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DNA sequence of the *Xenopus laevis* mitochondrial heavy and light strand replication origins and flanking tRNA genes

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ABSTRACT

We have determined the primary structure of the two regions of the Xenopus laevis mitochondrial genome which encompass the origins of heavy (H) and light (L) strand replication. The first segment, which consists of 2398 nucleotides, contains the displacement loop (D-loop), the tRNA genes for threonine, proline and phenylalanine, the origin of H-strand replication, and the promoters of H- and L-strand transcription. The second segment, which consists of 447 nucleotides, contains the L-strand replication origin flanked by the tRNA genes for tryptophan, alanine, asparagine, cysteine, and tyrosine. A comparison of the sequences of the Xenopus laevis mitochondrial L-strand replication origin region and the eight tRNA genes with their counterparts from the mammalian mitochondrial genomes reveals that these regions are quite homologous, while its D-loop region shows only slight homology with those of the mammalian mitochondrial genomes.

INTRODUCTION

All eucaryotes examined to date contain a double stranded mitochondrial specific DNA which codes for several mitochondrial components. In higher vertebrates, the mitochondrial DNA ranges in size from 14.5 to 19.5 kb (1) and codes for the 22 tRNAs and 2 ribosomal RNAs required for the mitochondrial protein synthesizing system. In addition, these genomes code for at least five known polypeptides (3 subunits of cytochrome C oxidase, ATPase subunit 6, and cytochrome b) and may code for an additional eight polypeptides in the other available unidentified open reading frames (URFs) (2-4).

The complete nucleotide sequences of the human, bovine, and mouse mitochondrial genomes (2-4), and the restriction maps and partial nucleotide sequences of several other higher eucaryote mitochondrial genomes have been reported (5-12). These studies reveal that higher eucaryote mitochondrial DNAs have common elements of overall gene organization but differ slightly in their nucleotide sequences. In contrast, the Drosophila yakuba mitochondrial ribosomal RNA genes and the origin of replication occupy similar relative positions (13-15), but the overall gene order differs from that observed for

the mammalian mitochondrial genome (16).

Higher eucaryote mitochondrial DNAs replicate by a mechanism in which each strand contains its own, physically distinct replication origin (17). Heavy strand (H-strand) replication begins in the displacement loop (D-loop) region while light strand (L-strand) replication does not begin until approximately two-thirds of the H-strand has been replicated (18-19). This asynchronous mechanism of mitochondrial DNA replication requires the displacement of the H-strand by synthesis of a complementary DNA fragment to form a D-loop structure which is flanked by the genes for $tRNA^{Phe}$ on one side and the genes for $tRNA^{Thr}$ and $tRNA^{Pro}$ on the other. The H-strand replication proceeds from this D-loop region without concomitant L-strand replication until it encounters a cluster of five tRNA genes with the first two separated from the last three by a G-C rich stem and T rich loop. It is here that the L-strand replication begins (20).

The Xenopus laevis mitochondrial genomic D-loop region contains a hybridized 14S single stranded DNA initiation segment, while the mammalian mitochondrial genomic D-loop regions contain a 7S single stranded DNA initiation segment. The amphibian 14S DNA consists of at least two species, 1350 and 1510 nucleotides in length (21). The mammalian 7S DNAs are smaller than their amphibian counterparts and are in the size range of between 500 and 630 nucleotides (21-22). Sequence analysis (2-4,9), electron microscopy (23), and direct isolation of expanded D-loop structures (20) have shown that the mammalian mitochondrial D-loop regions map in the segment flanked by the genes for $tRNA^{Pro}$ and $tRNA^{Phe}$, and span 1122 nucleotides in the human, 910 in the bovine, 879 nucleotides in the mouse, and at least 717 nucleotides in the rat mitochondrial genome (2-4,9). In the Xenopus laevis mitochondrial genome the origin of H-strand replication has been located by electron microscopy studies (24) and restriction enzyme mapping (5).

In this report we present the complete nucleotide sequence of the region of the Xenopus laevis mitochondrial genome which begins at the $tRNA^{Thr}$ and $tRNA^{Pro}$ genes, continues through the 2134 nucleotide D-loop region and extends into the 12S ribosomal RNA gene immediately following the $tRNA^{Phe}$ gene. In addition, we present the complete nucleotide sequence of the region which begins in URF2 and extends into the cytochrome C oxidase subunit I (CoI) gene. This region also contains the $tRNA^{Trp}$ and $tRNA^{Ala}$ genes, the origin of L-strand replication, and the $tRNA^{Asn}$, $tRNA^{Cys}$ and $tRNA^{Tyr}$ genes. Although there is little homology among the D-loop regions of the Xenopus laevis mitochondrial genome and those of other higher eucaryotes, the few

regions of sequence similarities could represent segments which perform similar functions. In contrast, the regions encoding the eight tRNA genes, a portion of the 12S ribosomal RNA gene, a portion of the cytochrome C oxidase subunit I gene and the origin of L-strand replication show significant homology with their counterparts from other higher eucaryotes.

MATERIALS AND METHODS

A clone containing the entire Xenopus laevis mitochondrial genome inserted into the BamHl site of pBR-322 (pXlm-31) and transvected into E. coli strain Hb-101 was obtained from Dr. I. Dawid (NIH, Bethesda, Md.). The complete mitochondrial genomic insert was excised by restriction endonuclease cleavage with BamHl, purified by preparative electrophoresis on low melting 0.7% agarose gels, eluted by a modified freeze-thaw method (25), and concentrated by ethanol precipitation. After further digestion with selected restriction endonucleases, fragments of the Xenopus laevis mitochondrial genome were ligated into either M13-mp8, -mp9, -mp10 or -mp11 (26). In some instances the linearized genome was treated with EcoRl to cleave the DNA into three fragments, one of which encompassed the D-loop region (5,24). This fragment was gel purified as described above, treated with nuclease Bal-31 for short time periods, and blunt end ligated into the SmaI site of M13-mp9. After transvection into E. coli strain JM-101, the single stranded recombinant phage DNAs containing fragments of the Xenopus laevis

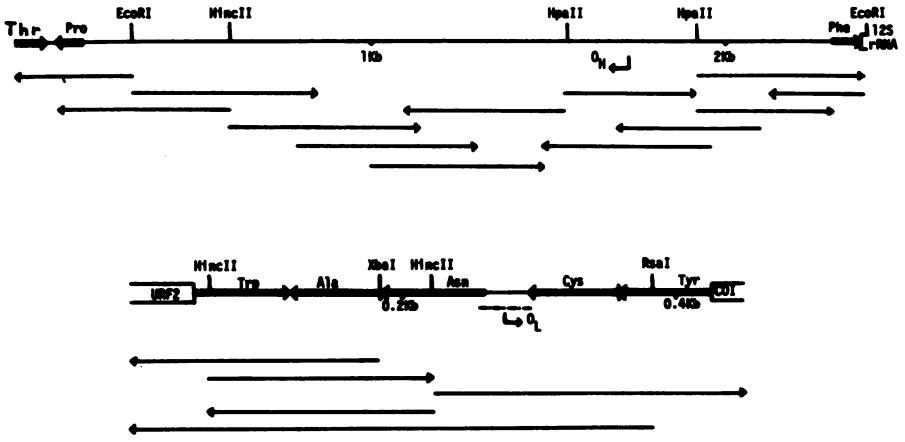
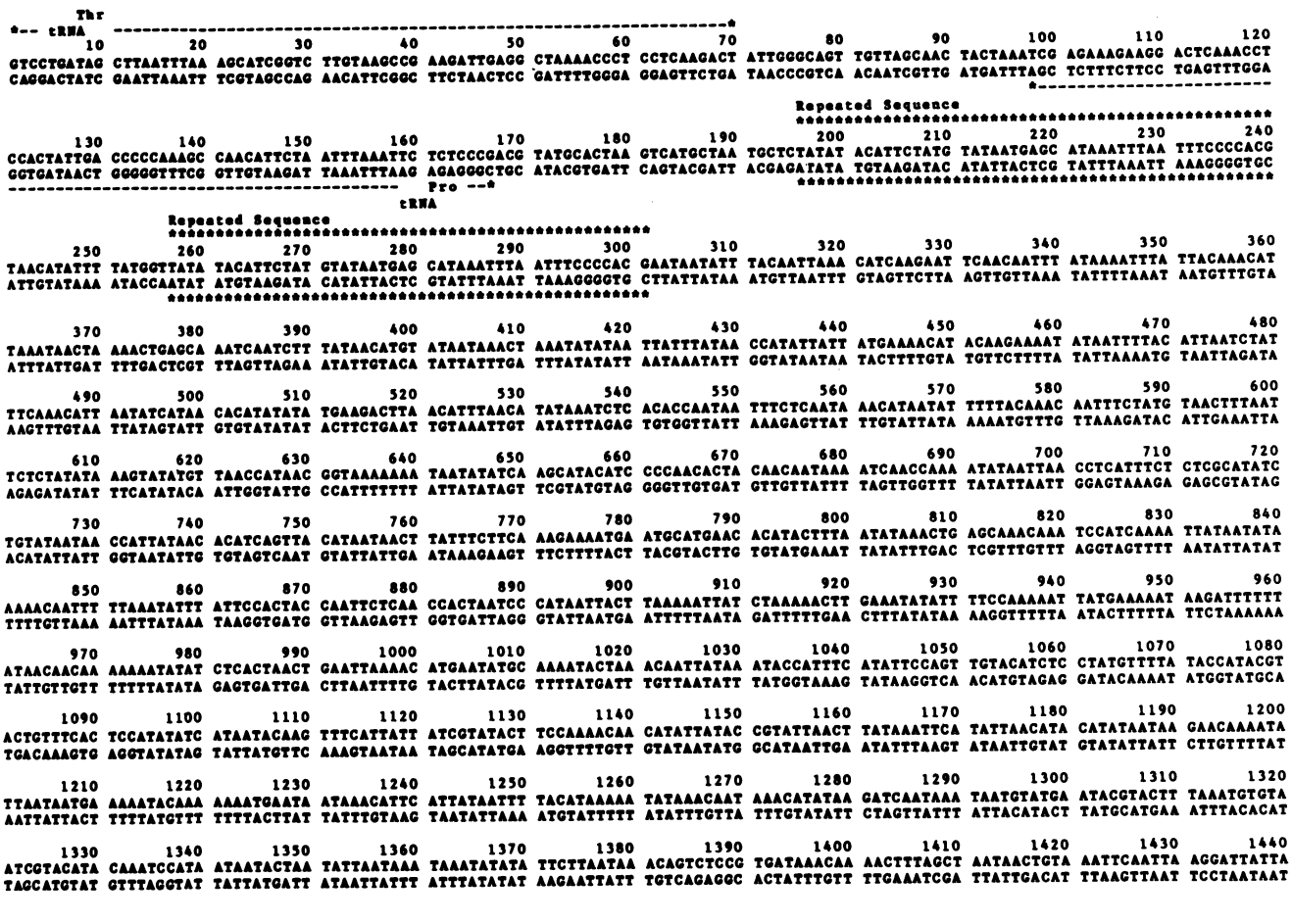


Figure 1. The restriction sites and sub-clones used for sequencing the regions of Xenopus laevis mitochondrial genomes which encompass the origins of H- and L-strand replication and their flanking genes.


Thr
tRNA
Pro
tRNA
Repeated Sequence
Repeated Sequence
Repeated Sequence

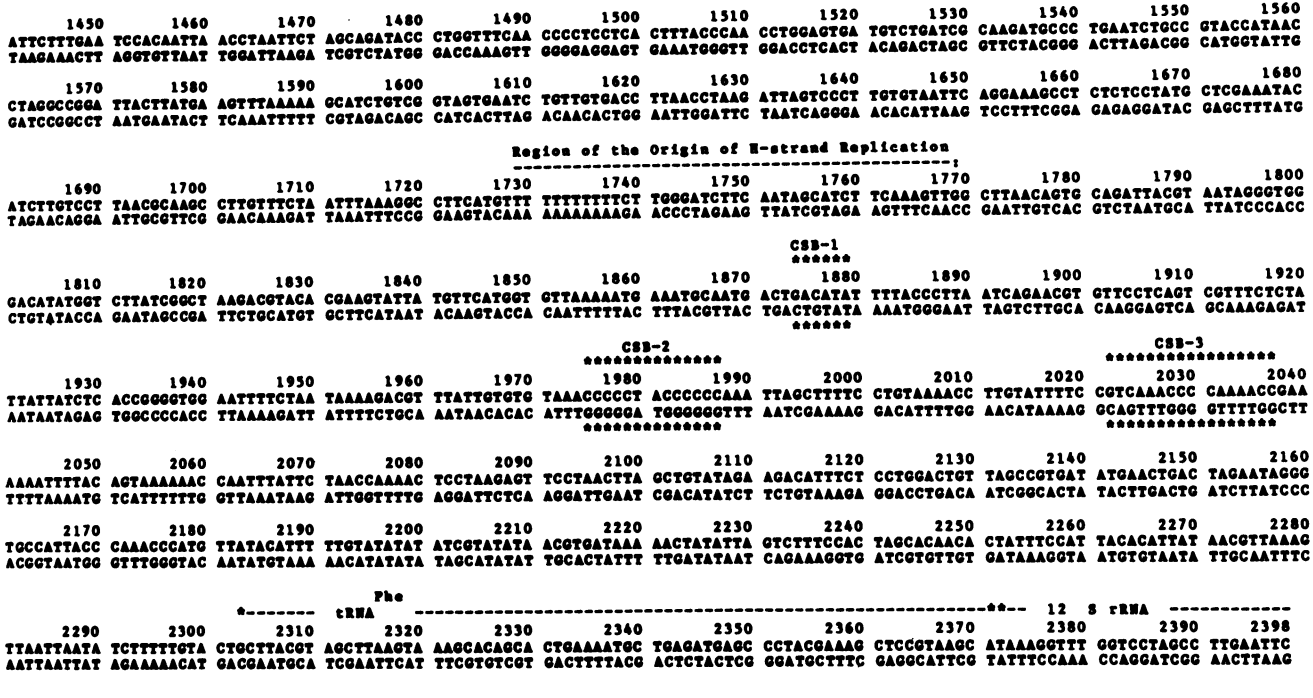


Figure 2. DNA sequence of the region of the Xenopus laevis mitochondrial genome encompassing the D-loop and its flanking genes.

mitochondrial genome were isolated by phenol extraction of the polyethylene glycol concentrated phage (27).

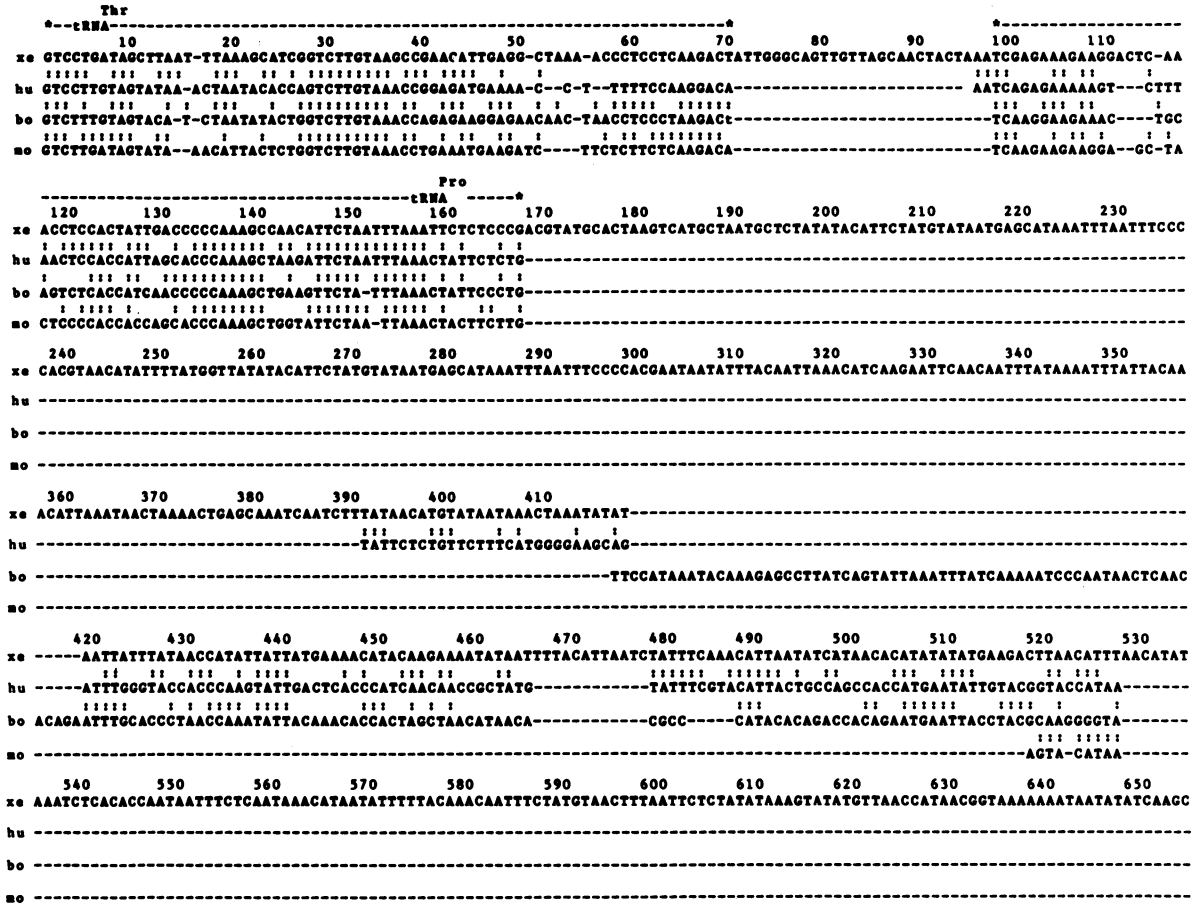
All DNA sequences were obtained by the dideoxynucleotide chain termination method (27-28). The M13 sub-clones were selected first at random and later by hybridization to overlapping fragments cloned in M13-mp8 or M13-mp9 in the opposite orientation. All inserts were sequenced using a flanking universal primer (29). The complete sequence of contiguous regions was assembled from individual, overlapping sub-clones containing fragments in either orientation, using the programs described by Staden (30-32) but modified to run on an IBM-3081 computer. Copies of these modified programs are available to others upon request. The optimal nucleotide sequence homology among the higher eucaryote mitochondrial DNA D-loop regions was determined by the NUCALN program of Wilbur and Lipman (33). All recombinant DNA experiments were performed in accordance with NIH guidelines.

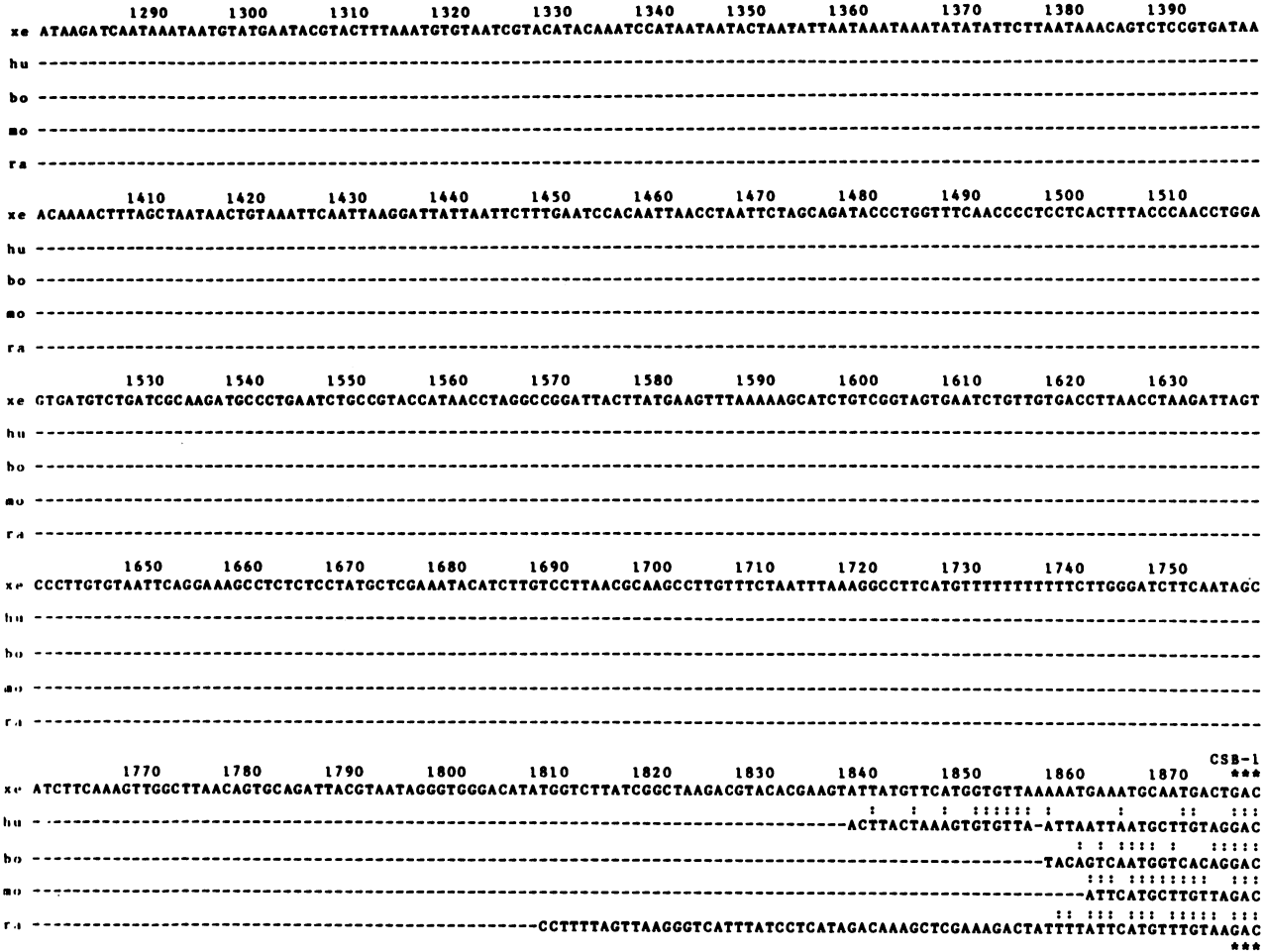
RESULTS AND DISCUSSION

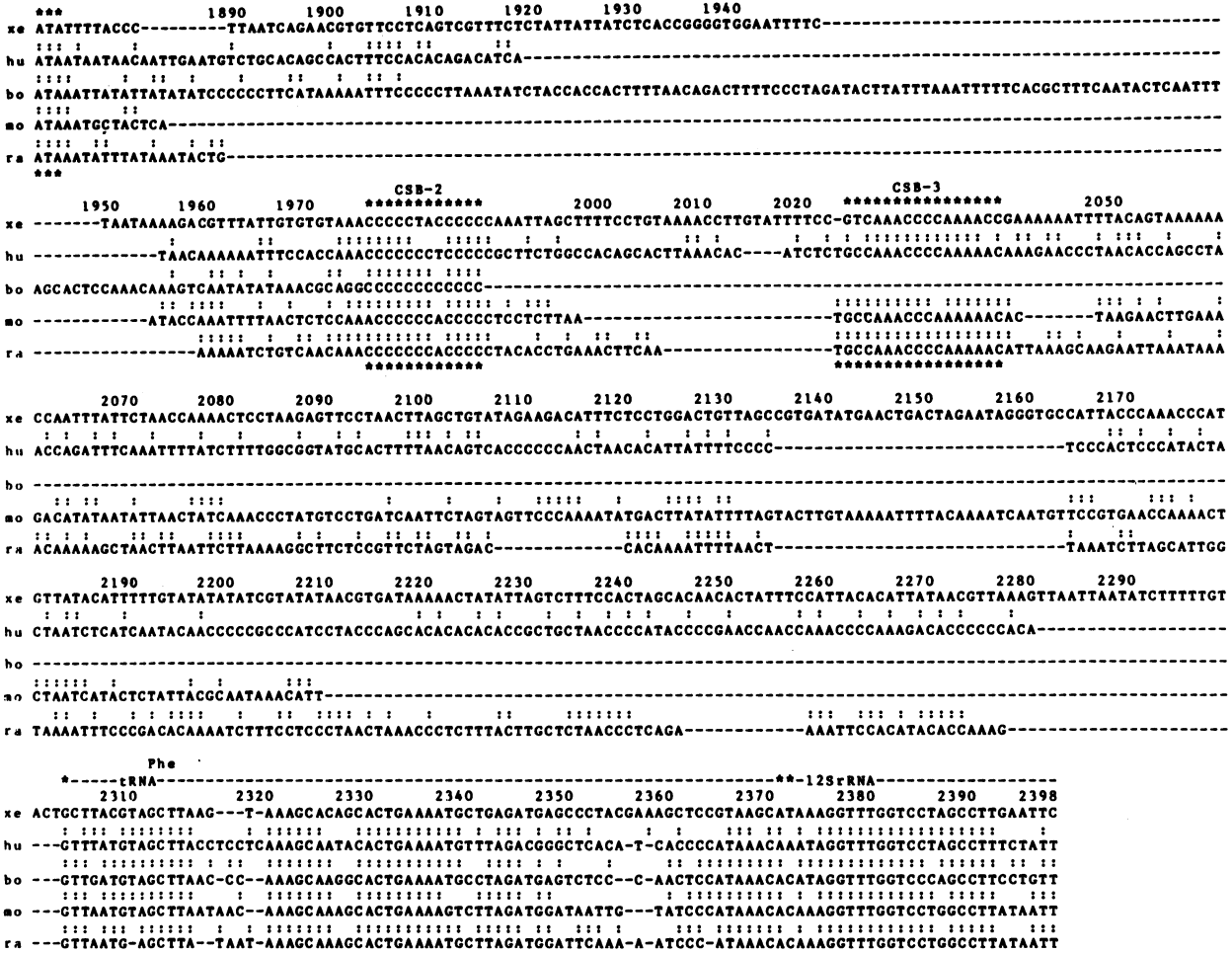
Sequencing approach. The restriction maps and sequencing strategies for the segments of the Xenopus laevis, whose sequences now are reported, are shown in Figure 1. The restriction endonuclease digestion sites predicted by the nucleotide sequence data shown in Figures 2 and 4 were confirmed by restriction enzyme mapping experiments (data not shown) and are in agreement with those presented in Figure 1 and reported earlier (5).

H-strand replication origin and its flanking genes. The complete nucleotide sequence of the Xenopus laevis mitochondrial DNA segments corresponding to the region surrounding the origin of H-strand replication is shown in Figure 2. Here the genes for $tRNA^{Pro}$ and $tRNA^{Phe}$ are separated by 2134 nucleotides, an amount almost twice as much as that separating these genes in other vertebrate mitochondrial genomes (2-4). The genes for $tRNA^{Thr}$ and $tRNA^{Pro}$ at the 5' end of this region are separated by 27 nucleotides in the Xenopus laevis mitochondrial genome, while they either overlap or are separated by only two nucleotides in the mammalian mitochondrial genomes (2-4). The additional nucleotides separating these two tRNA genes and the larger size of the non-coding region between the $tRNA^{Pro}$ and the $tRNA^{Phe}$ genes account for over half of the increased size of the Xenopus laevis mitochondrial genome, when compared to the mammalian mitochondrial DNAs (2-4).

An analysis of the D-loop region also reveals the presence of two large repeated sequences. The first occurrence of this repeated sequence begins 28


Thr
*--tRNA
Pro
tRNA -----*
xe
hu
bo
mo


1290 1300 1310 1320 1330 1340 1350 1360 1370 1380 1390
xe ATAAGATCAATAAATAATGTATGAATACGTACTTTAAATGTGTAATCGTACATACAAATCCATAATAATACTAATATTAATAAATAAATATATATTCTTAATAAACAGTCTCCGTGATAA
hu
bo
mo
ra
1410 1420 1430 1440 1450 1460 1470 1480 1490 1500 1510
xe ACAAAACTTTAGCTAATAACTGTAAATTCAATTAAGGATTATTAATTCTTTGAATCCACAATTAACCTAATTCTAGCAGATACCCTGGTTTCAACCCCTCCTCACTTTACCCAACCTGGA
hu
bo
mo
ra
1530 1540 1550 1560 1570 1580 1590 1600 1610 1620 1630
xe GTGATGTCTGATCGCAAGATGCCCTGAATCTGCCGTACCATAACCTAGGCCGGATTACTTATGAAGTTTAAAAAGCATCTGTCGGTAGTGAATCTGTTGTGACCTTAACCTAAGATTAGT
hu
bo
mo
ra
1650 1660 1670 1680 1690 1700 1710 1720 1730 1740 1750
xe CCCTTGTGTAATTCAGGAAAGCCTCTCTCCTATGCTCGAAATACATCTTGTCCTTAACGCAAGCCTTGTTTCTAATTTAAAGGCCTTCATGTTTTTTTTTTTCTTGGGATCTTCAATAGC
hu
bo
mo
ra
CSB-1
1770 1780 1790 1800 1810 1820 1830 1840 1850 1860 1870 ***
xe ATCTTCAAAGTTGGCTTAACAGTGCAGATTACGTAATAGGGTGGGACATATGGTCTTATCGGCTAAGACGTACACGAAGTATTATGTTCATGGTGTTAAAAATGAAATGCAATGACTGAC
hu ----------ACTTACTAAAGTGTGTTA-ATTAATTAATGCTTGTAGGAC
bo ----------TACAGTCAATGGTCACAGGAC
mo ----------ATTCATGCTTGTTAGAC
ra ----------CCTTTTAGTTAAGGGTCATTTATCCTCATAGACAAAGCTCGAAAGACTATTTTATTCATGTTTGTAAGAC
***


CSB-2
CSB-3
Phe
*-----tRNA----------**-12SrRNA----------
xe
hu
bo
mo
ra

nucleotides from the 5' terminus of the $tRNA^{Pro}$ and extends to position 240. The identical sequence also occurs at position 257 through 301 (see Figure 2). It has been proposed that a series of repeated sequences in the mammalian mitochondrial genome, which are also within 100 nucleotides of the 5' end of the $tRNA^{Pro}$ gene, may serve as terminators for synthesis of the mammalian 7S D-loop DNA (22). The Xenopus laevis mitochondrial 14S D-loop DNA's synthesis has been reported to begin at position 1730 to 1770 (24). Since the two major 14S DNAs are approximately 1350 and 1510 nucleotides long (21), the two repeated sequences beginning at positions 196 and 257 may be the terminators for 14S DNA synthesis.

Figure 3 shows an alignment of the Xenopus laevis, human, bovine and mouse mitochondrial genome sequences (2-4) in the regions from the $tRNA^{Thr}$ gene through the initial portion of the 12S ribosomal RNA gene. In addition, the sequenced region of the rat mitochondrial genome (9) is also aligned with its mammalian and amphibian counterparts. The optimal alignment of each sequence pair was obtained using the NUCALN program described by Wilbur and Lipman (33) and aligned with the human sequence to produce the results shown in Figure 3. This alignment of the human and bovine sequences differs slightly from that reported earlier (34), as it is based on five different mitochondrial DNA sequences rather than two. As shown in Figure 3, all five DNAs have regions of high homology in their coding sequences for the tRNA and rRNA genes. Although these four mammalian mitochondrial DNAs have some sequence homology near the center of the non-coding D-loop region, the greatest homology is observed in the region between the putative origin of H-strand replication and the $tRNA^{Phe}$ gene. In contrast, the Xenopus laevis and mammalian mitochondrial DNAs have low homology in the central region between the $tRNA^{Pro}$ gene (position 167) and the putative origin of H-strand replication (approximately position 1750). However, some sequence homologies can be observed in the region between the origin of H-strand replication and the $tRNA^{Phe}$ gene. These regions of sequence homology include the conserved sequence blocks (CSB) described earlier (3,22) and indicated in Figures 2 and 3.

The human, mouse, rat and Xenopus laevis mitochondrial genomes contain all three CSBs, while the bovine genome lacks CSB-3 in the region 3' to the

Figure 3. Alignment of the Xenopus laevis, human (2), bovine (3), mouse (4), and rat (9) DNA sequences in their D-loop regions. A colon (:) indicates that an identical nucleotide is present in the human DNA sequence while a dash (-) represents a gap introduced to maximize the sequence alignment.

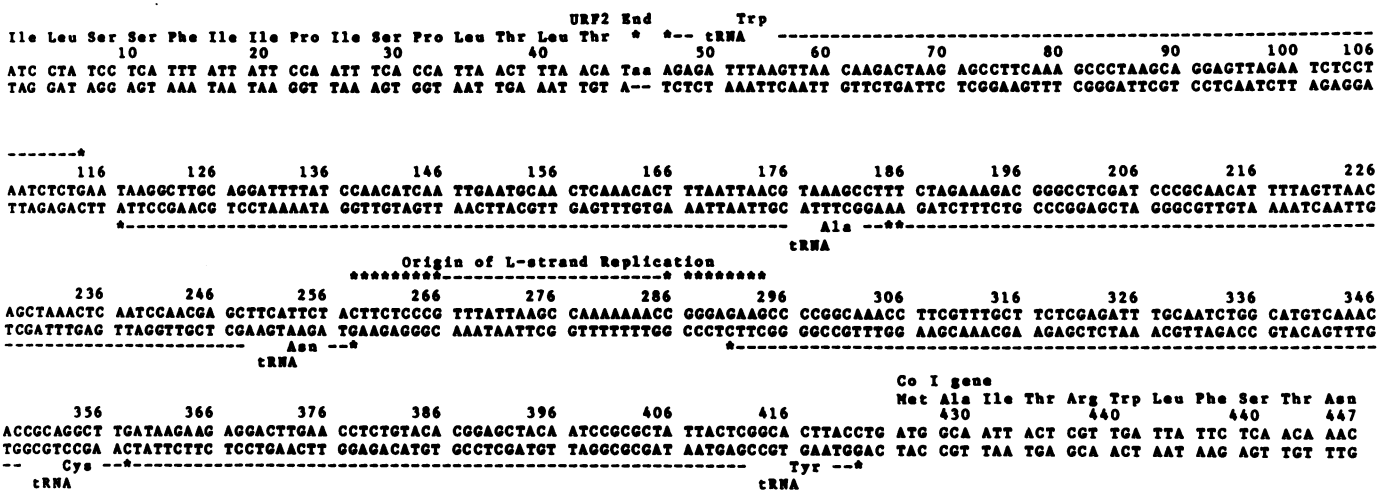
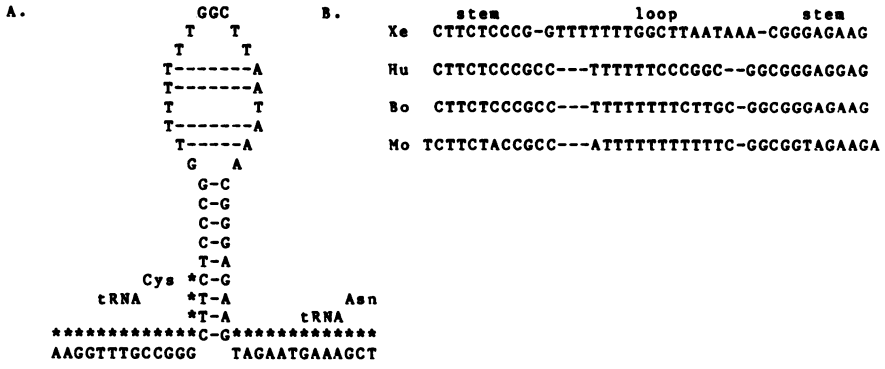


Figure 4. The region of the Xenopus laevis mitochondrial genome which encompasses the origin of L-strand replication and its flanking genes.

reported origin of H-strand replication. The occurrence of homologous sequences in this region of the D-loop may represent initiation points for the RNA primer implicated in the synthesis of the single stranded D-loop DNA initiation segment (21). Finally, these conserved sequence blocks occur in regions which can be drawn into very large hairpin structures (22,34). Since these regions in the Xenopus laevis mitochondrial D-loop segment can also be drawn as large hairpin structures, they may be important in the D-loop's biological function.

Recent evidence demonstrates that both strands of the vertebrate mitochondrial genome are completely transcribed from points at or near the $tRNA^{Phe}$ gene (35,36,37). After transcription, the large polycistronic RNA is processed to its shorter mature RNA species by a series of steps which is not well understood. The promoters for Xenopus laevis mitochondrial transcription most likely are contained in the regions adjacent to the $tRNA^{Phe}$ gene (37). Although these regions contain several AT rich segments, an examination of the sequence similarities (see Figure 3) does not immediately reveal which regions may function as the mitochondrial transcription promoters.

Origin of L-strand replication and its flanking genes. The segment of the Xenopus laevis mitochondrial genome shown in Figure 4 most likely encompasses the putative origin of L-strand replication because its surrounding tRNA and protein coding segments have high sequence homology with the other vertebrate mitochondrial genomes. As shown in Figure 5A, this region may form the hairpin loop structure similar to other vertebrate mitochondrial origins of L-strand replication. Its stem may be either nine nucleotides long with continuous base pairings, or fifteen nucleotides long but with two mismatched base pairings. The former structure would contain a nineteen nucleotide long A and T rich loop while the latter structure would contain a smaller T rich loop, as indicated by the additional dashes in the loop region of Figure 5A. This putative origin of L-strand replication is flanked on the 5' side by the genes for $tRNA^{Tyr}$ and $tRNA^{Cys}$ and on the 3' side by the genes for $tRNA^{Trp}$, $tRNA^{Ala}$ and $tRNA^{Asn}$. As shown in Figure 5B, this loop and stem region has a high sequence homology with its mammalian counterparts. The spacing between genes in this region is summarized in Figure 5C. Here the distance between the $tRNA^{Asn}$ and $tRNA^{Cys}$ genes flanking the putative L-strand replication origin is the same as in the mammalian mitochondrial genomes. In all but the human mitochondrial genome, the distances between the other structural genes are very similar.



C.

Species	URF2	-- tRNA Trp	-- tRNA Ala	-- tRNA Asn	Ori	tRNA Cys	-- tRNA Tyr	-- CoI
Xe	-2	2	0	32		-1	2	
Hu	-2	7	1	31		-1	12	
Bo	-2	1	1	32		0	1	
Mo	-2	1	2	32		2	1	

Figure 5. A. Postulated secondary structure in the region of the origin of L-strand replication. B. Comparison of the primary structure of the Xenopus laevis, human, bovine and mouse L-strand replication origin regions. C. Distances between genes in this region.

As presented in Figure 4, the region we have sequenced begins with the last 45 nucleotides of the putative URF2 reading frame. Here, as has been reported for several other mitochondrial reading frames, the 3' end of the URF2 overlaps the $tRNA^{Trp}$ gene. Thus, once the full length tRNA has been cleaved from the primary transcript, the URF2 mRNA must be polyadenylated to produce the required stop codon. This mechanism for post-transcriptional introduction of stop codons originally described for the human mitochondrial system (2) and later observed for other mammalian mitochondrial systems (3-4) may also occur in the Xenopus laevis system. Thus, the use of post-transcriptional polyadenylation to produce stop codons in mitochondrial m-RNAs may be a universal phenomenon in vertebrate mitochondria.

tRNA genes. The nucleotide sequences of the regions corresponding to the eight sequenced Xenopus laevis mitochondrial tRNA genes are shown in their cloverleaf forms in Figure 6 and are compared to their mammalian mitochondrial DNA counterparts in Figure 7. As has been observed with other vertebrate mitochondrial tRNAs (2-4,9,11,34,41-46), the Xenopus laevis mitochondrial tRNAs lack many of the features usually associated with cytoplasmic tRNAs. Only four of the eight tRNA genes reported here contain a T at position 8, and only three contain the two T's at the 5' end of loop IV.

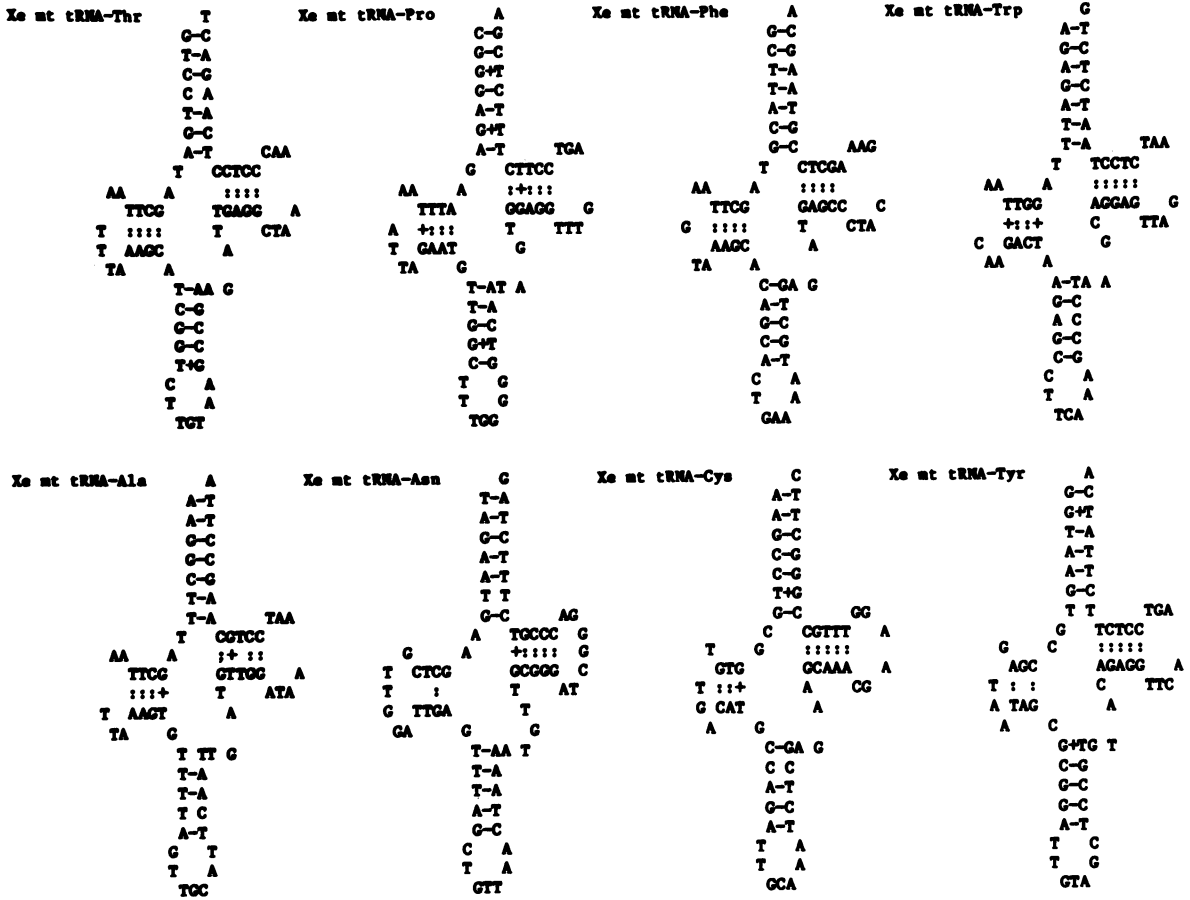


Figure 6. Xenopus laevis mitochondrial tRNA genes presented in their cloverleaf structure.

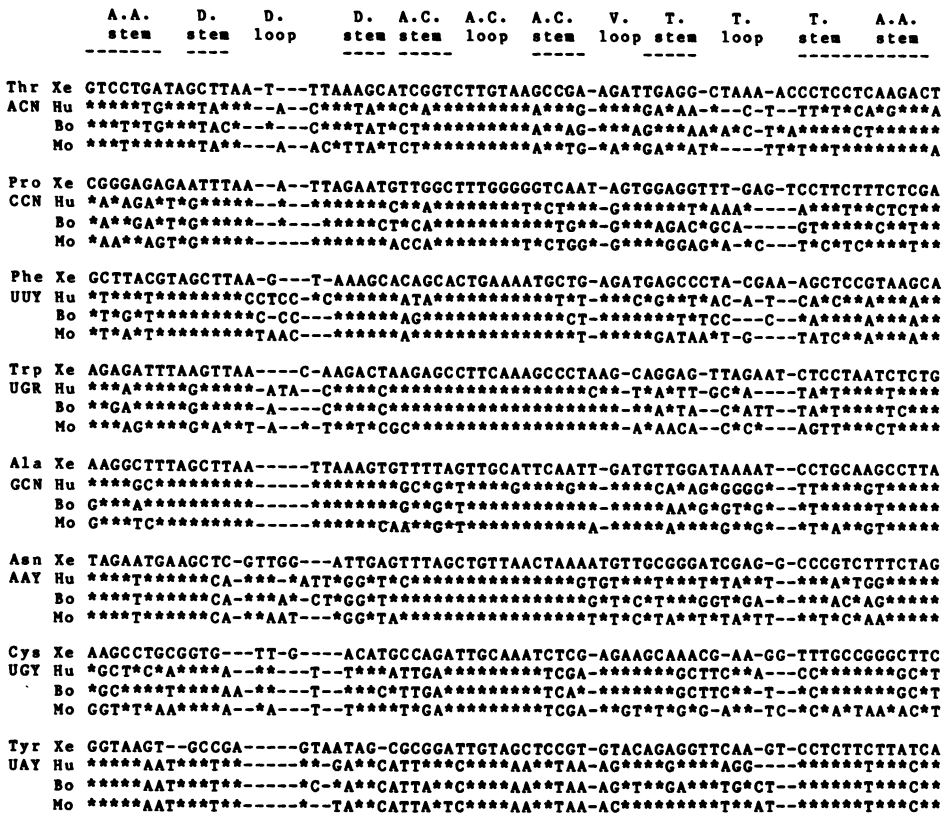


Figure 7. Nucleotide sequence comparison of vertebrate mitochondrial tRNA.

As with other mitochondrial tRNAs, the Xenopus laevis mitochondrial tRNAs also have several instances of mis-matched base pairings in their stem regions. The most prevalent mis-match is the G+T pairings which do not cause significant distortions in these helical regions (47) and are allowed under the wobble hypothesis (48). Other mismatched pairings occur in the stem of loop I in $tRNA^{Asn}$ and $tRNA^{Tyr}$. These loosely paired regions may be stabilized by base stacking and/or tertiary structural interactions, rather than by classical base pairings, as in the case of the truncated mammalian $tRNA^{Ser}$ (AGY) (46).

As can be seen in Figure 7, the primary sequences of these eight Xenopus laevis mitochondrial tRNA genes are quite similar to those of the mammalian mitochondria. The greatest homology occurs in the anticodon loop and stem and in the amino acid acceptor stem regions. In contrast, the least

conservation of sequence homology occurs in loops I and IV and in their corresponding stem regions. It might be that the lack of conserved elements in loops I and IV is due partially to the removal of any evolutionary pressure for maintaining the internal promoters required for eucaryote tRNA gene transcription (38), since the mitochondrial tRNAs are transcribed as large polycistronic species from a single promoter on each strand which is located in the D-loop region (39,40).

In summary, the primary structures of the Xenopus laevis mitochondrial DNA segments reported in this communication show regions which are homologous to the mammalian mitochondrial genomes. This observation indicates that elements of the amphibian and mammalian mitochondrial DNAs nucleotide sequences and their overall gene organization have been conserved during evolution.

ACKNOWLEDGEMENTS

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