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Genetics

Bizarre tRNAs inferred from DNA sequences of mitochondrial genomes of nematode worms

(*Ascaris suum*/*Caenorhabditis elegans*/tRNA genes)

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ABSTRACT **The complete nucleotide sequence of the mitochondrial DNA (mtDNA) molecule of the parasitic nematode worm *Ascaris suum* has been determined. This molecule lacks genes for tRNAs of the standard form. Instead, 21 sequences are found that can be folded into structures that resemble tRNAs in which the TΨC arm and variable loop are missing and replaced with a single loop of between 4 and 12 nucleotides. Considerations of various properties of these sequences, including the number, predicted anticodons, conserved nucleotides, direction of transcription, base composition, and relative gene arrangements are consistent with the interpretation that they are genes for a different sort of tRNA. Transfer RNA genes with a similar potential secondary structure are found in mtDNA of the free-living nematode *Caenorhabditis elegans*, suggesting that this unusual form of tRNA is used by all nematode mitochondria.**

Complete nucleotide sequences and gene contents have been determined for the circular mitochondrial DNA (mtDNA) molecule of human (1–3), mouse (4), cow (5), *Xenopus laevis* (6), and *Drosophila yakuba* (7, 8). All of these metazoan mtDNA molecules contain the genes for 2 rRNAs and 22 tRNAs of the mitochondrion's protein synthesizing system and for 13 proteins: cytochrome *c* oxidase (CO) subunits I, II, and III; ATPase subunits 6 and 8; respiratory chain NADH dehydrogenase (ND) components 1–6 and 4L, and cytochrome *b*. There are some differences in the relative arrangements of genes between *D. yakuba* and vertebrate mtDNAs (8).

The secondary structures of both vertebrate and *D. yakuba* mt tRNAs resemble those of prokaryotic tRNAs and eukaryotic nuclear-encoded tRNAs. However, there is considerable variation with regard to both size and sequence of the dihydrouridine and TΨC loops of metazoan mt tRNAs and also in the occurrence among these different mt tRNAs of nucleotides that are invariant or semi-invariant in prokaryotic and eukaryotic nuclear-encoded tRNAs (8–12). We have recently completed the nucleotide sequence of the mtDNA molecule of the parasitic nematode worm *Ascaris suum*. This molecule does not contain sequences that can be folded into the characteristic secondary structure of known tRNAs. However, between the different protein and rRNA genes are found sequences that have a common potential secondary structure that resembles a tRNA in which the TΨC arm and variable loop are replaced with a loop of between 4 and 12 nucleotides. In this report, we present arguments that support the view that these sequences encode the functional mt tRNAs of nematode worms.

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MATERIALS AND METHODS

Adult *A. suum* were obtained from the intestines of pigs at a local slaughterhouse. Mitochondria were isolated as described (13) from body wall muscle.

Caenorhabditis elegans [Bristol N2 strain (14)] were maintained, amplified, and purified as described (14, 15), except that *Klebsiella aerogenes* was used as the bacterial food source. Worms were ruptured by using a Dounce homogenizer A, and mitochondria were isolated as described for *A. suum* except that mannitol was used in place of sucrose and 0.1–0.2% bovine serum albumin was present in all solutions.

Mitochondria from both *A. suum* and *C. elegans* were lysed with 10% sarkosyl and mtDNAs were isolated by cesium chloride/ethidium bromide centrifugation (13).

Restriction fragments of *A. suum* and *C. elegans* mtDNAs were cloned into pBR325 or pUC9 using as host *E. coli* K-12 HB101 and JM83, respectively. Details regarding preparation and identification of primary clones; restriction enzyme digestions; electrophoresis; cloning of fragments into M13mp8, M13mp9, M13mp18, and M13mp19; purification of single-stranded and double-stranded M13 DNAs; and preparation of viral DNAs containing partial deletions of cloned restriction fragments of mtDNAs are given or referred to in refs. 16 and 17.

DNA sequences were obtained from M13 cloned fragments using the Sanger procedure (18) and assembled and analyzed as described in ref. 8. Nematode mt rRNA genes were identified by nucleotide sequence comparisons, and nematode mt protein genes were identified by amino acid sequence and hydropathic profile comparisons to mouse and *D. yakuba* mt protein genes (4, 8).

RESULTS AND DISCUSSION

We have sequenced the entire mtDNA molecule of the nematode worm *A. suum* (unpublished data). A map of the genes contained in this molecule is given in Fig. 1. The molecule is 14,284 nucleotide pairs (ntp) long and contains the genes for 12 proteins and 2 rRNAs. The protein genes are the same as 12 of the 13 protein genes found in vertebrate and *Drosophila* mtDNAs. The gene for ATPase 8, which in other metazoan mtDNAs examined always precedes the ATPase 6 gene, has not been located in the *A. suum* mtDNA molecule.

In vertebrate and *Drosophila* mtDNA molecules, tRNA genes occur either singly or in groups of up to six between many of the protein and rRNA genes (1, 4–8). Between many of the protein and rRNA genes of *A. suum* mtDNA there are

Abbreviations: nt, nucleotide(s); ntp, nucleotide pair(s); COI, -II, and -III, cytochrome *c* oxidase subunits I, II, and III; ND1 to -6, NADH dehydrogenase subunits 1–6.

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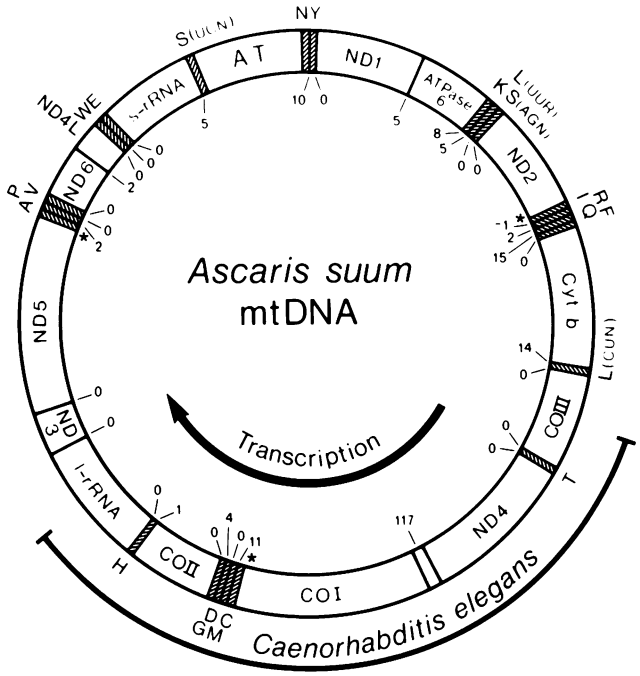

FIG. 1. Gene map of the *A. suum* mtDNA molecule (unpublished data). Each tRNA gene (hatched area) is identified by the single letter amino acid code. All genes are transcribed in the direction shown by the arrow. The numbers of apparently noncoding nucleotides that occur between the different genes are shown at the gene boundaries on the inner side of the map. The −1 between the $tRNA^{Ile}$ and $tRNA^{Arg}$ genes indicates a 1-nt overlap. An asterisk indicates an incomplete termination codon (T or TA). The AT region identifies a sequence of 899 nt, which includes up to 18 repeats of AT, and in which no genes have been identified. The 117 nt between the *ND4* and *COI* genes include a sequence with the potential to form a stable hairpin structure with a stem of 9 ntp and a loop of 8 nt, but again this region lacks genes. The segment of the *C. elegans* mtDNA molecule sequenced is indicated.

sequences of up to 247 nucleotides (nt), but these sequences cannot be folded into the secondary structures characteristic of known tRNAs. However, these sequences can be folded into 21 separate structures with common secondary sequence characteristics resembling tRNAs in which the variable loop and TΨC arm are replaced with a loop of between 4 and 12 nt (designated the TV-replacement loop; Fig. 2). Each of these structures, which are between 52 and 62 nt in size (mean, 57 nt), has an aminoacyl stem of 7 ntp, a dihydrouridine (D-) stem of 4 ntp (3 ntp in $tRNA^{Asn}$), a D-loop of between 5 and 9 nt, an anticodon stem of 5 ntp, and an anticodon loop of 7 nt. A further single structure that resembles a tRNA in which the D-arm is replaced with a loop of 5 nt and that contains a TCT anticodon is also found (Fig. 2).

The following considerations support the hypothesis that the structures described above are the complete set of tRNA genes of the *A. suum* mitochondrial genome.

Anticodons. The number of putative tRNA genes in *A. suum* mtDNA, 22, is the same as that found in vertebrate and *D. yakuba* mtDNAs. The tRNAs transcribed from these vertebrate and *D. yakuba* genes appear to be sufficient to decode the mt protein genes (1, 4, 6, 8, 19). In the *A. suum* mt protein genes, all codons are used except 4 that end in C (CTC, CCC, TGC, and CGC). As in other metazoan mtDNAs and fungal mtDNAs, TGA specifies tryptophan rather than functioning as a translation termination signal (9, 20–22). Of the 64 possible trinucleotides in the anticodon position of the *A. suum* putative tRNA genes, the 22 that are found are unique and compatible with codon usage in *A. suum* mtDNA. Nineteen of these anticodons correspond to anticodons found in tRNA genes of both vertebrates and *D. yakuba*. One of the *A. suum* mt tRNA genes has a TTT anticodon, which is the same as that found in the $tRNA^{Lys}$ gene of vertebrate mtDNAs. A CTT anticodon is found in the $tRNA^{Lys}$ gene of both *Drosophila* and mosquito mtDNAs (23–25). In both vertebrate and insect mitochondria, there is a tRNA gene and a corresponding tRNA, which contains a D-arm replacement loop (refs. 6, 8, and 26, and references therein). These tRNAs all contain a GCU anticodon, which recognizes AGT and AGC serine-specifying codons in vertebrate mtDNAs [AGA and AGG are either absent or are believed to be stop codons in vertebrate mtDNAs (1, 4–6)] and AGT, AGC, and AGA serine-specifying codons in insect mtDNAs (23, 24, 27); AGG codons are not found in *Drosophila* mtDNAs (7, 8). The anticodon of the putative mt tRNA gene of *A. suum* that contains a D-arm replacement loop has a TCT anticodon. This is consistent with the unique genetic code modification of *A. suum* mitochondria in which all AGN codons are used to specify serine (unpublished data). The putative mt $tRNA^{Arg}$ gene of *A. suum* contains an ACG anticodon, which is in contrast to the TCG anticodon of all other metazoan mt tRNA genes reported to date. However, tRNA genes with an ACG anticodon have been reported from a variety of sources, including yeast mtDNA (28).

Constant Nucleotides. Within mtDNAs from vertebrates and *D. yakuba*, the tRNA genes differ among themselves in regard to the presence or absence of the various nucleotides that are highly conserved in prokaryotic and eukaryotic nuclear-encoded tRNAs. In contrast, 8 nucleotides or nucleotide combinations that are considered invariant in prokaryotic and eukaryotic nuclear-encoded tRNAs (see refs. 28–31) are conserved with a frequency between 91% and 100% in the putative *A. suum* mt tRNA genes (Fig. 3). A further 9 nucleotides or nucleotide combinations that are considered semi-invariant in prokaryotic and eukaryotic nuclear-encoded tRNAs occur in the *A. suum* mt tRNA genes with a frequency of 81–100% (Fig. 3). In addition, of the 21 *A. suum* mt tRNA genes that contain a TV-replacement loop, 20 have an A·T pair or a T·A pair in the D-arm at positions 12–23 (Fig. 3), and a purine occurs at positions L2 and L3 in the TV-replacement loop in 100% and 91%, respectively, of these genes. The $G^{11}{\cdot}C^{24}$ pair (rather than the conserved pyrimidine^{11}·purine^{24} pair) found in *A. suum* $tRNA^{fMet}$ and $tRNA^{Trp}$ genes is a constant feature of the corresponding tRNA genes of other metazoan mtDNAs.

As is the case in other metazoan mt tRNA genes, the trinucleotide CCA, which occurs at the 3′ end of prokaryotic and eukaryotic nuclear-encoded tRNA genes, is absent from the *A. suum* mt tRNA genes.

Transcription, Base Composition, and Gene Arrangement. All of the *A. suum* putative mt tRNA genes would be transcribed from one strand of the molecule, which is the same strand from which all protein and rRNA genes are transcribed (Fig. 1). There is a peculiar base bias between the complementary strands of the *A. suum* mtDNA molecule; the sense strand is 49.8% thymine, 20.4% guanine, 22.2% adenine, and 7.7% cytosine. This base bias, which among mt tRNA genes is 42.3% thymine, 20.8% guanine, 28.8% adenine, and 8.1% cytosine, permits secondary structure formation in the corresponding RNA molecules since the plenitude of guanine and uracil permits pairing. However, RNA transcribed from the strand complementary to that from which tRNAs are transcribed would be very low in secondary structure potential since this RNA contains an abundance of nonpairing cytosine and adenine residues.

In the *A. suum* mtDNA molecule (Fig. 1), as in both vertebrate and *Drosophila* mtDNA molecules, individual genes are separated by few or no nucleotides. The only possible overlap between tRNA and protein genes involves

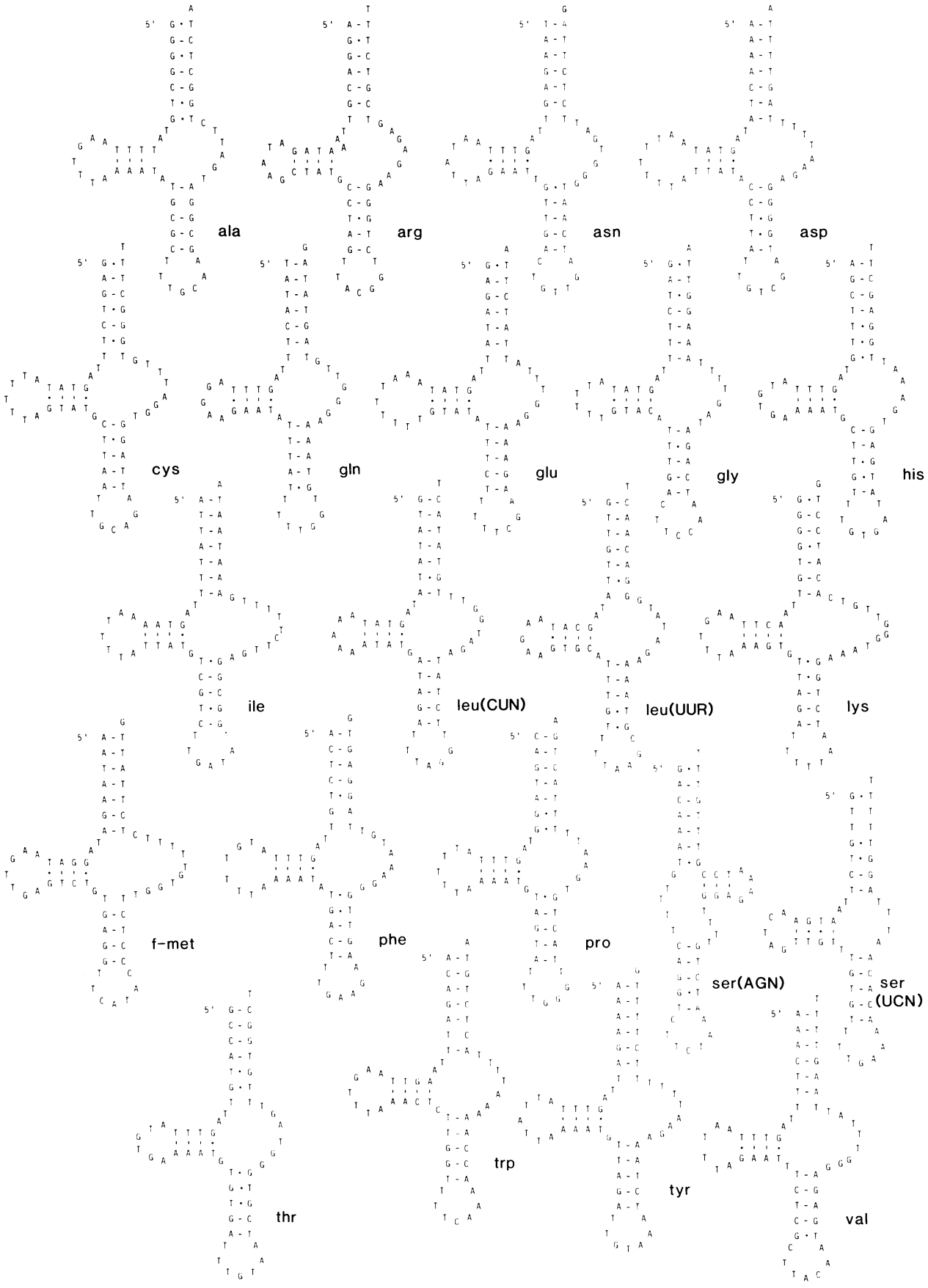

FIG. 2. The 22 tRNA genes of *A. suum* mtDNA shown in the presumed secondary structural form of the corresponding tRNAs. In all but one [Ser(AGN)] of these tRNA genes, the TΨC arm and variable loop are replaced with a loop of between 4 and 12 nt. The $tRNA^{Ser}_{AGN}$ gene resembles the $tRNA^{Ser}_{AGY}$ gene of mammalian mtDNA and the $tRNA^{Ser}_{AGY/A}$ gene of *D. yakuba* mtDNA: the TΨC arm and the variable loop are present but the D-arm is replaced with a loop of 5 nt.

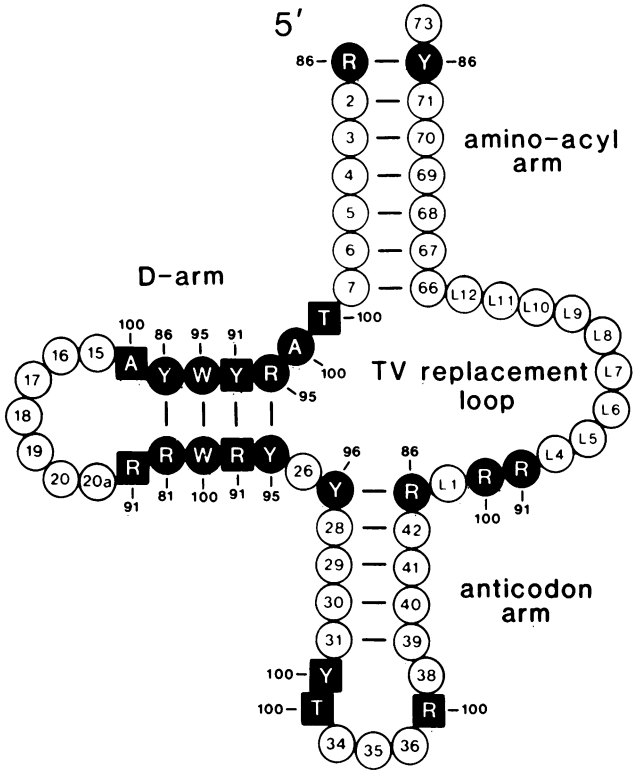

FIG. 3. Diagram of the consensus secondary structure of *A. suum* mt tRNA genes. The number of nucleotides shown in the D (dihydrouridine)-loop and the TV (TΨC arm-variable loop)-replacement loop are the maximum numbers observed. Letters in solid squares identify nucleotides or nucleotide combinations that occur with a frequency (accompanying numbers) of >90% among *A. suum* mt tRNA genes and are also constant nucleotides in prokaryotic and eukaryotic nuclear-encoded tRNAs. Letters in solid circles identify other nucleotides or nucleotide combinations that occur with a frequency (accompanying numbers) of >80% in *A. suum* tRNA genes. Nine of these nucleotides (1, 9, 10, 13, 22, 25, 27, 43, and 72) are considered semi-invariant in prokaryotic and eukaryotic nuclear-encoded tRNAs (29, 30). The numbering system used (1–43 and 66–73, as given in the open circles) reflects the conventional numbering system used for yeast $tRNA^{Phe}$ (31). L1–L12 denote the maximum of 12 nt observed in the TV-replacement loop of the *A. suum* mt tRNA genes (Fig. 2). A, adenine; T, thymine; R, adenine or guanine; Y, cytosine or thymine; W, adenine or thymine.

the first guanine of the $tRNA^{Cys}$ gene, which is the terminal nucleotide of the TAG termination codon of the *COI* gene. In vertebrate and *Drosophila* mtDNAs, some protein genes end in either T or TA, and it has been argued that these nucleotides are converted to a complete TAA termination codon by polyadenylylation of individual gene transcripts following precise cleavage of these transcripts from polycistronic primary transcripts (2, 8, 32). It is therefore possible that the terminal TA dinucleotide of the *A. suum COI* gene represents a similar situation and that, in fact, there is no overlap with the $tRNA^{Cys}$ gene. This view is strengthened by our finding that the *ND2* and *ND5* genes of *A. suum* mtDNA end with a single thymine nucleotide. There does, however, appear to be a single nucleotide overlap between the 3′ end of the $tRNA^{Ile}$ gene and the 5′ end of the $tRNA^{Arg}$ gene for which we have no alternative explanation (Fig. 1).

Computer analyses of the 899-ntp AT region, and the 117-ntp region between the *ND4* and *COI* genes (see legend to Fig. 1), and of both strands of the remainder of the molecule have failed to reveal sequences that can form secondary structures characteristic of vertebrate and *Drosophila* mt tRNA genes.

Transfer RNA Genes in *C. elegans* mtDNA. Respiration in adult *A. suum*, from which the mtDNA used in this study was isolated, is totally anaerobic, although early larval stages in the life cycle of *A. suum* involve aerobic respiration. It seemed plausible, therefore, that the peculiar tRNA genes of *A. suum* mtDNA are in some way associated with this organism's mode of respiration or some other aspect of its parasitic lifestyle. To test this possibility, we sequenced a segment of the mtDNA molecule from the free-living soil nematode *C. elegans*, an obligate aerobe. The segment sequenced contains the same genes as the equivalent segment of the *A. suum* mtDNA molecule (Fig. 1), including six tRNA genes, for aspartic acid, cysteine, glycine, histidine, methionine, and threonine. Each of these tRNA genes could be folded into secondary structures similar to those of the corresponding *A. suum* mtDNA genes with a TV-replacement loop. Differences in nucleotide sequence between the six corresponding tRNAs of *A. suum* and *C. elegans* are shown in Fig. 4. Overall similarities of sequences range from 58% ($tRNA^{Asp}$) to 85% ($tRNA^{fMet}$). There are a total of 95 differences in sequence, of which 86 are nucleotide substitutions and 9 are deletion/insertions. All of the deletion/insertions occur in D-loops and TV-replacement loops. Of the 50 substitutions that occur in stems, 44 (88%) are either compensated by a substitution in the complementary strand or involve changes such that G·C, G·T, or A·T pairing is still possible, thus maintaining secondary structure stability. The remaining six stem substitutions all involve a nucleotide pair that is a mismatch in a tRNA of one species but not in the other. These data add strength to the hypothesis that the TV-replacement loop-containing structures are, in fact, functional tRNA genes and indicate that these genes are a characteristic of nematode mtDNA rather than being limited to parasitic or partially anaerobic organisms.

Concluding Remarks. The observations and arguments presented above provide exceptionally strong support for the occurrence of a set of structurally unusual functional tRNAs in nematode mitochondria. However, direct evidence that this is the case must await isolation of actual tRNAs, their characterization by direct sequencing, and a demonstration that these tRNAs can be specifically charged. It is known that, at least in *C. elegans*, nuclear-encoded tRNAs are normal (33).

tRNAs are central to the deciphering of genetic information. As a group, these molecules must be able to interact with specific regions of rRNA molecules within the ribosome and at the same time maintain their individuality with regard to specific amino acid charging. Maintenance of a constant structure of tRNAs among living organisms suggests that this structure is extremely important to function. It has been suggested that the variations in size and sequence of the D-loops and TΨC-loops of vertebrate and of *Drosophila* mt tRNAs reflect how various tRNAs achieve their final tertiary structure (8). The dramatic structural change found in nematode mt tRNAs suggests that, in this case, function of the tRNAs may require major changes in structure and/or function of one or more of the other molecules with which tRNAs interact to effect information transfer. In this regard, it is interesting to note that the mt rRNA genes of *A. suum* and *C. elegans* are considerably smaller than the mt rRNA genes of *Drosophila* and vertebrates and that this results from loss of secondary structural elements (unpublished data).

The nematode mt tRNAs are a further addition to a growing list of genetic novelties found in mitochondria. In this case, the extensive loss of specific sequence makes it unlikely that reversion to a normal tRNA could occur. Thus, this bizarre tRNA form is likely to be diagnostic for groups of individuals showing a common lineage with nematodes.

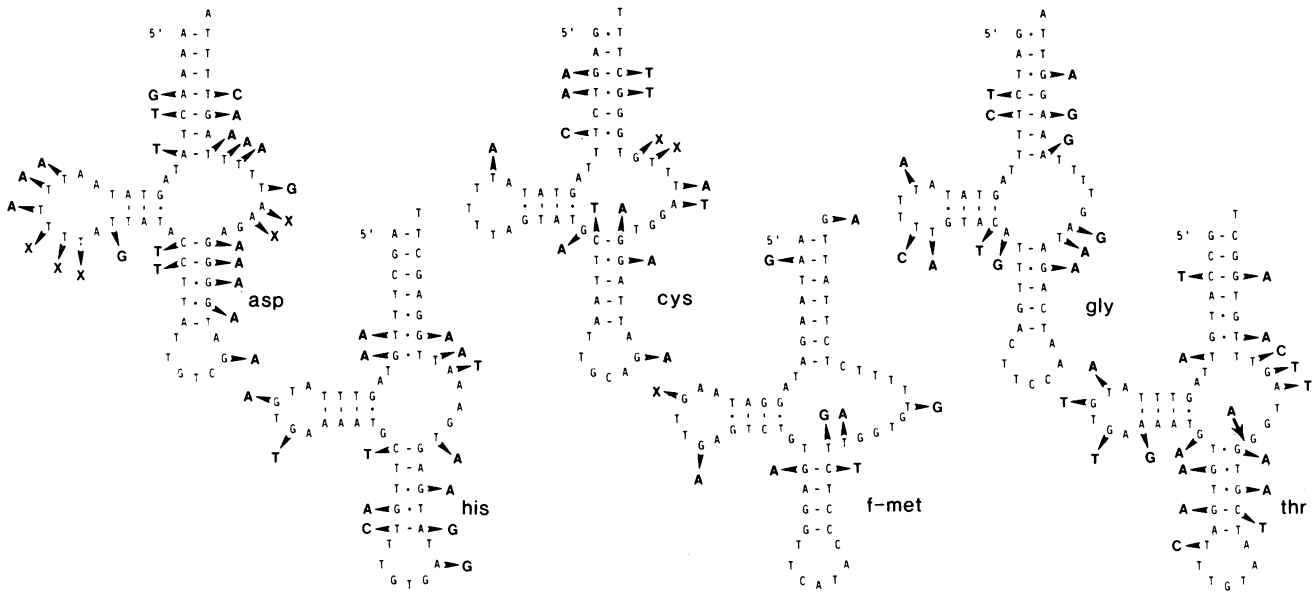

FIG. 4. Nucleotide differences between corresponding tRNA genes in mtDNAs of *A. suum* and *C. elegans*. Six mt tRNA genes of *A. suum* are shown in the presumed secondary structures of the corresponding tRNAs. Larger letters and arrowheads indicate substitutions found in the corresponding *C. elegans* tRNA genes. The letter A accompanying an arrow pointing between 2 nt in the $tRNA^{Thr}$ gene indicates an insertion; the letter X indicates a deletion.

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