coli*[65] or yeast[66] nucleotidyl transferase. The significantly more sensitive assay for amino acid incorporation[67] could not be used.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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