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Molecular Characterization of a Cloned Dolphin Mitochondrial Genome

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Summary. DNA clones have been isolated that span the complete mitochondrial (mt) genome of the dolphin, *Cephalorhynchus commersonii*. Hybridization experiments with purified primate mtDNA probes have established that there is close resemblance in the general organization of the dolphin mt genome and the terrestrial mammalian mt genomes. Sequences covering 2381 bp of the dolphin mt genome from the major noncoding region, three tRNA genes, and parts of the genes encoding cytochrome b, NADH dehydrogenase subunit 3 (ND3), and 16S rRNA have been compared with corresponding regions from other mammalian genomes. There is a general tendency throughout the sequenced regions for greater similarity between dolphin and bovine mt genomes than between dolphin and rodent or human mt genomes.

Key words: D-loop sequence — Similarity blocks — Sequence evolution — Conservation of genome organization

Introduction

Valuable information about phylogeny and the speciation process in primates (Ferris et al. 1981; Brown et al. 1982), ungulates (Higuchi et al. 1984; Watanabe et al. 1986), rodents (Ferris et al. 1983; Lansman et al. 1983), birds (Shields and Wilson 1987), reptiles (Wright et al. 1983), and amphibians (Spolsky and Uzzell 1984) has been provided by analysis of sequence variability in the animal mitochondrial DNA (mtDNA). The complete nucleotide sequences of human, murine, and bovine mtDNAs have identified unique characteristics of mammalian mtDNA including compact and efficient gene arrangements, specific codon usage, and a high mutation rate (Brown et al. 1979; Anderson et al. 1981, 1982; Bibb et al. 1981). In addition to the structural genes, the animal mitochondrial (mt) genome contains a large noncoding region, including the displacement loop or D-loop, that comprises 5–7% (approximately 800–1200 bp) of the genome. Length variations in the D-loop region account for virtually all of the size differences between human, murine, and bovine mtDNAs. The noncoding region appears to encode regulatory functions for the expression of mt structural genes and replication of the heavy strand (Ojala et al. 1980; Olivo et al. 1983; Chang and Clayton 1984; Chomyn et al. 1985). However, at present, there is only limited information relating to the structural organization of putative regulatory signals within the D-loop region and the controlling mechanisms for mt replication and gene expression.

We have studied two dolphin species: *Cephalorhynchus commersonii* (Commerson's dolphin) and *Delphinus delphis* (common dolphin). The Commerson's dolphin is found exclusively in the southern coastal waters of Argentina and Chile and around the Falkland Islands in the Atlantic Ocean as well as the Kerguelen Islands in the Indian Ocean, whereas the common dolphin is found in temperate and tropical waters around the world. The *C. commersonii* mtDNA described here is representative of the mt genome of an individual animal living in a natural environment. The nucleotide and amino acid

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sequence comparisons with terrestrial mammalian mtDNAs have provided information about the conservation of regulatory signals and functional domains in proteins from a mammal adapted for life in a marine environment.

Materials and Methods

Preparation of mtDNA. Samples of *Delphinus delphis* liver were obtained from a dolphin that had been stranded on a California beach. The *Cephalorhynchus commersonii* liver sample was obtained through the Hubbs Marine Research Institute and Sea World in San Diego and came from the natural population of Commerson's dolphins living off Tierra del Fuego. The dolphin mtDNAs described here can therefore be regarded as representative of wild-type genomes.

Supercoiled mtDNA was prepared from frozen liver tissue of *Cephalorhynchus commersonii* and *Delphinus delphis*. The tissue was disrupted in a high-EDTA buffer (10 mM Tris-HCl, pH 7.3, 0.25 M sucrose, 75 mM EDTA) using an Omnimixer blender and a Dounce homogenizer, followed by removal of whole cells and nuclei by low-speed centrifugation. Mitochondria were pelleted by high-speed centrifugation, lysed with 2% SDS, and the supercoiled mtDNA was purified by propidium di-iodide–CsCl equilibrium density gradient ultracentrifugation. A typical yield was 5–10 μg of supercoiled mtDNA from 20 g of liver tissue.

Construction and Characterization of mt Clones. Preparations of purified supercoiled mtDNA were digested with *Eco*RI or *Bam*HI and ligated with predigested phage (Charon 28) and plasmid (pBR322, pRK404, and pACYC184) vectors. In each case, the vectors were treated with calf intestinal alkaline phosphatase to minimize the re-ligation of vector molecules without insert sequences. Standard transfection and transformation procedures were used to regenerate phage and bacterial stocks. Following antibiotic selection, clones were screened for the presence of mtDNA inserts by plaque and colony hybridization techniques using purified dolphin mtDNA as a probe. Some regions of the mt genome were not recovered when unfractionated restriction digests were ligated directly to the vectors. We therefore purified individual restriction fragments by preparative agarose gel electrophoresis and electro-elution prior to ligation with the stringent, low-copy number plasmids pRK404 and pACYC184. This approach allowed the cloning of restriction fragments covering the entire *C. commersonii* mt genome.

Hybrid plasmid DNAs were purified from bacterial cell cultures using techniques of Triton X-100/lysozyme lysis and equilibrium density gradient centrifugation with CsCl and ethidium bromide. Detailed restriction maps of the mt sequences were derived, and this information was used in the design of nucleotide sequencing experiments. We used the chemical sequencing technique of Maxam and Gilbert (1980) in conjunction with 5′ and 3′ end-labeled restriction fragments. All sequences were derived independently from both DNA strands.

Hybridization Reactions with Primate mt Probes. Purified restriction fragments of primate mtDNAs were labeled by nick translation in the presence of α^{32}P-dATP and α^{32}P-dCTP to obtain specific activities of 3–5 × 10^8 cpm/μg. The probes were cloned fragments of mtDNA from gorilla and pygmy chimpanzee (gift from Dr. W.M. Brown, Laboratory of Molecular Systematics, University of Michigan, Ann Arbor, Michigan 48109, USA). Samples of mtDNA were digested with restriction enzymes, electrophoresed in 1.5% agarose gels, and then DNA fragments within the gel were depurinated and transferred to nitrocellulose filters by capillary flow of buffer [20× standard saline citrate (SSC: 0.15 M NaCl, 0.015 M sodium citrate)]. The filters were air-dried, baked in a vacuum oven at 80°C, prehybridized for 1 h, and then hybridized with 1 × 10^7 cpm of probe in a buffer containing 6× SSC, 40% formamide, and 10% dextran sulfate overnight at 22°C, washed extensively in 2× SSC and then 0.1× SSC at 22°C, and autoradiographed with an intensifying screen for 1–2 days. The low stringency conditions of hybridization and washing were necessary to allow hybrid formation between the primate and dolphin sequences that are significantly diverged and moreover have a high A+T content. After hybridization, washing, and autoradiography, the probe was stripped from the filter by washing at 70°C for 60 min in 0.1× SSC, 0.1% SDS, prior to recycling with the next probe.

Results

Supercoiled mtDNA was purified by density gradient ultracentrifugation from long-term frozen liver tissue of the two dolphin species. Restriction enzyme cleavage maps were established for the two dolphin mt genomes (Fig. 1), and, assuming similar lengths for the two genomes (approximately 16,330 bp), we have used the analytical approach of Nei and Li (1979) to estimate a sequence divergence of 7%. Subsequently, the positions of several specific genes (cytochrome b, cytochrome oxidase subunit II, and D-loop) were determined in both the *C. commersonii* and *D. delphis* mtDNAs by Southern blot analysis using cloned restriction fragments of primate mtDNAs as hybridization probes (Fig. 2).

Restriction fragments, generated by digestion of supercoiled mtDNA from the dolphin *C. commersonii* with the enzymes *Bam*HI or *Eco*RI, were cloned into several vectors including pACYC184, pRK404, pBR322, and Charon 28 and propagated in *Escherichia coli* (Fig. 3). The whole mtDNA, linearized with *Eco*RI, *Bam*HI, or *Hind*III, could not be stably propagated by ligation with any of the vectors. The vectors pBR322 and Charon 28 allowed only stabilization of one *Bam*HI fragment (fragment B, Fig. 3). The stringent, low-copy plasmids pRK404, an R2 derivative (gift of Dr. G. Ditta, Biology Department, University of California, San Diego, La Jolla, California 92093, USA) and pACYC184, a p15A replicon (Fig. 3), appeared to be more suitable for stable recombination with the dolphin mt fragments. Separation of individual mt restriction fragments was necessary to obtain clones of several of the fragments. Similar difficulties were experienced in the isolation of human mtDNA fragments from similar regions (Drouin 1980). The recombinant clones that cover the entire dolphin mt genome are summarized in Fig. 3.

We have determined the nucleotide sequences of several regions of the dolphin mt genome (Fig. 1) using cloned fragments of the *C. commersonii* mtDNA and the chemical sequencing method of

Maxam and Gilbert (1980). The sequenced portions, comprising 2381 bp, contain the entire large noncoding region of 900 bp (Fig. 4), the $tRNA^{Thr}$, $tRNA^{Pro}$, and $tRNA^{Phe}$ genes (Fig. 5), partial sequence of the 16S rRNA gene (Fig. 6), and partial sequences of the genes encoding cytochrome b and ND3 (a subunit of the NADH dehydrogenase enzyme complex previously referred to as URF 3, Chomyn et al. 1985) (Fig. 7). The locations and orientations of these genes in the dolphin mt genome are identical to bovine mtDNA (Fig. 1; cf. Anderson et al. 1982).

The dolphin mtDNA sequences were compared with matching sequences from human (Anderson et al. 1981), mouse (Bibb et al. 1981), and cow (Anderson et al. 1982) in order to assess amino acid conservation and the nature and frequency of nucleotide changes. The pattern of amino acid conservation in the dolphin cytochrome b and ND3 proteins (Fig. 7b) is very similar to that in human, cow, and mouse. There is also similarity in the codon usage, and, as in the other mammals, the dolphin cytochrome b translation reading frame ends without a termination codon, which is presumably

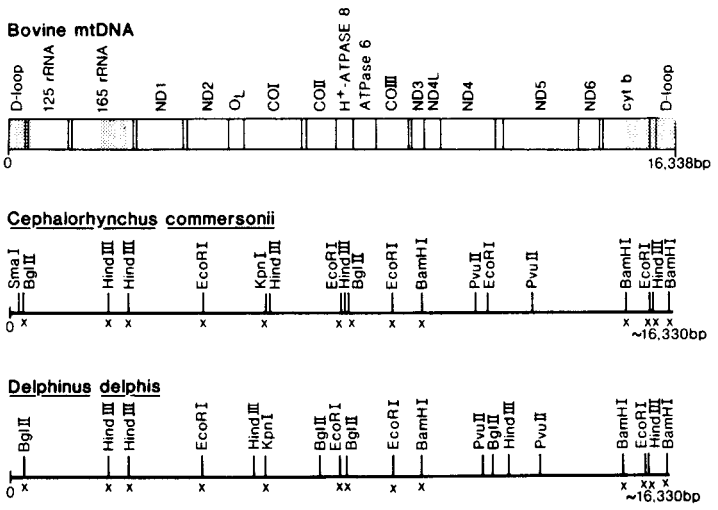

Fig. 1. A schematic representation of the dolphin mt genome aligned with bovine mtDNA. Restriction maps of the circular mt genomes from two dolphins, *C. commersonii* and *D. delphis*, are shown in a linear fashion, with the L-strand running 5′ to 3′. Common restriction sites in the two genomes are indicated by x's. The dolphin mt genomes are aligned with the bovine mt genome whose genetic content is shown schematically using conventional abbreviations to indicate coding regions. The order of restriction fragments, orientation of the restriction maps, and similarity of genetic organization between dolphin and cow was verified by genetic mapping and by sequence analysis of dolphin mtDNA. The regions in the *C. commersonii* mtDNA that have been sequenced are indicated by shading of the homologous areas in the bovine mtDNA.

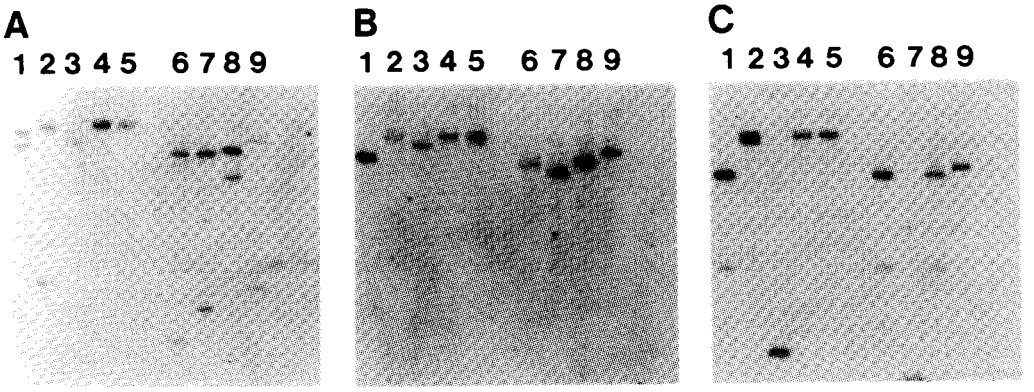

Fig. 2. Mapping of specific genes in mtDNA *D. delphis* using primate mt genes as hybridization probes. Restriction fragments of purified supercoiled mtDNA were generated by single and double digests and separated by gel electrophoresis: lane 1, *Eco*RI; 2, *Bam*HI; 3, *Bgl*II; 4, *Pvu*II; 5, *Kpn*I; 6, *Eco*RI + *Bam*HI; 7, *Eco*RI + *Bgl*II; 8, *Eco*RI + *Pvu*II; 9, *Bam*HI + *Kpn*I. The filter was sequentially hybridized with three different primate mtDNA fragments as genetic probes: **A** the cytochrome b gene, **B** D-loop, and **C** the cytochrome oxidase subunit II gene. The precise location of the specific genes in the mtDNA from another dolphin (*C. commersonii*), predicted by the mapping, was subsequently verified by DNA sequencing.

generated by polyadenylation of the transcript (Anderson et al. 1981, 1982; Bibb et al. 1981). The last codon in the dolphin sequence is indicated by an asterisk. The mutation rate of codons increased in the position order: 2, 1, 3. The protein genes appear to evolve by point mutations with preference for changes C–T and A–C (Table 1). The amino acids with the highest positional correlation (Tyr, Phe, Trp, Arg, His, Glu, Gly) include aromatic hydrophobic and charged residues. The amino acids Val, Met, Thr, and Ser are the least conserved ones and seem to be interchangeable in the protein sequence.

The nucleotide sequences of $tRNA^{Thr}$, $tRNA^{Pro}$, and $tRNA^{Phe}$ (Fig. 5a) were compared in schematic cloverleaf configurations allowing assessment of conserved features in the tRNA secondary structure (Fig. 5b). A generalized cloverleaf tRNA structure derived from individual patterns of nucleotide conservation shows considerable variability in the mt tRNA sequences and in the size of single-stranded loops. The position of invariant nucleotides and intrastrand bonds indicates that the most conserved structural features are the anticodon loop, the D.H.U. stem, nucleotides 8 and 9, and the tip of the aminoacyl stem, whereas the T.Ψ.C. loop and the D.H.U. loop appear to be the most variable regions.

The evolution of mt 16S rRNA genes in the compared mammals appears to involve both point mutations with a strong bias toward specific changes C–T, C–A, and A–T, resulting in a high positional conservation of G (Table 1), and length variations similar in size to the rearrangements occurring dur-

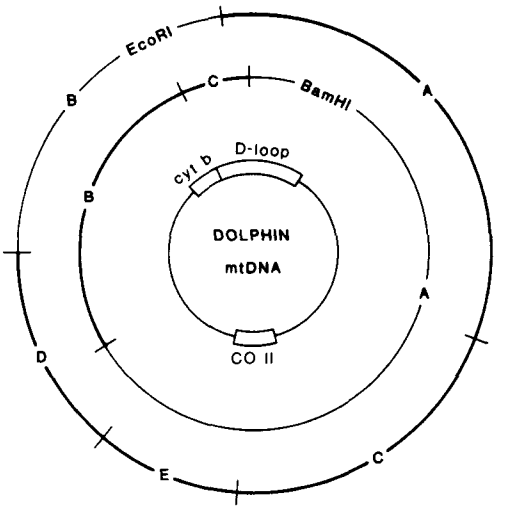

Fig. 3. Summary of recombinant dolphin mtDNA clones in the pACYC184 vector. Restriction fragments generated by digestion of supercoiled mtDNA from the dolphin *C. commersonii* with the enzymes *Bam*HI or *Eco*RI were cloned into the pACYC184 vector. Size-ordered restriction fragments are named in alphabetical order. Available cloned mtDNA fragments are identified by heavy lines. The inner circle indicates the orientation of the dolphin mt genome with the L-strand running 5′ to 3′. We used the mtDNA inserts from the recombinant pACYC184 plasmids to prepare a recombinant dolphin mtDNA probe. Six gel-purified DNA fragments (*Eco*RI: A, C, D, and E and *Bam*HI: B and C) were mixed in approximately equimolar ratios and labeled with α^{32}P-dATP and α^{32}P-dTTP using nick-translation or random primer extension techniques. This recombinant probe hybridized with the entire dolphin mtDNA sequence.

Table 1. Nature and frequency of specific base substitutions in D-loops and the sequences encoding proteins, tRNAs, and 16S rRNAs in mtDNA of dolphin, cow, human, and mouse

Type of change	Protein genes (total number of specific substitutions)[a]		Average bias[b]	tRNA genes (total number of specific substitutions)			Average bias	16S rRNA gene (total number of specific substitutions)	Average bias	Conserved D-loop[c] (total number of specific substitutions)		Average bias
	cyt B	ND3		$tRNA^{Thr}$	$tRNA^{Pro}$	$tRNA^{Phe}$				In blocks A–K	Between blocks B–F	
Transitions												
A ↔ G	47	14	1.33	23	41	17	1.83	95	0.91	19	27	0.75
C ↔ T	120	62	2.25	31	41	37	2.42	169	1.31	59	68	2.07
Transversions												
A ↔ C	103	45	1.83	5	5	15	0.56	155	1.49	24	37	1.04
A ↔ T	32	30	0.77	13	6	11	0.74	144	1.39	29	49	1.27
G ↔ C	14	5	0.23	—	1	—	0.02	28	0.27	11	12	0.37
G ↔ T	12	4	0.20	5	9	3	0.39	34	0.33	13	21	0.55

[a] Substitutions resulting from pairwise comparison of dolphin, bovine, human, and murine mitochondrial sequences as indicated in Table 2

[b] Average bias = 6 × (total number of the specific type of substitutions)/(total number of all types of substitutions). Bias 1.0 represents the case of random frequency of substitutions (probability 1/6)

[c] Conserved sequences in the central region of D-loops (similarity blocks B through F), blocks A, K and H, I, J (when present) were analyzed

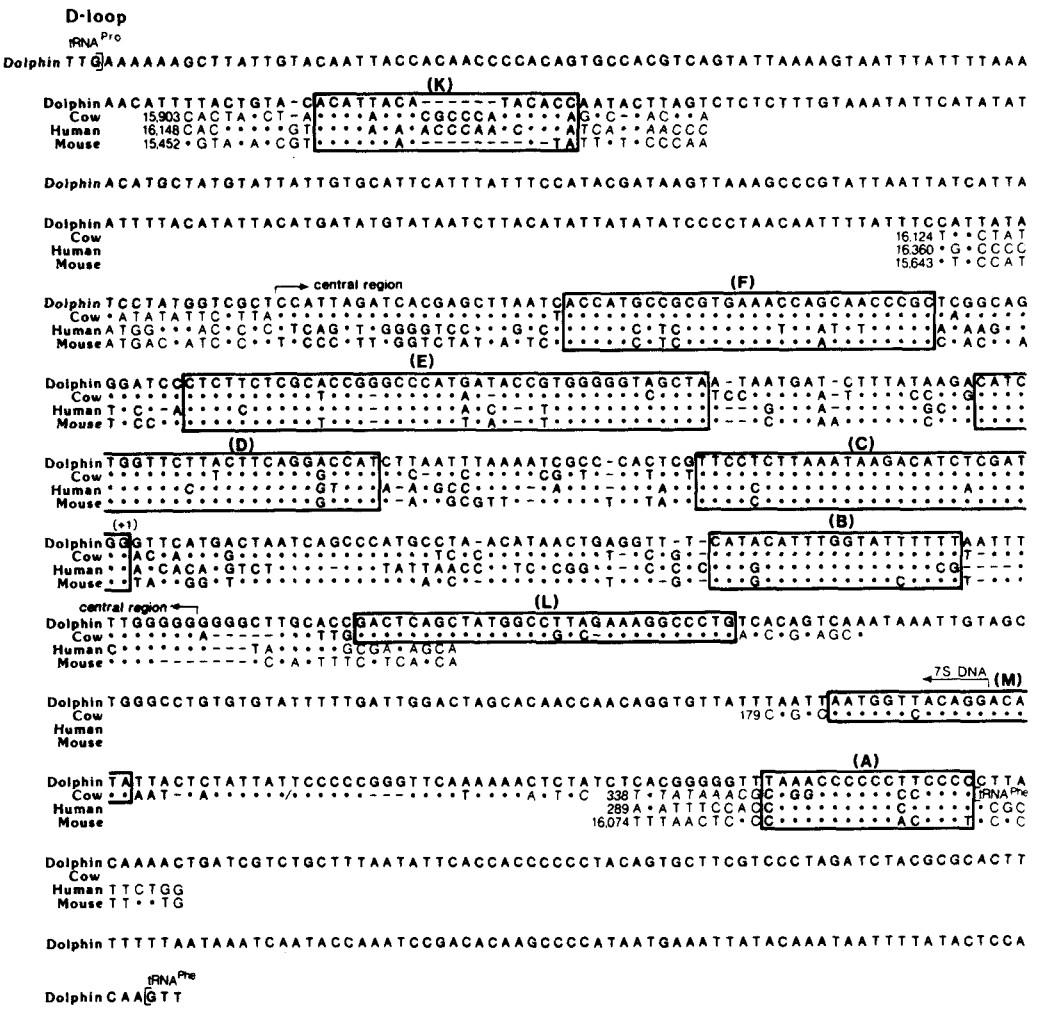

Fig. 4. The nucleotide sequence of the entire dolphin D-loop and the alignment of mammalian mt sequences. The D-loop, comprising 900 bp located between the $tRNA^{Pro}$ and $tRNA^{Phe}$ genes, is shown. The sequence, analogous to the beginning of the published human and bovine mtDNA sequences, is indicated as (+1). Sequence similarity among D-loops of the compared mammals is clustered in a central region of less than 280 bp (marked in the figure) and in the similarity blocks A through M. In addition to the pairwise alignment of the similar sequences, short stretches of adjacent sequences are shown to demonstrate the sharp termination of the clusters of similarity by runs of unique sequences. The start of the bovine 7S DNA is indicated by an arrow; this site has not yet been mapped in the dolphin D-loop. A single deletion of three nucleotides in the bovine sequence (indicated ·/·) has been introduced to maximize the sequence alignment. The mt sequences of the L-strand from the four mammals are aligned to obtain maximum similarity. Positions identical with the dolphin sequence are indicated by dots, and deleted positions and gaps in the sequence, by dashes. In order to facilitate the identification of sequences that have been aligned with the dolphin sequence, nucleotide numbers are included from the published sequences (Anderson et al. 1981, 1982; Bibb et al. 1981).

ing divergence of the mt tRNA genes. Interpretation of the visualized pattern of the structural conservation has to await elucidation of the mt 16S rRNA structure.

Information derived from this comparative analysis of dolphin mt genes shows that the degree of sequence similarity between dolphin and cow is higher than for any other pair of mammals that have been compared at both the nucleotide and amino acid levels (Table 2).

Comparison of D-loop regions of human, cow, and mouse provided information on the presence

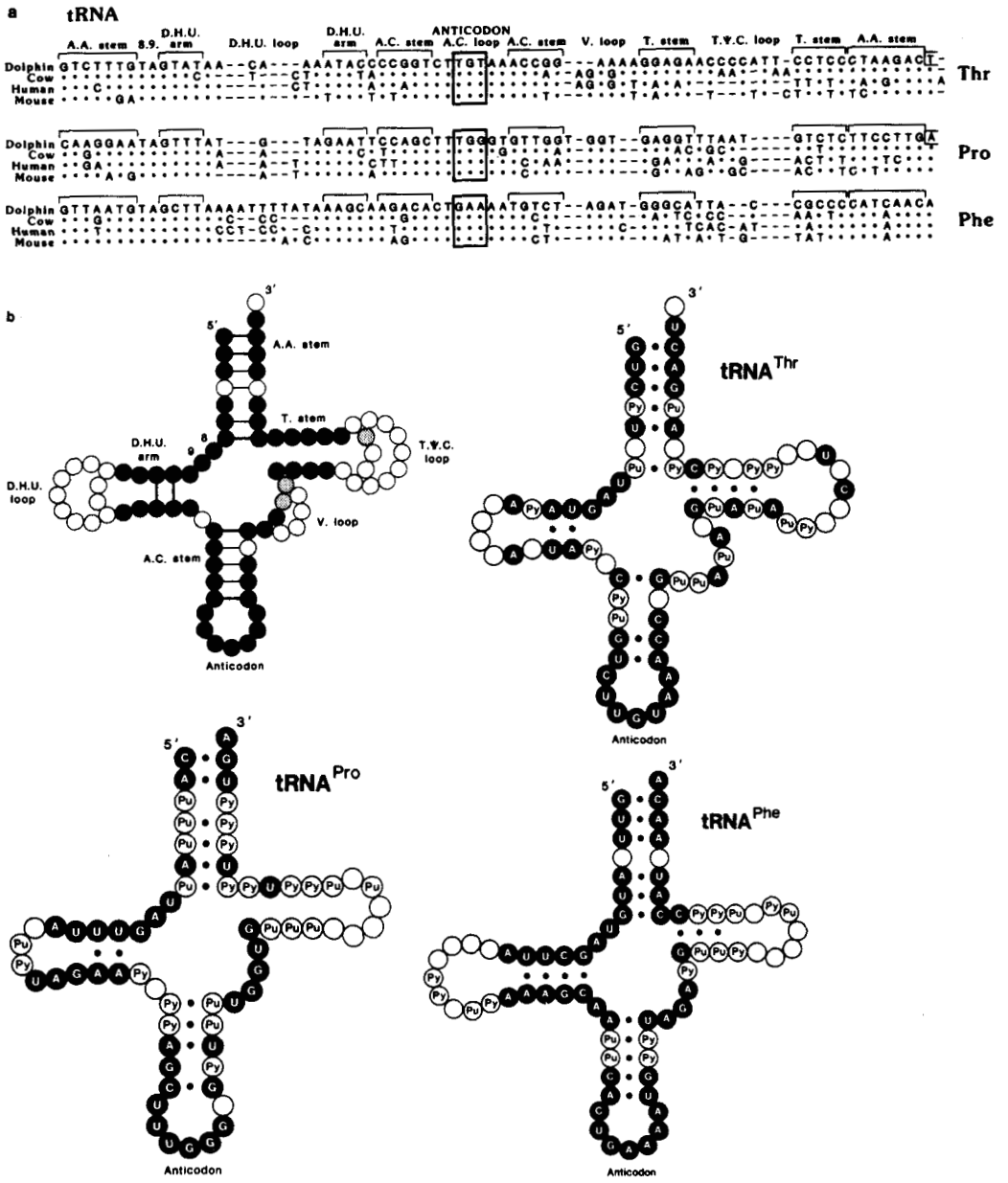

Fig. 5. Sequences of mt tRNA^{Thr}, tRNA^{Pro}, and tRNA^{Phe} genes. **a** The three tRNA genes are shown in a pairwise alignment with other mammalian mt sequences (as in Fig. 4) and also aligned with each other vertically to demonstrate the similarity between the mt tRNAs and the classical tRNA structure, whose various loops and stems are marked by conventional abbreviations along the top of the figure. The anticodon triplets are enclosed in boxes. **b** The tRNA sequences also are compared in schematic cloverleaf configurations to allow assessment of secondary structure conservation. The sequences of the dolphin tRNA^{Thr} and tRNA^{Pro} genes overlap for the terminal nucleotide (indicated in the figure). A composite tRNA structure has been derived from the individual 12 sequences in order to visualize the consensus pattern of structural conservation in the mt tRNA. The degree of conservation of the individual nucleotide positions and the base-pairing pattern are indicated by color intensity of the symbols representing bases and intrastrand bonds. A black circle represents a fully conserved nucleotide, whereas a white circle indicates that the position can be occupied by any nucleotide. In order to indicate variability in the D.H.U. and T.ΨC. loops, all alternative loop sizes are shown.

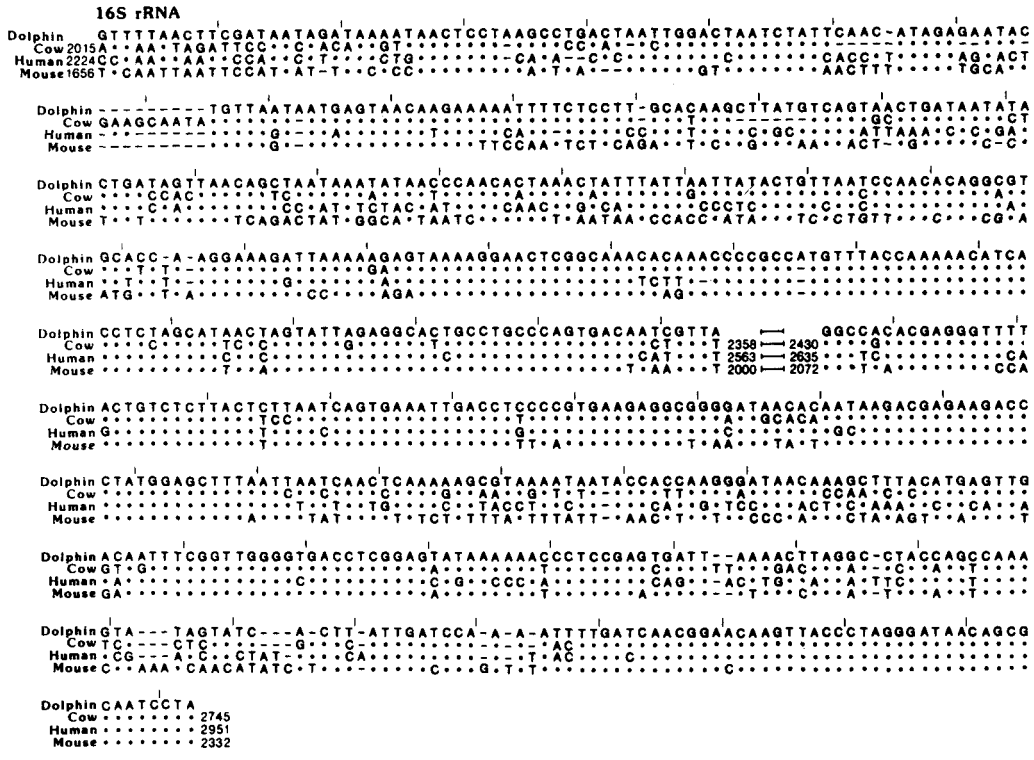

Fig. 6. Comparisons of sequences from mt 16S rRNA genes. The mammalian mt sequences are aligned as described in Fig. 4.

of several clusters of conserved sequences, referred to as similarity blocks A through K, in an otherwise highly divergent sequence (Anderson et al. 1982). The dolphin D-loop region, comprising 900 bp (Fig. 4), is very similar in size to that of the cow (910 bp, Anderson et al. 1982). However, the patterns of similarity blocks in dolphin and cow are different (Fig. 8), and the bulk of the dolphin D-loop sequence is unique. The middle of the D-loop, covered by the 7S DNA, was shown to be the most conservative region in D-loops of human (Anderson et al. 1981), cow (Anderson et al. 1982), goats and sheep (Upholt and Dawid 1977), mouse (Bibb et al. 1981), and rat (Sekiya et al. 1980), and appears to be well conserved also in dolphin. This central D-loop region (Figs. 4 and 8), spanning less than 300 bp, contains a compound group of five similarity blocks (B, C, D, E, F) with extremely high sequence conservation in all four mammals (85–96%, Table 2). These central blocks of similarity are separated by short segments of variable length and sequence (40–56% sequence conservation). In contrast, dolphin and cow show very high similarity throughout the entire central region (the interblock sequences are 70% conserved) and beyond. We identified two similar sequences, termed blocks L and M (83% and 94% conserved), that are present only in dolphin and cow (Figs. 4 and 8). In cow, the block M spans the putative start of bovine 7S DNA. Two additional blocks of similarity shared by all four mammals, A and K, are less conserved than the central blocks (79% and 58%), and they are flanked by long stretches of highly divergent sequences. The blocks G, H, and J are absent from the dolphin D-loop (Fig. 8), but their sequences are highly conserved when present in other mammals (Anderson et al. 1981, 1982; Bibb et al. 1981). Except for the 13 blocks of sequence similarity and the conserved compound structure of the central region, up to 70% of the D-loop sequences in dolphin and other mammals are unique.

Discussion

The sequence comparisons of dolphin, bovine, human, and murine mt genes presented here confirm

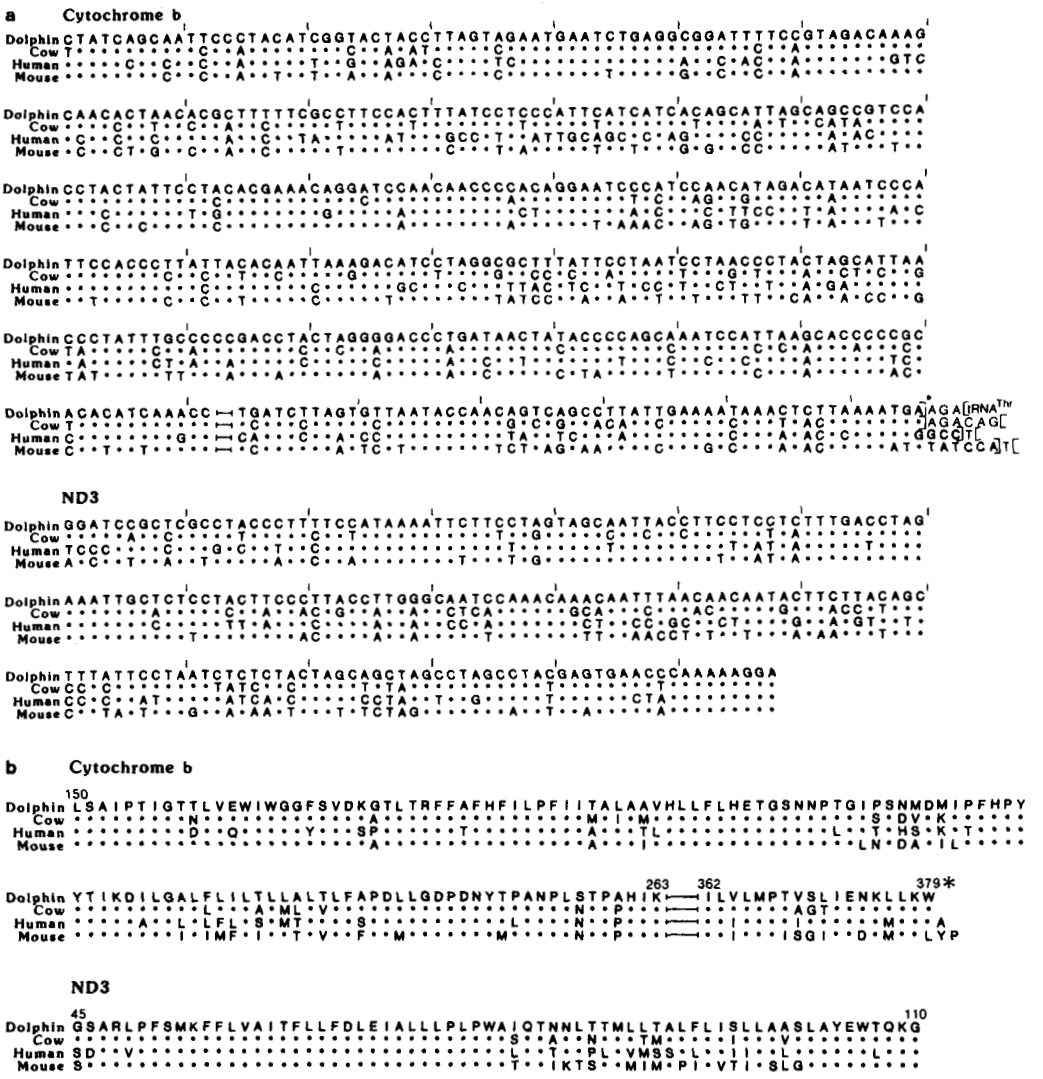

Fig. 7. Comparisons of sequences from mt proteins cytochrome b and ND3. Both the nucleotide sequences of the protein genes (a) and the inferred amino acid sequences (b) are compared with other published mt sequences. The sequences are aligned as described in Fig. 4.

that the mammalian mt genome has not evolved in the same way and at the same rate in all regions. Several distinct mechanisms seem to contribute to mtDNA variability. Evolution by point mutation has occurred throughout the genome, although there are differences in the probabilities of specific types of nucleotide changes (Table 1). This gene-specific pattern of nucleotide substitutions probably reflects functional constraints on the products of these genes and on the thermodynamic stability of mtDNA conformations. Point mutations are the principal mechanism for divergence of the protein genes, whereas sequence changes in the tRNA and rRNA genes also involve short insertions and deletions. Large sequence rearrangements including recombination events appear to occur in the D-loop region, which is the most dynamically evolving portion of the mt genome.

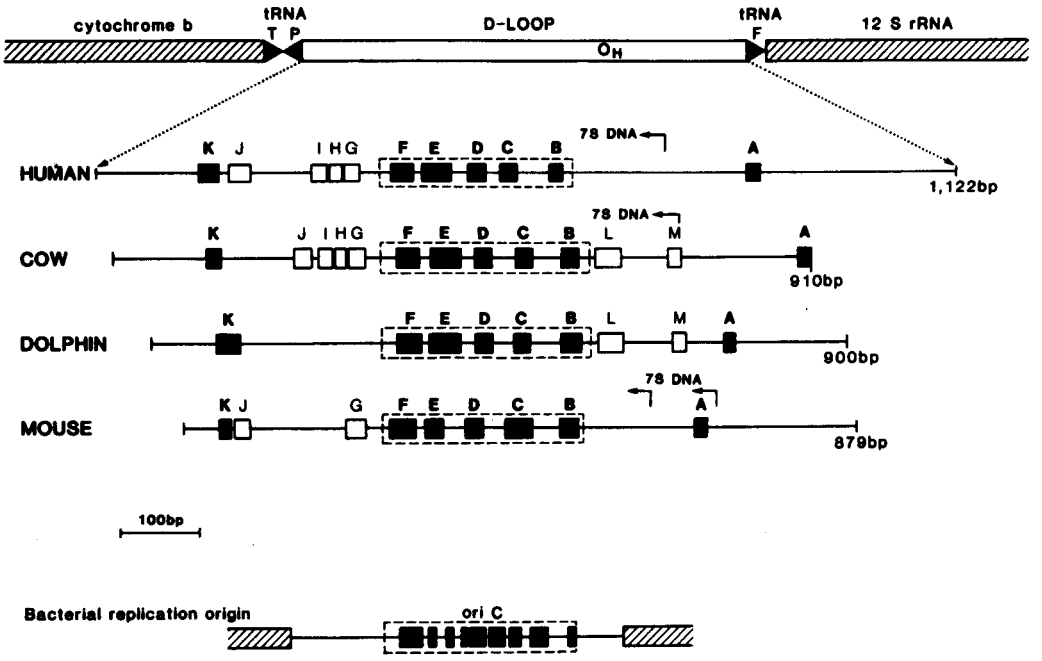

Fig. 8. Generalized pattern of sequence similarities in mammalian mt D-loops and possible relations to bacterial *ori*C. The mammalian D-loops are aligned using the sequence similarity (see Fig. 4) of a highly conserved central region (enclosed by a dashed line). Similarity blocks present in all mammalian D-loops examined (Anderson et al. 1981, 1982; Bibb et al. 1981) are represented by shaded boxes, whereas blocks only present in some mammals are shown by open boxes. Length variation within the D-loop arises from unique sequences that alter the relative position of some similarity blocks with respect to the central region and the tRNA genes designating the extremities of the D-loop region. The origin of DNA replication for the H chain is represented by O_H. The mapped starting positions of 7S DNAs, associated with H-chain replication, are shown by arrows. The 245-bp minimal bacterial origin of chromosomal replication (*ori*C, enclosed in a dashed box) contains short blocks of conserved sequence, which are present in six species of enterobacteria (Zyskind et al. 1983).

The additive effect of various mutational events results in different characteristics and rates of sequence change in distinct functional regions of the mt genome. Consequently, only a complex statistical representation that considers regions with different modes of evolution separately would appear to be appropriate for analysis of mtDNA divergence.

Considering possible relationships between DNA structure and function, it is interesting to note a similarity between the mt D-loop regions (Fig. 8) containing regulatory signals for mtDNA replication (Anderson et al. 1981, 1982; Bibb et al. 1981) and the bacterial chromosomal replication origin, *ori*C (Zyskind et al. 1983). The consensus structures of the D-loop and *ori*C both consist of interspersed blocks of conserved sequences (Fig. 8). These bacterial similarity blocks are not related by sequence to the mt blocks, but the general organization of *ori*C resembles the mt D-loop. this may reflect the prokaryotic origin of the mt genetic system (the endosymbiotic hypothesis), or the pattern of interspersed conserved and variable sequences may represent an efficient structural organization of a genomic replication origin that has evolved independently in separate genetic systems.

High sequence conservation in the similarity blocks of the D-loop region may indicate functional importance (Anderson et al. 1982); however, the absence of the blocks G, H, I, J, L, and M in several of the examined mammals (Fig. 8) poses questions about the nature of their function in the other mt genomes. It appears that while several of the similarity blocks may form a group of invariant sites present in all mammals and be crucial for the regulatory functions encoded in D-loops, other blocks, conserved only in certain species, may be involved in more specialized functions.

Although a certain degree of functional conservation can be inferred from the patterns of similarity blocks discussed above, we suggest that the D-loop has additional invariant features that are not apparent by direct comparisons of linear sequences but

Table 2. Similarity of mitochondrial genes in dolphin, cow, human, and mouse[a]

Species compared	Protein genes[b]				tRNA genes[c] nucleotide similarity (%)			16S rRNA[d] nucleotide similarity (%)	D-loop[e] nucleotide similarity (%)		
	Nucleotide similarity (%)		Amino acid similarity (%)								
	Cyt b	ND3	Cyt b	ND3	$tRNA^{Thr}$	$tRNA^{Pro}$	$tRNA^{Phe}$		Within blocks B–F	Between blocks B–F	Unique sequences
Dolphin, cow	79	79	86	88	77	79	73	81	96	77	26
Dolphin, human	70	70	78	76	68	63	71	74	86	43	31
Dolphin, mouse	72	73	78	74	67	72	71	69	90	56	20
Cow, human	69	73	78	77	68	65	71	70	88	40	22
Cow, mouse	77	73	80	76	63	60	70	66	90	53	28
Human, mouse	69	70	74	71	67	66	69	66	90	42	33

[a] Data on human, bovine, and murine mtDNA sequences were published previously
[b] Cytochrome b, 414 bp (138 codons) compared; ND3, 198 bp (66 codons) compared
[c] Dolphin $tRNA^{Thr}$, 71 bp; $tRNA^{Pro}$, 67 bp; $tRNA^{Phe}$, 72 bp
[d] 16S rRNA, 657 bp compared
[e] Central region of D-loop, comprising 263 bp and containing blocks of similarity B through F, was compared (Figs. 2 and 3) and 100 bp in the unique sequences flanking the central region

can be surmised by analysis of conformational properties (Brown et al. 1986) and physicochemical profiles (Mignotte et al. 1987) of animal D-loops. The less-apparent features involve local thermodynamical, stereochemical, and topological properties of the DNA molecule (Lukesová-Southern 1982). Distribution of the collective physicochemical properties within the D-loop determines the dynamic profile of the region: potential for occurrence of local changes in DNA conformation (confined to within 100 Å or 10–40 bp of linear sequence) such as B- to Z-DNA transition, strand separation, and formation of a gap or a hairpin loop. These localized and transient molecular deformations in supercoiled mtDNA constitute reactive sites with distinct stereochemical properties, which could participate in protein–DNA interactions or in formation of multistrand DNA structures (Lukesová-Southern 1982). Such molecular interactions in D-loops were proposed to be required in the process of transcription and replication of the mt genome, and therefore the physicochemical properties of D-loop DNA, which are necessary for these interactions, are likely to be conserved in evolution.

Our results establish a strong similarity between dolphin and bovine mtDNA sequences. The suggestion of a close evolutionary relationship between cetaceans and ungulates is consistent with current theory on cetacean origin (Barnes et al. 1985), inferred from biochemical, anatomical, and physiological comparisons of modern mammals and the fossil record relating to cetacean phylogeny (Gingerich et al. 1983). However, appropriate statistical analyses of mtDNA sequences from different cetacean and ungulate taxa are needed for an accurate assessment of the early evolution of dolphins and whales.

Addendum. In a related study, we (A.E.D., S.O.S., and others, manuscript in preparation) have used a mixture of recombinant mtDNA fragments as a hybridization probe to determine mtDNA genetic distances among and within species, populations, and schools of spinner and spotted dolphins. When supercoiled mtDNA, purified from frozen tissues, was used as a hybridization probe against total cell DNA, various nonspecific and satellite nuclear DNA fragments were often detected due to their copurification with mtDNA. The recombinant dolphin mtDNA probes have eliminated these problems.

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