Mitochondrial genes of *Schistosoma mansoni*†

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SUMMARY

Two clones, totalling 8068 bp and spanning over half of the coding region of the mitochondrial genome of *Schistosoma mansoni*, have been sequenced. Complete sequences are presented of the large and small ribosomal RNA subunits, CO2, ND3, ND4, ND6 and ATPase 6 genes. Incomplete sequences were found for the CO1, ND2 and CytB genes. At least 10 tRNAs were also detected and alternative structures for some of these discussed. The gene order of *S. mansoni* is unique and differs from that of *Fasciola hepatica*, the only other trematode for which any information is available.

Key words: mitochondrial genome, *Schistosoma mansoni*, ribosomal RNA genes, transfer RNAs.

INTRODUCTION

Mitochondrial genomes have attracted much interest in recent years. These small, circular genomes have evolutionary dynamics different from those of nuclear genomes and are more amenable to detailed characterization. They have been objects of intense comparative analysis among phyla and kingdoms (e.g. Boore & Brown, 1998) and sequences of their genes have been used in a multitude of studies in population genetics and phylogeny (reviewed by Avise, 1998). Very little information is available as yet for the mitochondrial genome of any member of the Platyhelminthes (flatworms), despite the fact that this is a large and ancient phylum. The largest fragment sequenced to date was derived from a clone (3·47 kb) spanning the ND1, ND3 and CO1 genes, as well as several tRNA genes, from the trematode *Fasciola hepatica* (see Garey & Wolstenholme, 1989). A cDNA clone from *F. hepatica*, characterized by Zurita *et al*. (1988), contained about 700 bp of the mitochondrial large subunit (LSU) ribosomal RNA gene. Virtually all other reported flatworm mitochondrial sequences were obtained by PCR using primers designed against the sequences mentioned above.

Here we present sequence from over half of the coding portion of the mitochondrial genome of an important parasite, *Schistosoma mansoni*, and discuss the properties that have enabled us to identify and characterize the genes present.

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† Note: sequences reported here are available from GenBank under the accession numbers AF130787 and AF130788.

MATERIALS AND METHODS

Després, Imbert-Establet & Monnerot (1993) purified mitochondrial DNA from a Brazilian strain (ms-BRE-1) of *Schistosoma mansoni* and digested it with a number of restriction endonucleases. They cloned into pBR322 two fragments of mtDNA from this strain. One fragment (2·8 kb) had been produced using the enzyme *Hin*dIII and the resultant clone was named pSmmH2-3 (referred to hereafter as the 2·8 kb clone). This clone was known to have an internal recognition site for *Hin*dIII. The second fragment (5·4 kb) was produced by digestion with the enzyme *Bcl*I. This was cloned into the (compatible) *Bam*HI site in pBR322 and the recombinant plasmid named pSmmBC2 (referred to hereafter as the 5 kb clone). We subcloned these inserts as follows. The 2·8 kb clone was digested with *Hin*dIII and the resulting fragments of 2·0 kb and 0·8 kb were separately cloned into pUC18 and sequenced using 'universal' sequencing primers and custom-made internal primers. That the 2 fragments were truly adjacent in the mitochondrial genome was confirmed by PCR across the fragment boundaries using genomic DNA from the NMRI strain of *S. mansoni* as substrate. The resulting PCR product was sequenced. The 5 kb clone was excised from the plasmid using the enzymes *Nhe*I and *Sph*I. These cut close to, and on either side of, the inactivated *Bam*HI site in pBR322. The excised insert was cloned into pUC18 to facilitate deletion cloning.

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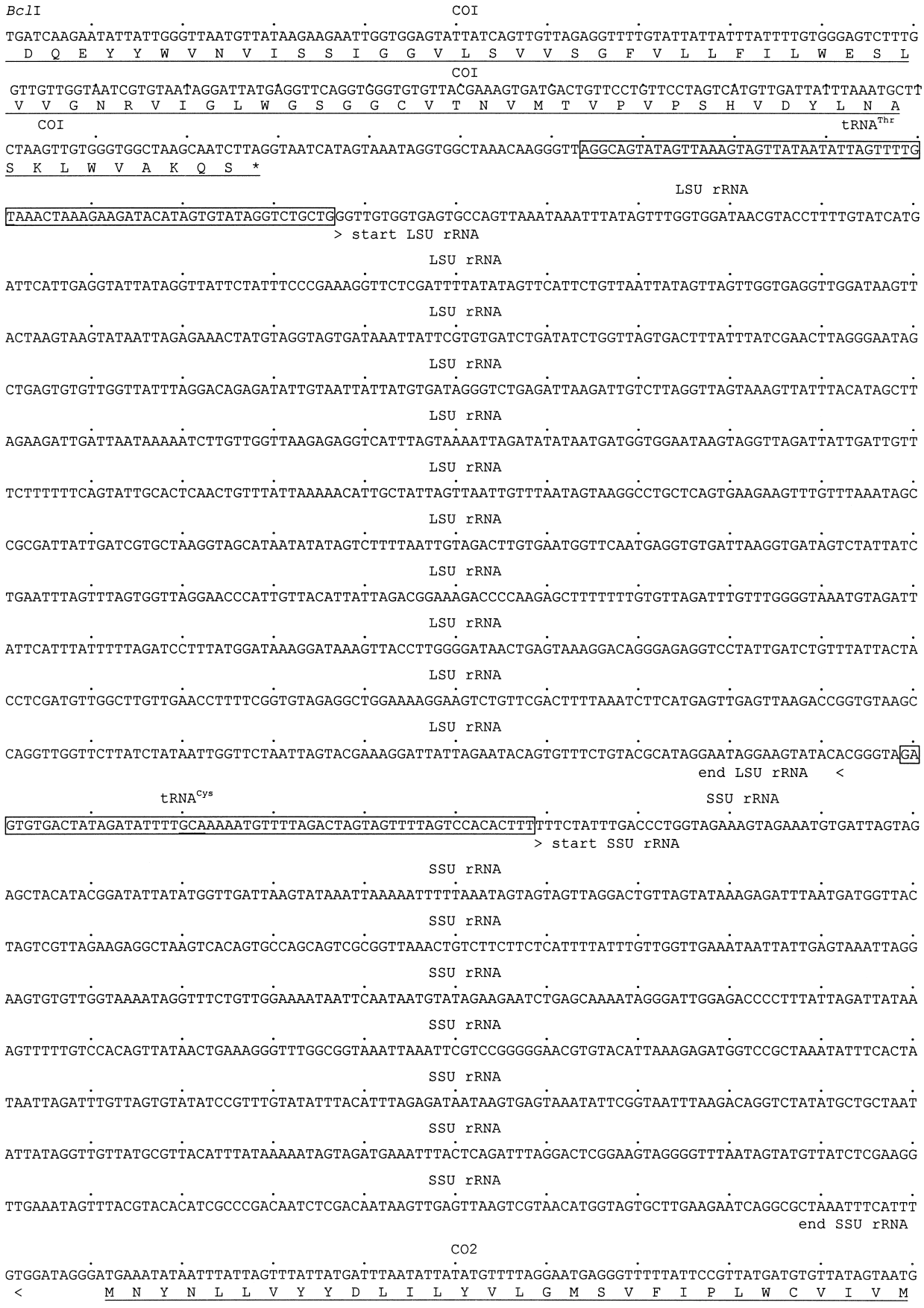

Fig. 1. For legend see p. 306.

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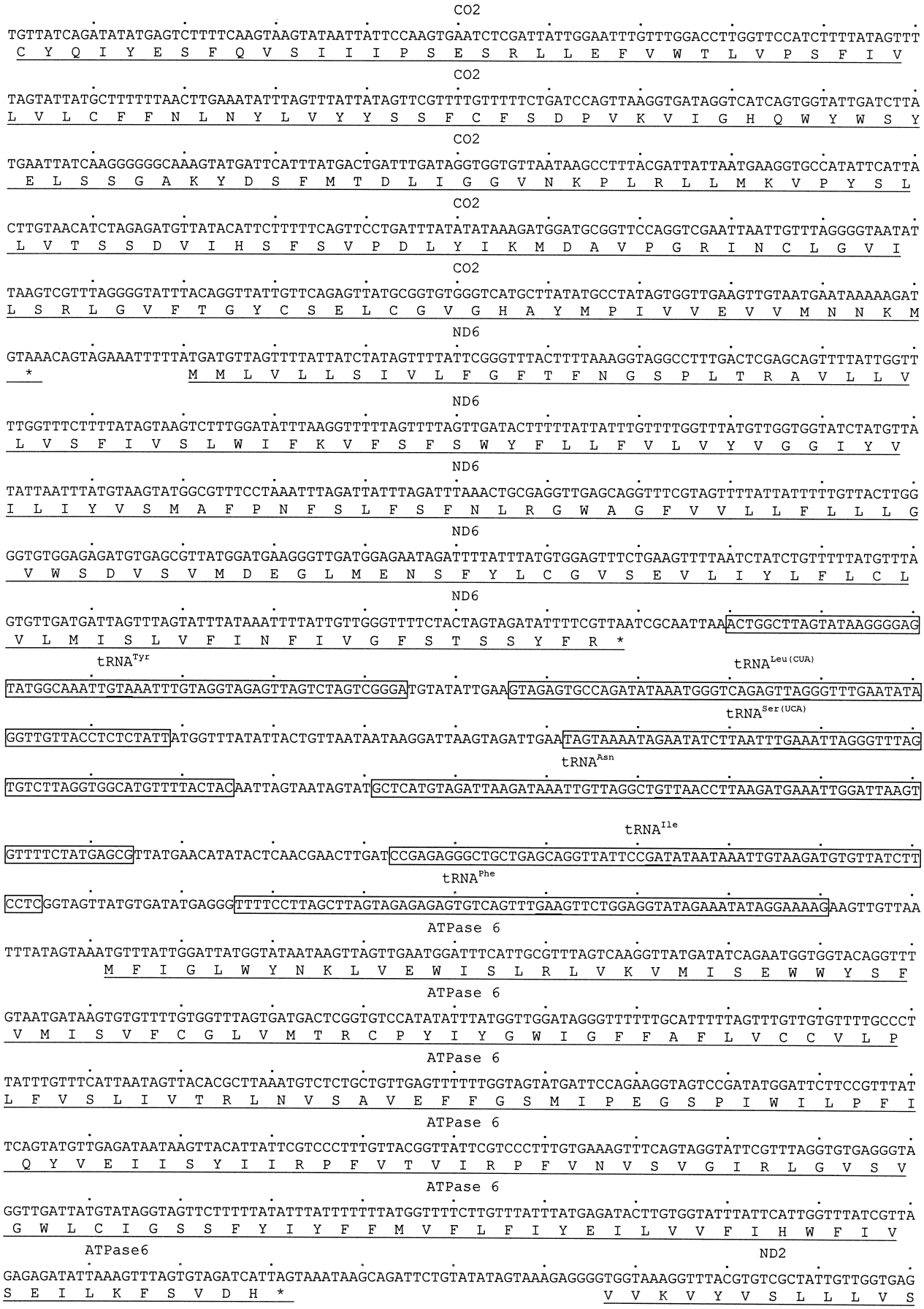

Fig. 1. For legend see p. 306.

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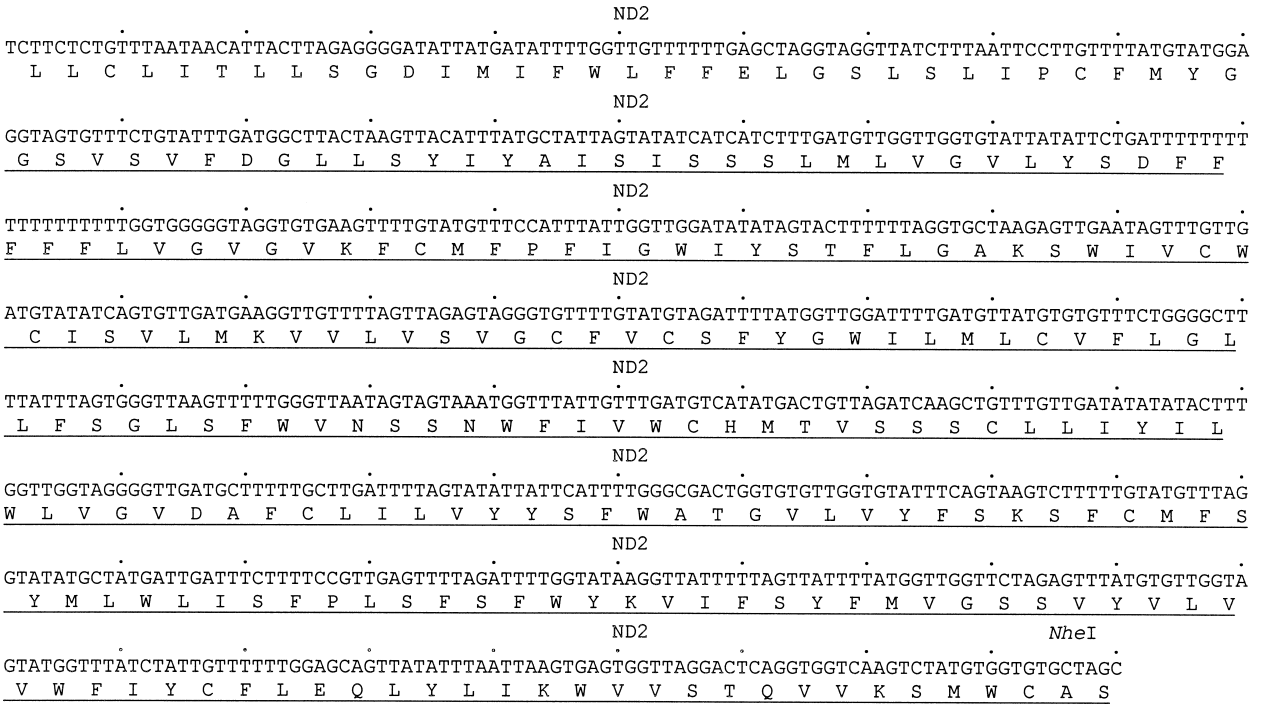

Fig. 1. Sequence of the 5 kb mitochondrial clone from *Schistosoma mansoni*. One hundred bases are shown per line, and a dot is placed over every tenth base. For protein coding genes, inferred amino acids are shown underlined below the DNA sequence. TAA is regarded as the stop codon for the CO2 and ND6 genes. Transfer RNA genes are boxed, and the anticodon underlined.

Sequencing of this insert was done by deletion cloning and by primer-walking using custom-made primers. After sequencing was completed, it became apparent that there must have been an internal site for *Nhe*I very close to one end of the insert. A small fragment (< 200 bp) of the insert and flanking pBR322 sequence was lost as a consequence.

Open reading frames were identified using ORF-Finder available through the GenBank web site and using the recognized genetic code for the trematode mitochondrial genome (GenBank Genetic Code 14). In most cases, Blast searches (Altschul *et al*. 1990) in GenBank identified each gene without difficulty. Start and stop codons were identified generally because the appropriate codons were found in the sequence.

Ribosomal rRNA genes were identified initially by similarities with published sequences. Their identities were confirmed and the approximate locations of their ends were found by working out secondary structure features. Secondary structure was determined mainly 'by eye' and the structures drawn using RNAVIZ (De Rijk & De Wachter, 1997). Helices were numbered according to Van de Peer *et al*. (1998) (small subunit – SSU) and De Rijk *et al*. (1998) (large subunit – LSU).

Various methods were used to identify transfer RNA genes. Some were identified by submitting the sequences to tRNAScan (Lowe & Eddy, 1997). However, this failed to identify all of the tRNA genes present. Additional genes were identified by searching candidate regions using a tRNA secondary structure template set up in MULTALIN (Corpet, 1988). This template was moved along the sequence until tRNA-like regions were found and then adjusted until a tRNA-like structure could be demonstrated. Potential tRNA structures were compared with known or suspected structures from other invertebrate mitochondria (Wolstenholme, 1992; Yamazaki *et al*. 1997).

RESULTS AND DISCUSSION

The 5 kb clone is 5190 bp (including the recognition sites for *Bcl*I and *Nhe*I) and contains, in order, the genes for CO1 (3′ end only), $tRNA^{Thr}$, LSU rRNA, $tRNA^{Cys}$, SSU rRNA, CO2, ND6, $tRNA^{Tyr}$, $tRNA^{Leu(CUA)}$, $tRNA^{Ser(UCA)}$, $tRNA^{Asn}$, $tRNA^{Ile}$, $tRNA^{Phe}$, ATPase 6, ND2 (incomplete) (Fig. 1).

The 2·8 kb clone is 2878 bp long and contains, in order, CytB (3′ end), ND4, $tRNA^{Aln}$, $tRNA^{Lys}$, ND3 and possibly $tRNA^{Pro}$ (see below) (Fig. 2).

These clones contain between them partial or complete sequences of 8 of the 12–13 protein-coding genes that typically occur in metazoan mt genomes, both of the ribosomal RNA genes and 10 or 11 of the 22 expected tRNAs. All genes code on the same strand within a single clone. However, we could not confirm that the 2 clones are in the same orientation relative to each other.

Base composition is comparable with that found in other invertebrates (Wolstenholme, 1992) in that

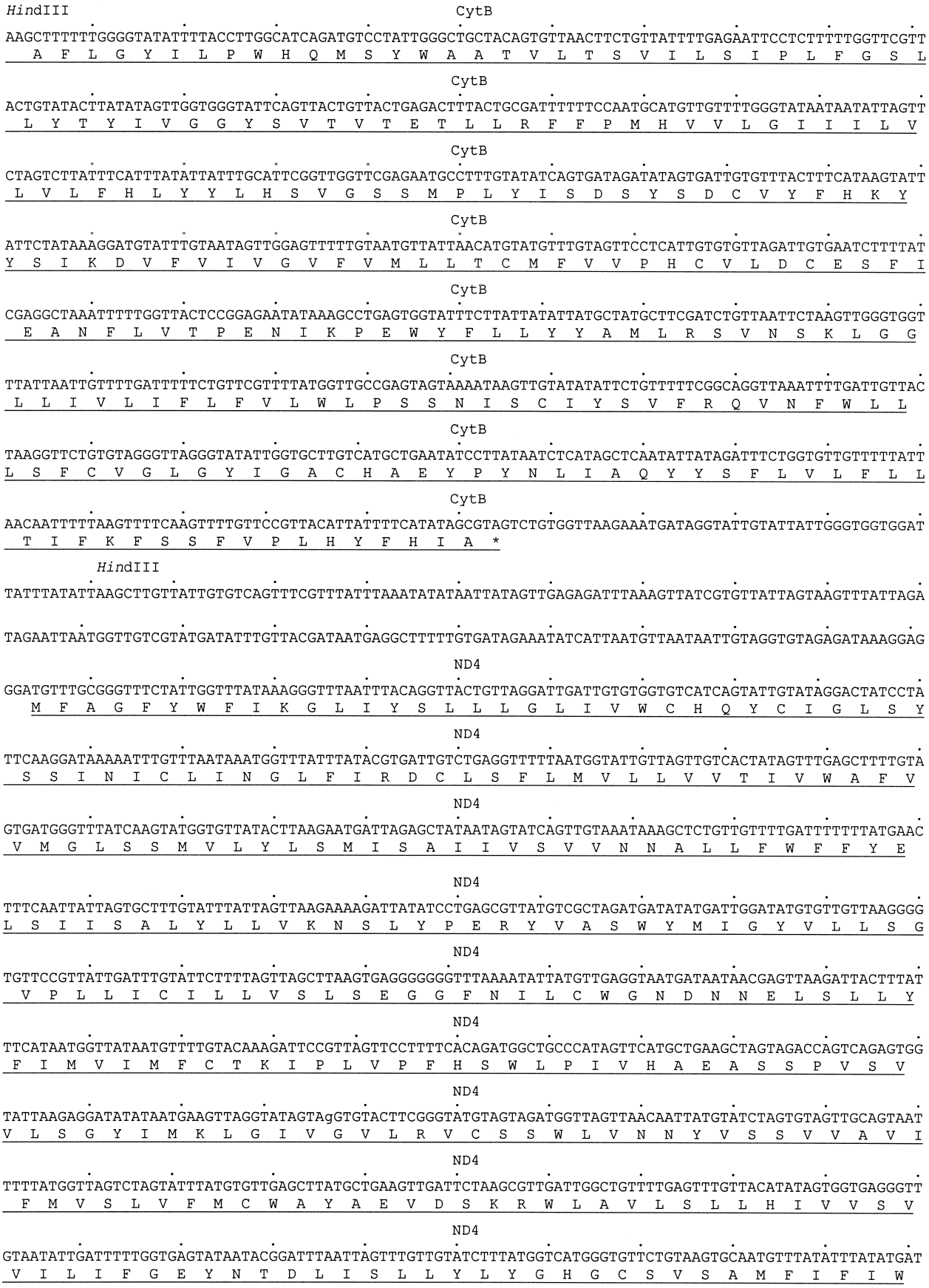

Fig. 2. For legend see p. 308.

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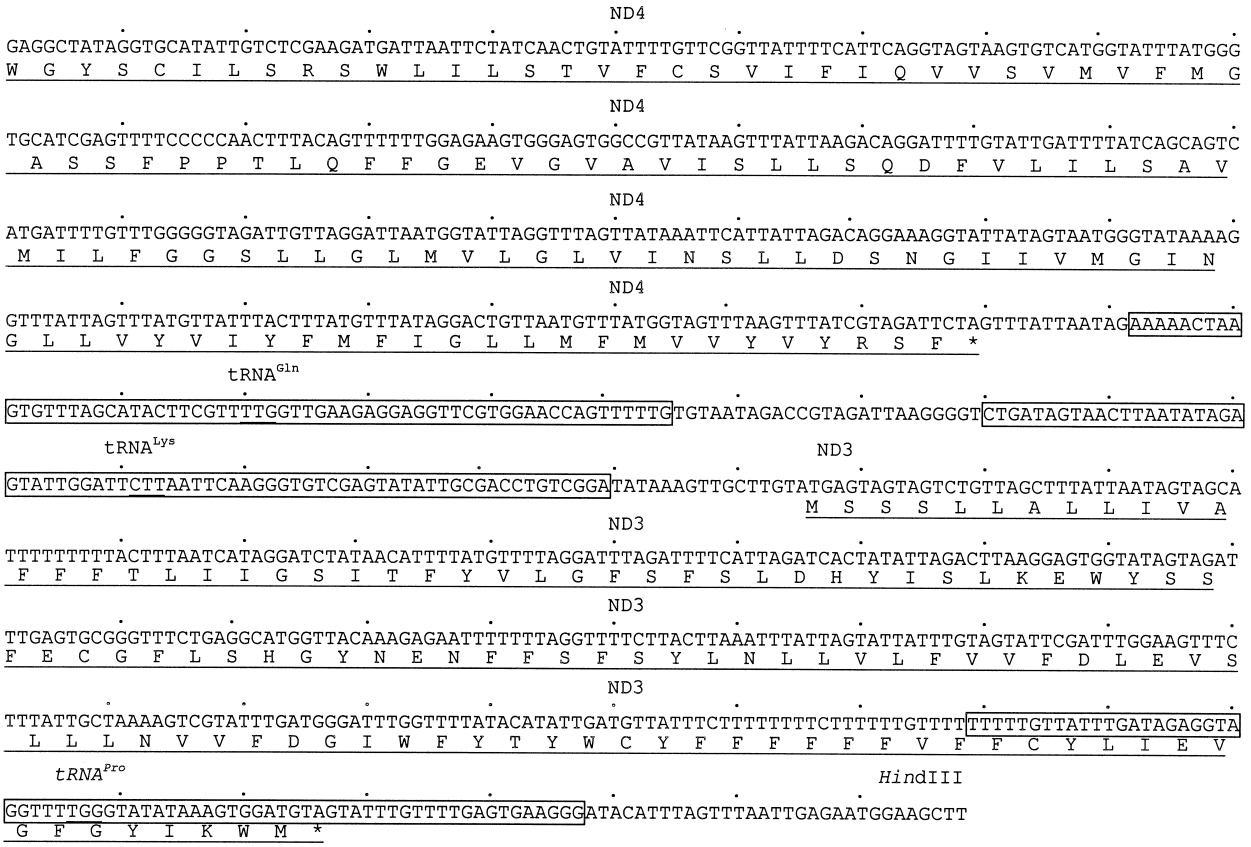

Fig. 2. Sequence of the 2·8 kb mitochondrial clone from *Schistosoma mansoni*. Details as for Fig. 1.

Table 1. Codon usage for mitochondrial protein-coding genes in *Schistosoma mansoni*

Codon	Count	Codon	Count	Codon	Count	Codon	Count
TTT-Phe	149	TCT-Ser	37	TAT-Tyr	95	TGT-Cys	46
TTC-Phe	14	TCC-Ser	2	TAC-Tyr	11	TGC-Cys	5
TTA-Leu	138	TCA-Ser	26	TAA-***	3	TGA-Trp	22
TTG-Leu	64	TCG-Ser	7	TAG-***	5	TGG-Trp	35
CTT-Leu	17	CCT-Pro	16	CAT-His	21	CGT-Arg	11
CTC-Leu	2	CCC-Pro	5	CAC-His	2	CGC-Arg	1
CTA-Leu	10	CCA-Pro	11	CAA-Gln	4	CGA-Arg	8
CTG-Leu	14	CCG-Pro	9	CAG-Gln	11	CGG-Arg	3
ATT-Ile	62	ACT-Thr	20	AAT-Asn	24	AGT-Ser	53
ATC-Ile	7	ACC-Thr	1	AAC-Asn	3	AGC-Ser	4
ATA-Ile	76	ACA-Thr	10	AAA-Asn	20	AGA-Ser	37
ATG-Met	61	ACG-Thr	3	AAG-Lys	32	AGG-Ser	18
GTT-Val	112	GCT-Ala	27	GAT-Asp	27	GGT-Gly	59
GTC-Val	10	GCC-Ala	1	GAC-Asp	1	GGC-Gly	2
GTA-Val	62	GCA-Ala	9	GAA-Glu	17	GGA-Gly	17
GTG-Val	38	GCG-Ala	5	GAG-Glu	22	GGG-Gly	26

there is a bias towards T. The nucleotide least represented is C. Base composition percentages for *S. mansoni* are (values for *Fasciola hepatica* reported by Garey & Wolstenholme (1989) are given in parentheses): A, 26·7 (15·3); C, 8·5 (9·5); G, 22·8 (27·9); T, 42·0 (47·3). The bias towards T is more marked in *F. hepatica* and A occurs much less frequently than in *S. mansoni*. However, these differences may not hold when the same regions of both species can be compared. Codon usages (Table 1) reflect the nucleotide bias. The frequency of C is very low and codons ending with this base are rare (4·3 % of total).

Assuming that it starts with the conventional ATG start codon, the ND2 gene would be preceded by an uncharacterized stretch of 105 bp immediately downstream of the ATPase 6 gene. No tRNA-like structure could be identified in this region. It is

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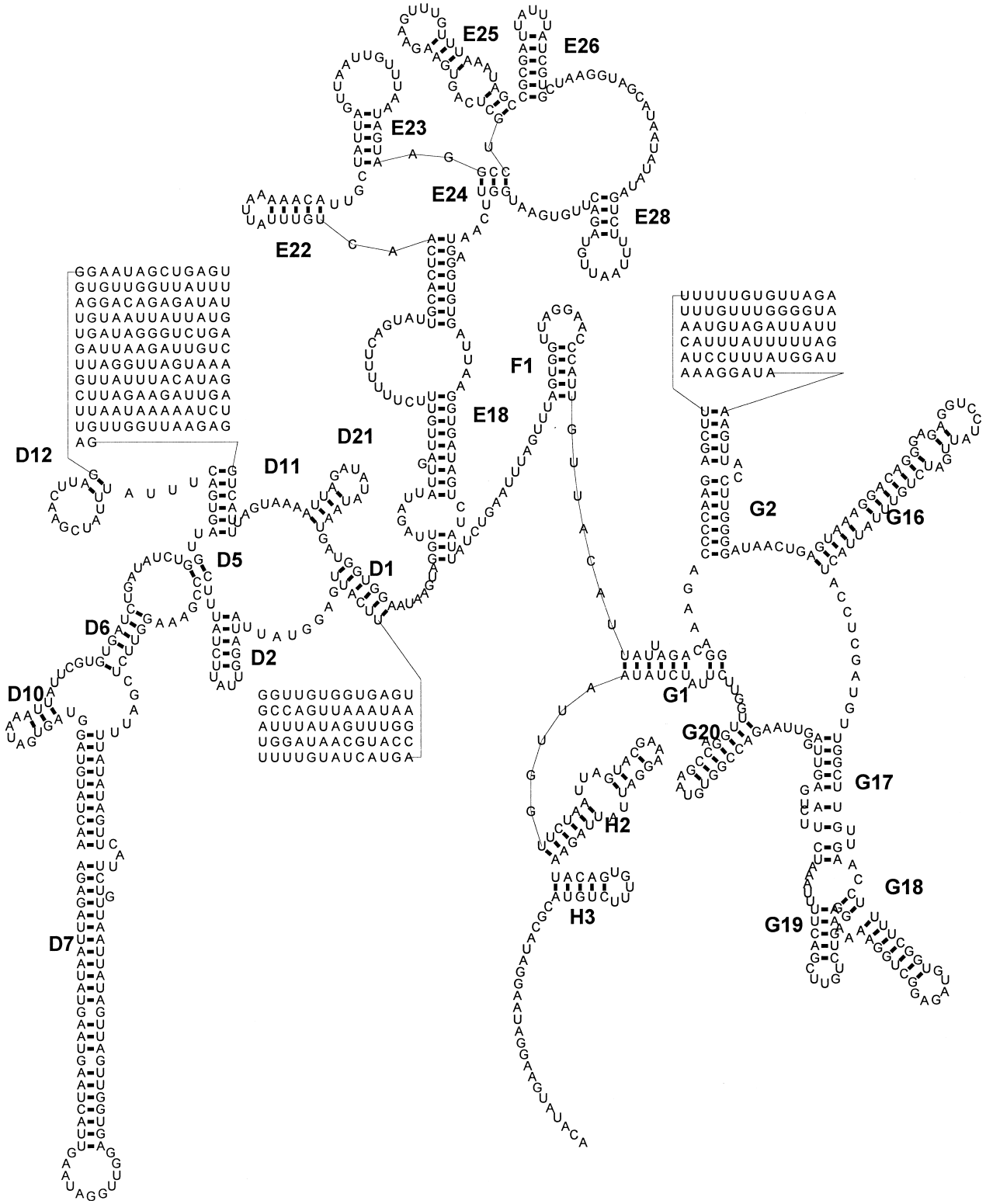

Fig. 3. Secondary structure of the LSU rRNA gene. Some portions were not folded and are shown unformatted.

likely that the ND2 gene in fact commences at an atypical start codon such as ATN, GTG or TTG (reviewed by Wolstenholme, 1992). Several such codons occur, in frame, in the region 5′ to the start of ND2. We have therefore assumed that the ND2 gene commences at the GTG codon commencing 34 bp downstream from the stop codon of the ATPase 6 gene.

The codon TAG functions as a stop codon in most cases. For 2 genes, this does not appear to be true: CO2 and ND6. Assuming TAG to be the actual stop codon, then these 2 genes would be copied onto a single transcript ending within $tRNA^{tyr}$. It is possible that this happens and that both genes are separated by later processing. BLAST searches in GenBank identified several expressed sequence tags

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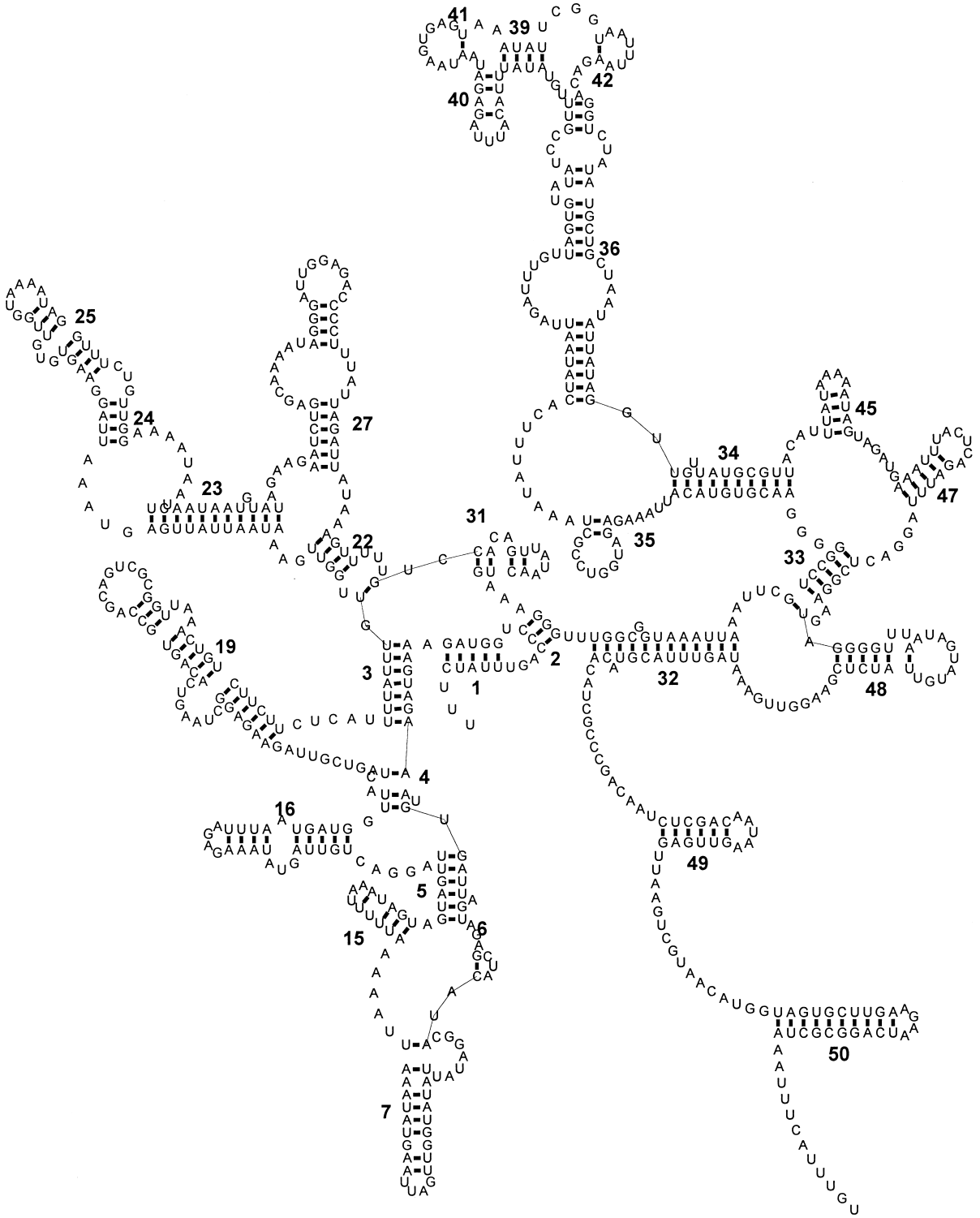

Fig. 4. Secondary structure of the SSU rRNA gene.

(ESTs) from *S. mansoni* that overlap both CO2 and ND6 coding regions by a substantial amount (over 100 bases) suggesting co-transcription. It is also possible that TAA functions as a stop codon in *S. mansoni*. This codon occurs only 3 times in our sequences. One occurrence is at a location corresponding to the 3′ end of the CO2 gene by comparison with other metazoan species, one at the 3′ end of the ND6 gene (18 codons upstream from the TAG in $tRNA^{Tyr}$ and separated by 10 bases from the start of this tRNA). The third occurs 7 codons upstream from the TAG at the end of the ND4 gene, so could represent the true stop codon there.

The ribosomal RNA genes are typical of those of eumetazoans. The lengths of the LSU (1056) and SSU genes (744) are rather shorter than in other eumetazoans except nematodes, in which the rRNA genes are particularly 'cut down' (Okimoto,

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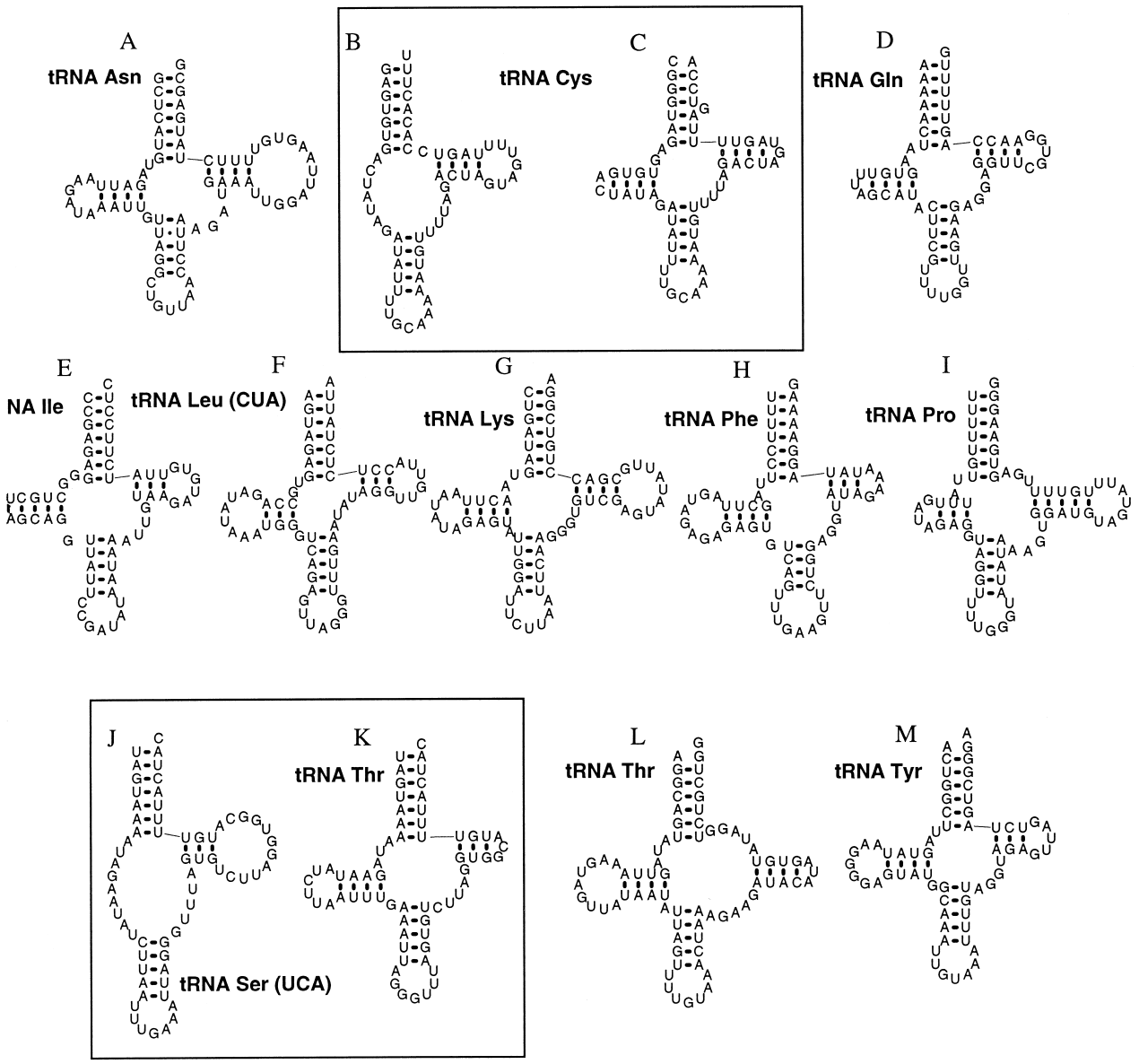

Fig. 5. Secondary structures of transfer RNA genes. (B and C) Alternative foldings for $tRNA^{Cys}$. (I) Putative $tRNA^{Pro}$ that overlaps the ND3 gene. (J and K) Alternative foldings for a stretch of sequence that could represent either $tRNA^{Ser(UCA)}$ or $tRNA^{Thr}$.

MacFarlane & Wolstenholme, 1994; Keddie, Higazi & Unnasch, 1998). The starts and ends of ribosomal genes are often hard to identify. Knowledge of secondary structure (Figs 3 and 4) provides some indication of where these might lie. In *S. mansoni*, there are other identified genes in close proximity at each end, thus narrowing possibilities further.

Mitochondrial transfer RNA genes are difficult to identify because of their small size and (especially among invertebrate taxa) considerable variability, and sometimes ambiguity, of structure. We identified 12 tRNA-like structures. Two of these overlapped completely in sequence but were not isoacceptors (see below), another had a substantial overlap with a protein-coding gene and a third could be folded into 2 different structures with the same anticodon. Until the complete mtDNA sequence for *S. mansoni* is known and it is possible to compare alternative structures, it will be difficult to resolve some of these ambiguities. Similar ambiguities and uncertainties have been reported for other invertebrates (e.g. by Boore & Brown, 1994; Yamazaki *et al.* 1997).

The $tRNA^{Ser(UCA)}$ gene sequence (Fig. 5J) can also be folded to produce a $tRNA^{Thr}$-like structure (Fig. 5K). However, a $tRNA^{Thr}$ (Fig. 5L) was also identified between the CO1 and LSU rRNA genes. Both of these threonine tRNAs are unusual in structure, but not beyond the range of possibilities seen among invertebrate mitochondria. For the moment, we regard the $tRNA^{Ser}$ as the correct structure.

Between the LSU and SSU rRNA genes occurs $tRNA^{Cys}$. The 2 alternative structures into which the

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sequence can be folded are shown in Fig. 5B, C. We cannot be sure which is the correct one. Absence of a dihydrouridine arm, as shown in Fig. 5B, is unusual for a cysteine tRNA. On the alignment in Fig. 1 we have boxed the sequence that forms this structure. The alternative form (Fig. 5C), while exhibiting all the usual stems seen in a tRNA, requires the presence of an unpaired base in the aminoacyl arm. Such unpaired bases have been reported for land snails by Yamazaki *et al*. (1997) and nematodes (e.g. Keddie *et al*. 1998).

A structure conforming to $tRNA^{Pro}$ could be recognized in *S. mansoni* (Fig. 5I), but it overlaps the 3′ end of the ND3 gene by 49 bases. An overlap of this magnitude appears to be unprecedented in mitochondrial genomes. It is possible that the ND3 gene ends earlier than shown in Fig. 2. However, the alignment of ND3 amino acid sequences from a number of organisms (not shown) does not support that possibility. In *F. hepatica* (see Garey & Wolstenholme, 1988) and several cestodes (Kokaze *et al*. 1997), $tRNA^{pro}$ lies in a cluster of tRNAs just 5′ of the ND3 gene.

Most of the tRNA structures are similar to those reported for *F. hepatica* by Garey & Wolstenholme (1989). The aminoacyl stem is 7 bp long (except in $tRNA^{Thr}$ (Fig. 5L), $tRNA^{Leu}$ (Fig. 5F and $tRNA^{Tyr}$ (Fig. 5M)) in which there are 6 bp). The dihydrouridine stem is 3–4 bp long, except in $tRNA^{Cys}$ (Fig. 5B) and $tRNA^{Ser(UCA)}$ (Fig. 5J), both of which lack this arm. The dihydrouridine loop varies from 3 to 9 bases. The anticodon stem is 5 bp in every case and the anticodon loop 7 bases long, except in $tRNA^{Phe}$ in which the stem is 4 bp long and the loop 9. The TΨC stem is 3–5 bp, with 3 and 4 bp being the commonest. The associated loop has 3–13 bases. The anticodon and TΨC stems are separated by 4 unpaired bases (5 in both possible $tRNA^{Thr}$ structures (Fig. 5K, L) and $tRNA^{Ser(UCU)}$). In most cases, the TΨC and aminoacyl stems are immediately adjacent, with no intervening unpaired bases. However, in $tRNA^{Thr}$ (Fig. 5L) there are 5 unpaired bases, and in the putative $tRNA^{Pro}$, there are 3. Both $tRNA^{Cys}$ (Fig. 5B) and $tRNA^{Ser(UCN)}$ have a loop replacing the dihydrouridine stem. A similar situation has been found for $tRNA^{Ser(AGN)}$ in many species. The $tRNA^{Ser(UCN)}$ lacks the dihydrouridine stem in nematodes (Keddie *et al*. 1998) and possibly also in some annelids and molluscs (Boore & Brown, 1994, 1995; Yamazaki *et al*. 1997).

Non-coding regions or regions otherwise uncharacterized are generally short (10–50 bp). The longest region (259 bp) is between the CytB and ND3 genes. This contains some short open reading frames, but no database matches could be found.

The gene order is different from that known in any other described mitochondrial genome. However, given the incomplete nature of the data and the fact that the orientation and location of the clones relative to one another are unknown, it is premature to attempt a detailed analysis. Gene orders are known only for fragments of the mt genome of other parasitic flatworms. In *F. hepatica*, the gene order in a 3466 bp segment is $tRNA^{Ala}$, $tRNA^{Asp}$, ND1, $tRNA^{Asn}$, $tRNA^{Pro}$, $tRNA^{Ile}$, $tRNA^{Lys}$, ND3, $tRNA^{Ser(AGN)}$, $tRNA^{Trp}$ and CO1 respectively (Garey & Wolstenholme, 1989). As is the case in *S. mansoni*, all genes identified in *F. hepatica* to date are encoded on the same strand. The complete genes in common with our sequences are ND3, $tRNA^{Asn}$, $tRNA^{Ile}$ and $tRNA^{Lys}$. The order is clearly different between the 2 species. In *F. hepatica*, the protein-coding gene preceding ND3 is ND1: in *S. mansoni*, ND3 is preceded by ND4. The ND3 in *S. mansoni* is preceded by $tRNA^{Gln}$ and $tRNA^{Lys}$ whereas in *F. hepatica* it is preceded by 4 tRNAs, of which $tRNA^{Lys}$ is the only one also found in this location in *S. mansoni*. Both $tRNA^{Asn}$ and $tRNA^{Ile}$, which lie between ND1 and ND3 in *F. hepatica* are located elsewhere in *S. mansoni*. Fernández *et al*. (1998) sequenced the ND3 gene for trematodes of the families Campulidae, Nasitrematidae and Dicrocoeliidae. They designed primers flanking the ND3 gene and based on the sequences of $tRNA^{Ile}$ and $tRNA^{Ser(AGN)}$ for *F. hepatica*. Presumably these tRNAs are present in the species they studied, as well as $tRNA^{Lys}$ which lies immediately 5′ of the ND3 gene as in *F. hepatica* and *S. mansoni*. In several cestode species (Kokaze *et al*. 1997), the ND3 gene is preceded by the same 4 tRNAs as found in *F. hepatica* and followed by $tRNA^{Trp}$. $TRNA^{Ser(AGC)}$ does not appear to occur in this location in the cestodes. Thus, *S. mansoni* appears to depart from a gene order around the ND3 gene that is otherwise largely conserved in cestodes and in other trematodes.

The information provided here is a major addition to our knowledge of the mitochondrial genome of parasitic flatworms.

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