The Marsupial Mitochondrial Genome and the Evolution of Placental Mammals

Axel Janke,* Gertraud Feldmaier-Fuchs,* W. Kelley Thomas,†,[1] Arndt von Haeseler* and Svante Pääbo*

**Zoologisches Institut, Universität München, PF 202136, D-80021 München, Germany, and †Division of Molecular Biology and Biochemistry, School of Biological Sciences, University of Missouri, Kansas City, Missouri 64110-2499*

Manuscript received June 17, 1993
Accepted for publication January 18, 1994

ABSTRACT

The entire nucleotide sequence of the mitochondrial genome of the American opossum, Didelphis virginiana, was determined. Two major features distinguish this genome from those of other mammals. First, five tRNA genes around the origin of light strand replication are rearranged. Second, the anticodon of $tRNA^{Asp}$ is posttranscriptionally changed by an RNA editing process such that its coding capacity is altered. When the complete protein-coding region of the mitochondrial genome is used as an outgroup for placental mammals it can be shown that rodents represent an earlier branch among placental mammals than primates and artiodactyls and that artiodactyls share a common ancestor with carnivores. The overall rates of evolution of most of the mitochondrial genome of placentals are clocklike. Furthermore, the data indicate that the lineages leading to the mouse and rat may have diverged from each other as much as 35 million years ago.

THE mitochondrial genome of vertebrates contains the genes for 13 proteins involved in oxidative phosphorylation, 2 ribosomal RNA (rRNA) genes and 22 transfer RNA (tRNA) genes. Except for the gene for the NADH dehydrogenase subunit 6 (*ND6*) and eight tRNAs, all genes are encoded on one strand (the H strand). A non-coding region, responsible for replicational and transcriptional control, contains regions of conserved sequence (WALBERG and CLAYTON 1981; SACCONE *et al.* 1991) as well as regions that differ in length and sequence between species. In marsupials, the arrangement of tRNA genes around the origin of light strand replication is altered (PÄÄBO *et al.* 1991) and an RNA editing process modifies the anticodon of a tRNA transcript (JANKE and PÄÄBO 1993). Here, the complete nucleotide sequence of the American opossum mitochondrial genome is presented. It reveals that the localization of all other genes is identical to that of placentals and that RNA editing seems to be confined to one tRNA.

The complete nucleotide sequence of the opossum was used to clarify the phylogeny of placental mammals, which have been repeatedly used to argue both for differences in evolutionary rates among lineages (WU and LI 1985; LI *et al.* 1987) and for equality of rates (BULMER *et al.* 1991; EASTEAL 1988; LI *et al.* 1990). The phylogenetic relationships among placental mammals are of great relevance for these arguments because they affect the interpretation of the observed sequence differences (EASTEAL 1992). For the elucidation of the mammalian phylogeny, sequences of entire mitochondrial genomes are available for human (ANDERSON *et al.* 1981), mouse (BIBB *et al.* 1981), cow (ANDERSON *et al.* 1982), rat (GADALETA *et al.* 1989), fin whale (ARNARSON *et al.* 1991) and harbor seal (ARNASON and JOHNSSON 1992). The only complete mitochondrial sequences from non-placental vertebrates are from an amphibian (ROE *et al.* 1985) and a bird (DESJARDINS and MORAIS 1990). Since these species have diverged from the mammalian ancestor about 350 and 300 million years ago, respectively (CARROLL 1988), multiple substitutions have erased much of the phylogenetic information on the lineages leading to the outgroups, making phylogenetic inferences difficult. For example, a recent study (ADACHI *et al.* 1993) using the combined protein sequences of all vertebrate mitochondrial proteins failed to resolve the branching order among the human, rodent and cow clades. Placentals and marsupials constitute a monophyletic group, which according to paleontological evidence diverged from a common ancestor early in the Cretaceous some 130 million years ago (CARROLL 1988; NOVACEK 1992). Thus, marsupials constitute the most appropriate outgroup for the placental radiation. The complete sequence of the mitochondrial genome of the North American opossum is here used to elucidate the phylogeny of placentals and to show that the majority of the genes in the mitochondrial genomes of placentals evolve at a constant rate.

MATERIALS AND METHODS

Liver was obtained from a fresh road-killed opossum, *Didelphis virginiana*, found near the University of California at Berkeley campus. Mitochondrial DNA was purified from liver by cesium chloride-gradient centrifugation (LANSMAN *et al.* 1981). Five *Eco*RI fragments (0.8, 3.1, 3.7, 4.2, 5.2 kb,

[1] Present address: Division of Biochemistry and Molecular Biology, University of California, Berkeley, California 94720-3202.

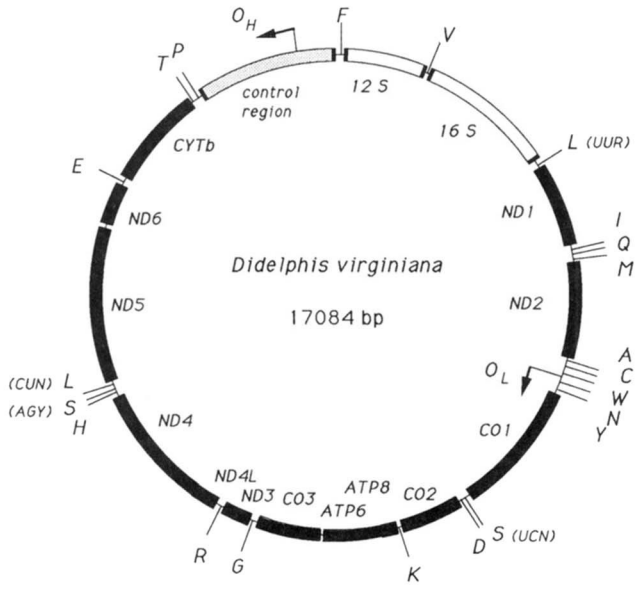


FIGURE 1.—Map of the *D. virginiana* mitochondrial DNA molecule. The location of origins of replication as well as the identity and arrangement of the various genes were determined by comparison of published mammalian sequences. Each tRNA is identified by its one-letter amino acid code. The tRNAs for serine and leucine are further identified by their codon family specificity. The *ATPase6* and *ATPase8* genes overlap by 46 nucleotides.

respectively) were ligated into the vector pBluescript SK$^+$ (Stratagene, La Jolla, California) and cloned in the *Escherichia coli* host strain DH5α. Some fragments were subcloned as *Hin*dIII fragments. Inserts were sequenced from the vector and by primer walking using the dideoxy chain termination technique. Both strands of the genome were sequenced and all overlaps between clones were confirmed by polymerase chain reaction (PCR) and direct sequencing from the purified mitochondrial DNA preparation.

Sequences were aligned by ESEE (CABOT and BECKENBACH 1989) and phylogenetic analyses were performed using the PAUP (SWOFFORD 1990) and the PHYLIP (FELSENSTEIN 1989) programs as well as programs designed by A.v.H. The alignment and a list of primers used for sequencing are available from A.J.

RESULTS AND DISCUSSION

Genome structure and organization: Like other mammalian mitochondrial genomes the marsupial genome appears to code for 22 tRNAs, 2 rRNAs and 13 proteins. The organization of the opossum mtDNA is shown in Figure 1 and the sequence of the L strand is presented in Figure 2. The length of the molecule is 17,084 bp.

Control region: The opossum control region is 1613 bp long. Three conserved sequence blocks (CSBs) have been identified in placentals (WALBERG and CLAYTON 1981) and two of these (CSBII and CSBIII) can be identified in opossum (Figure 2). By deletion analysis, these regions have been shown to constitute a bipartite recognition element for a RNase involved in processing of the RNA primer for DNA replication (BENNETT and CLAYTON 1990). Heterologous assays with human enzyme and mouse mitochondrial RNA indicate that essential sequences for substrate recognition are conserved among placentals (BENNETT and CLAYTON 1990). The sequence similarity for this region suggests that the functional conservation extends also to marsupials. Only a tentative identification of CSBI was possible due to limited sequence similarity. CSBI seems to be separated from the other sequence blocks by a region of repeats, as is the case in the rabbit mitochondrial control region (MIGNOTTE *et al.* 1990).

Additional regions of primary sequence conservation have been identified among placentals (SACCONE *et al.* 1991). These regions are located in the left and middle domain of the control region and are partially conserved in the marsupial. In particular, some stretches are highly conserved between placentals and opossum (Figure 2). One of these is located around position 15600 and displays similarity to a sequence that is associated with the termination of displacement loop-synthesis in humans and rodents (WALBERG and CLAYTON 1981).

The opossum control region contains several repeated sequence motifs. One stretch of 25 nucleotides adjacent to the putative termination-associated sequence exists in two almost perfect copies. Furthermore, a region of repeated sequence motifs is located between the putative CSBI and CSBII (positions 16361 to 16613). It is made up exclusively of adenine and thymine residues, that are repeated in two imperfect motifs (AAATA-AAAAAAA(A)TAAAATT and TA(A)TAATAAA), with additional As and Ts interjected. The above two motifs are repeated 3 and 12 times, respectively, creating a 253-bp region of As and Ts. A further region of repeats is located between CBSIII and $tRNA^{Phe}$, starting at position 16772. This region contains 8 copies of the motif (A/G)T(A/C)AAATTAT-AAAATTT(T)A(A/G), two of which are partial.

The two regions containing multiple repeated motifs in the opossum mitochondrial genome were amplified from DNA from three animals from Berkeley and Santa Cruz, California, and Sweeny, Texas, as well as from cloned mitochondrial DNA. Both regions vary in length among the animals and two of the animals were heteroplasmic with respect to the number of AT-rich repeats (A. JANKE, unpublished observation). Thus, the copy numbers of these repeats vary both between and within individuals as is the case in other animals displaying repeats in the control region (*e.g.*, SOLIGNAC *et al.* 1983; HAUSWIRTH *et al.* 1984; SOLIGNAC *et al.* 1986; WILKINSON and CHAPMAN 1991; ARNASON and RAND 1992; BROWN *et al.* 1992).

Transfer RNAs: As in other animals, 22 tRNA genes are found in the mitochondrial genome of the opossum. Mitochondrial tRNAs show more structural variation than nuclear tRNAs, particularly in their dihydrouridine (DHU) and TΨC loops. The marsupial gene sequence allows the evolutionary timing of some of these structural changes. Unlike the chicken (DESJARDINS and

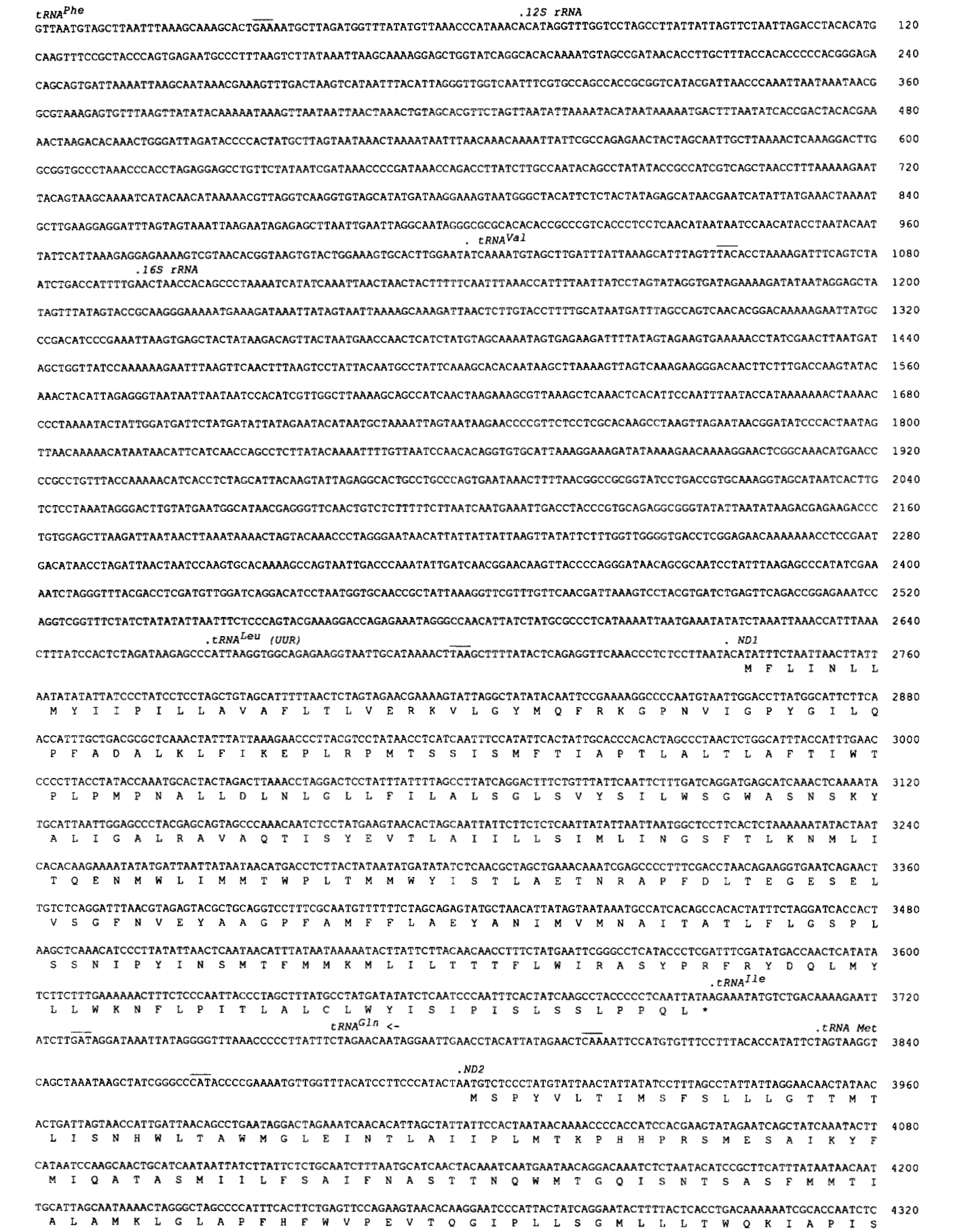


FIGURE 2.—(Continued on next four pages)

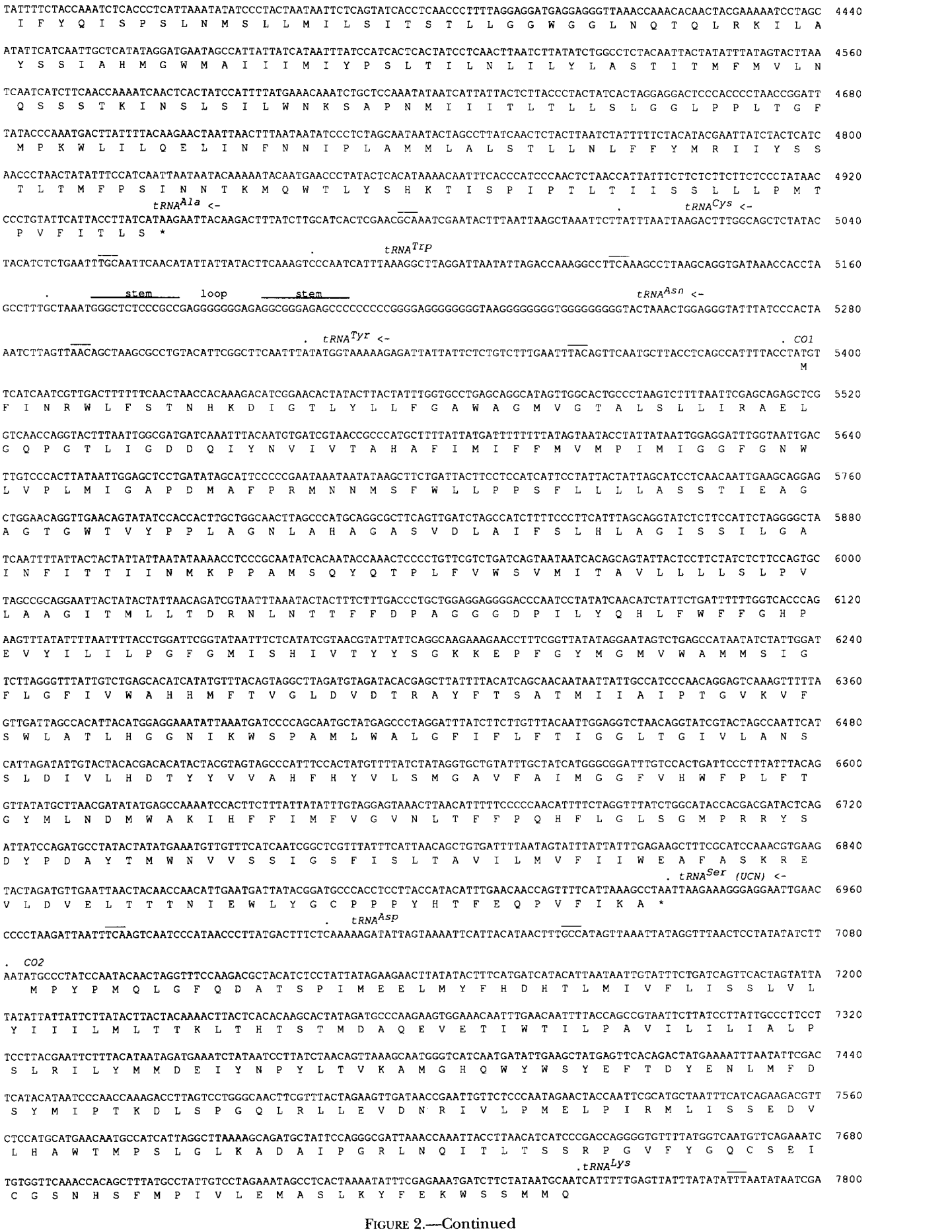


FIGURE 2.—Continued

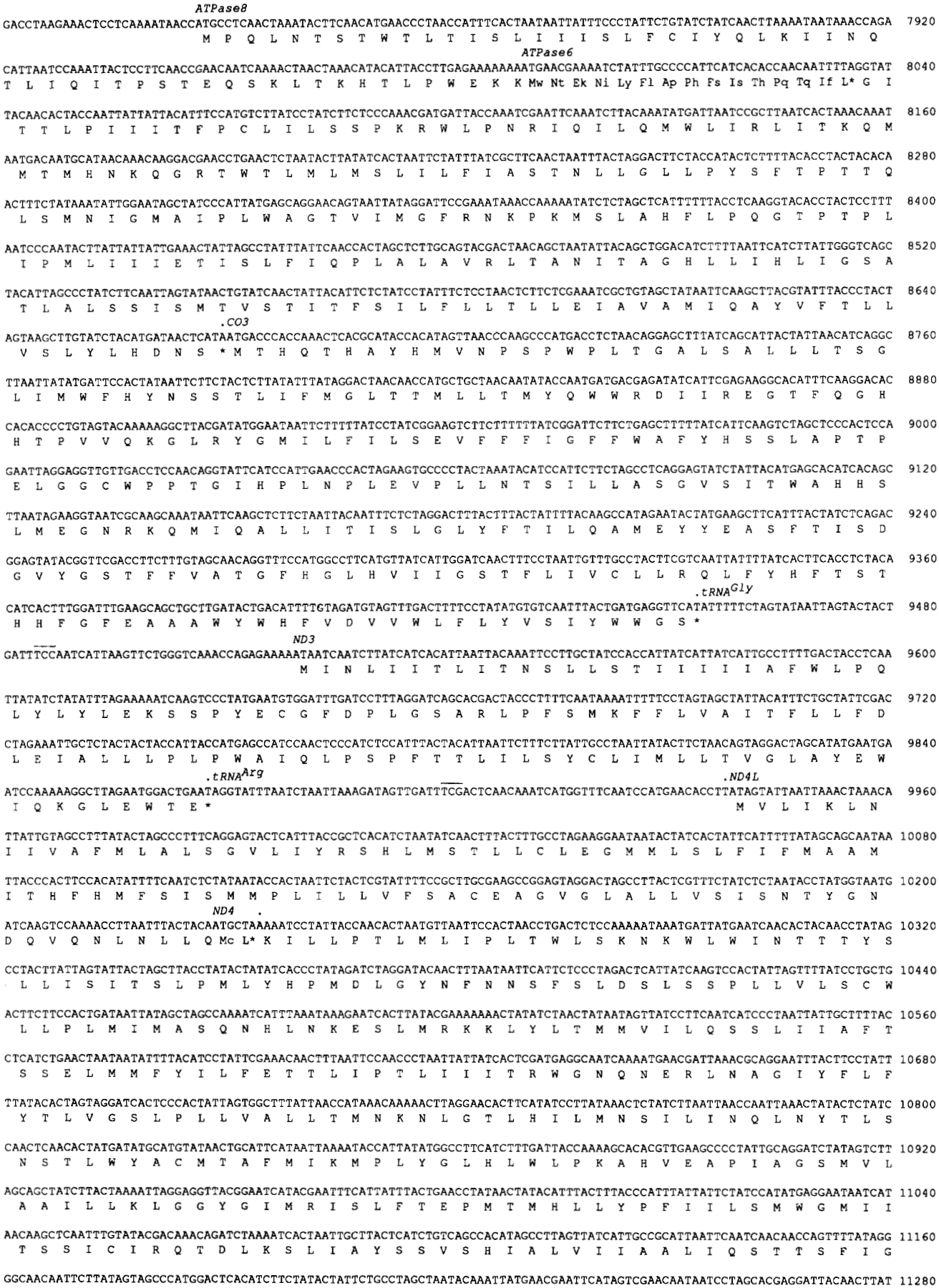


FIGURE 2.—Continued

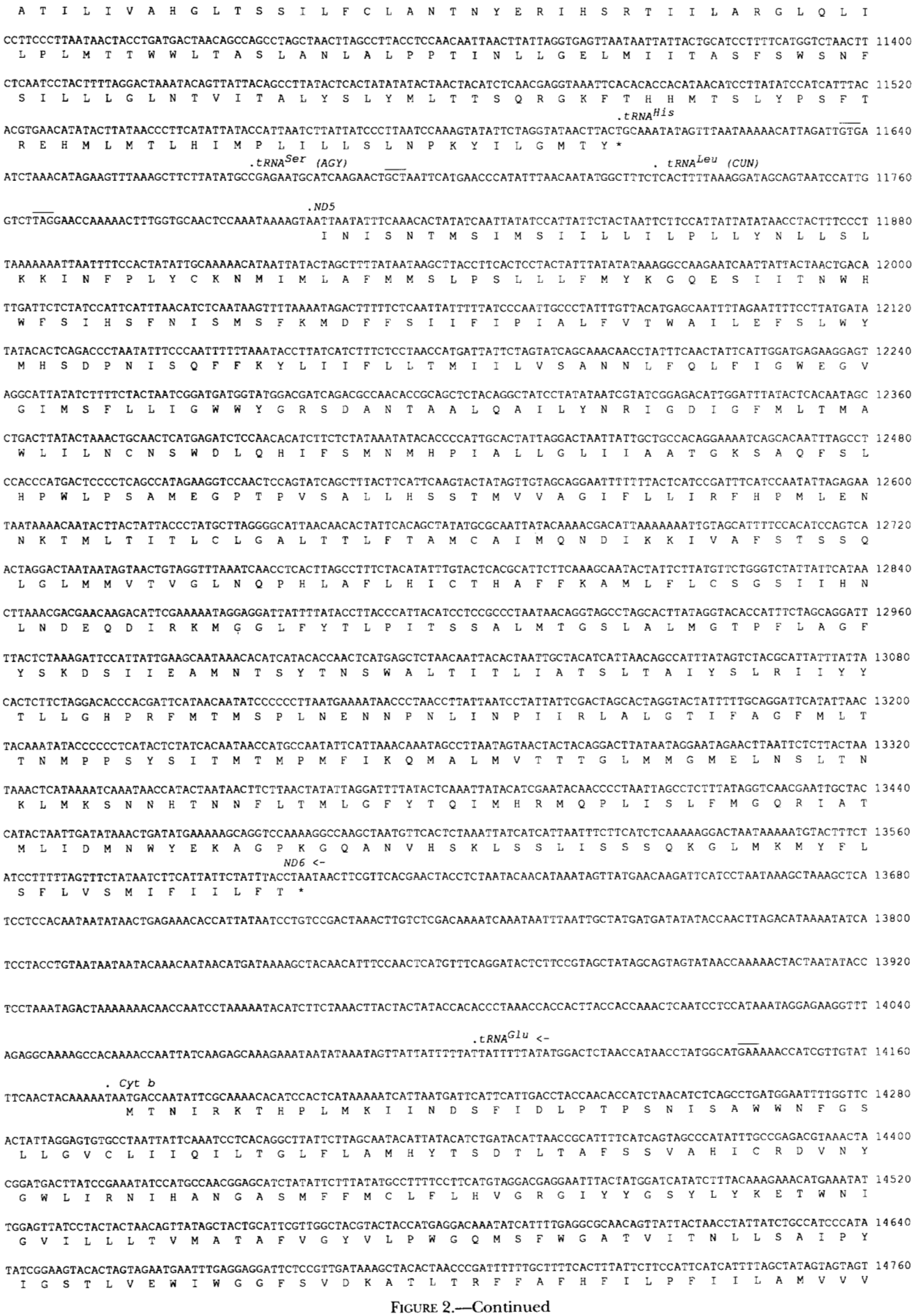


FIGURE 2.—Continued

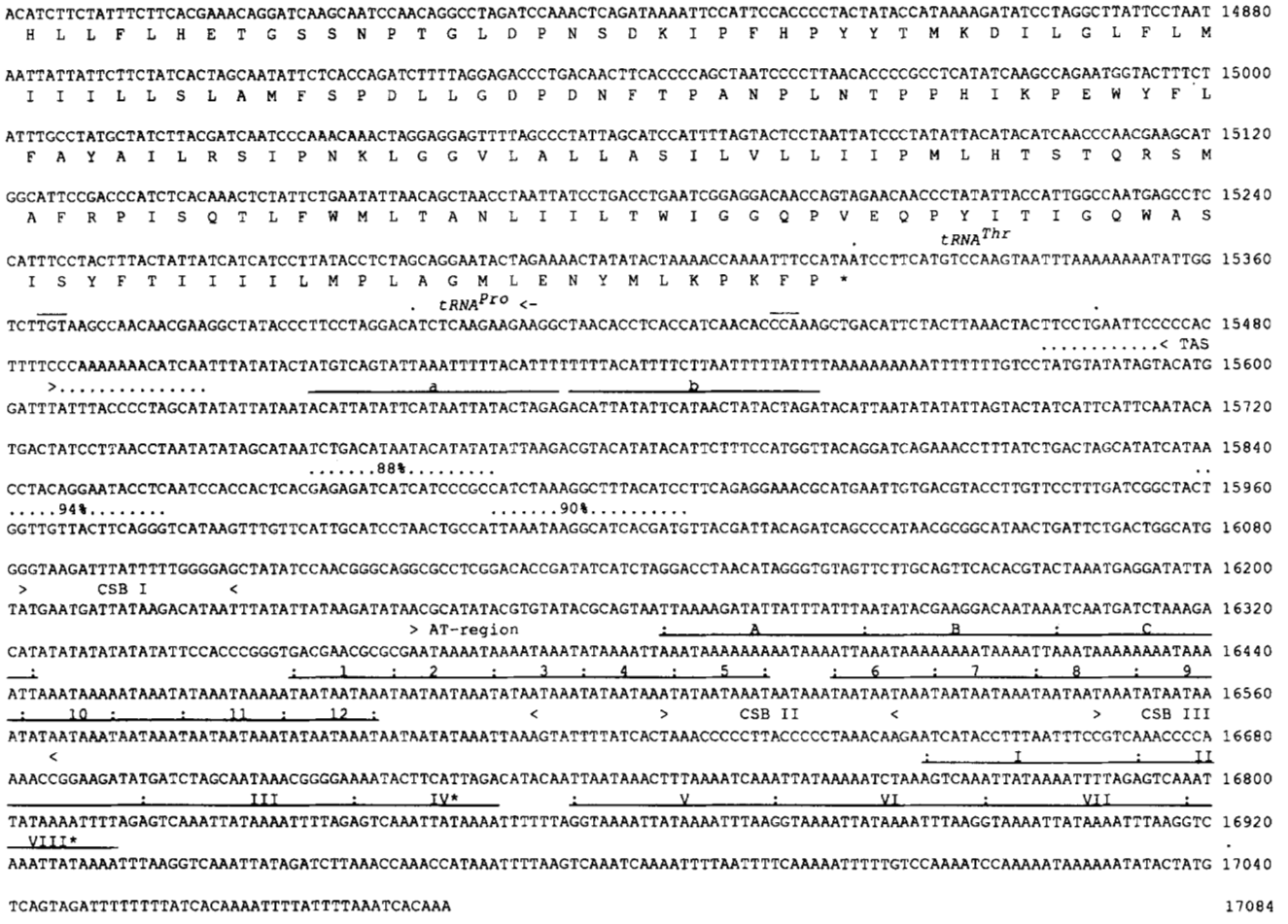


FIGURE 2.—The 17084 nucleotide sequence of the *D. virginiana* mitochondrial genome. Position 1 is at the 5′-end of the *tRNA*^{Phe} gene. Genes that are encoded with H as the sense strand are indicated by arrows. The first characters of the names of genes indicate their inferred first base and their last nucleotides are indicated by a dot. The predicted amino acid sequence (one-letter code, asterisks denote termination codons) of protein-coding genes is shown under the sequence. Where genes overlap, lower case is used for the first gene. The anticodons of tRNAs are overlined as well as the stem of the origin of light strand replication. In the control region, repeats are overlined and indicated by numbers and letters and conserved sequence blocks (CSB) are delimitated by arrow heads (< >). Regions of high sequence similarity among mammals are indicated by dots over the sequence and by the percent of positions where the opossum and all six placental sequences are identical. A partially deleted repeat is marked by an asterisk.

MORAIS 1990) and the frog (ROE *et al.* 1985), the tRNA for lysine of both placentals and the opossum has a reduced DHU arm (Figure 3). Thus, the reduction of the DHU arm is inferred to have taken place in an ancestor common to marsupials and placentals.

In placentals, the tRNA gene for serine (UCN) has an anticodon stem which consists of 6 instead of 5 base pairs and only one nucleotide is found between the acceptor stem and the DHU stem (YOKOGAWA *et al.* 1991). These unusual features, which do not exist in chicken and frog, are found in the inferred structure of the opossum tRNA^{Ser}(UCN) (Figure 3). Thus, these structural changes have occurred in a common ancestor of placental and marsupial mammmals. One other unusual structural feature exists in the the opossum tRNA^{Ser}(AGY) gene product, which lacks a DHU arm (Figure 3). This feature is, however, conserved among the vertebrates.

A rearrangement of tRNA genes around the replication origin of the L strand has been described in marsupials from South America, Australia and New Guinea (PÄÄBO *et al.* 1991). This arrangement is also found in the North American opossum genome. It involves an apparent transposition of the tRNA genes for alanine, asparagine and cysteine and can be explained by a duplication followed by deletions of tRNA genes and the recruitment of a new origin of light strand replication. The rearranged tRNA genes have longer intergenic flanking regions than those seen for other tRNA genes, and the hairpin loop of the origin of light strand replication is substantially longer than in placental animals and may represent a vestige of tRNA genes lost after the putative deletion.

RNA editing: The tRNA gene for aspartic acid carries the anticodon GCC instead of the normal anticodon for aspartic acid (GTC) (Figure 3). The second position of

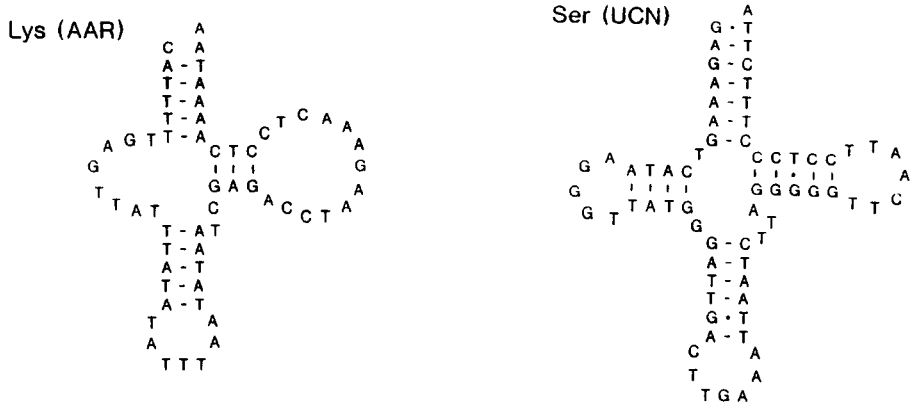
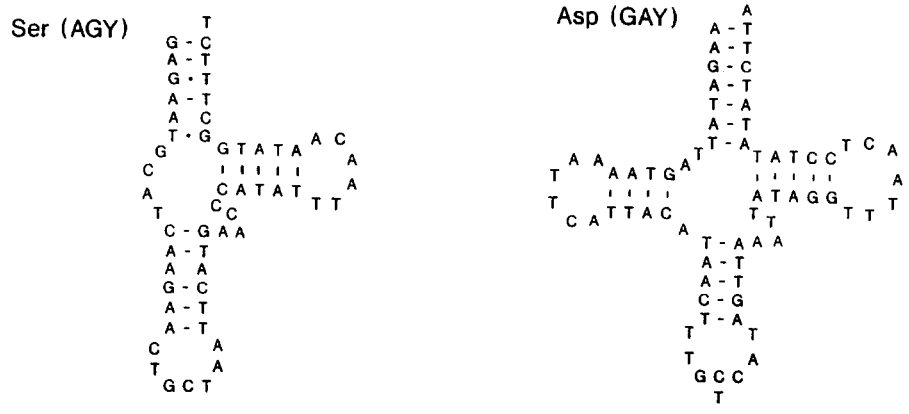


FIGURE 3.—Inferred secondary structures of four unusual tRNA genes in the opossum mitochondrial genome.



the codon in the transcription product is posttranscriptionally changed to be recognized as an uridine residue (JANKE and PÄÄBO 1993). Thus, the anticodon of this tRNA is generated by an RNA editing process, a phenomenon shown to occur also in the $tRNA^{Asp}$ gene product of Australian marsupials (M. DÖRNER, personal communication). However, the gene for $tRNA^{Asp}$ of monotremes carries the anticodon GTC (unpublished observation) as do placentals. Thus, the editing of $tRNA^{Asp}$ evolved in the common ancestor of marsupials. All other tRNA genes carry the expected anticodons and no other sequence position in the opossum mitochondrial genome is an obvious candidate for RNA editing in that it would give rise to a nonfunctional gene product. Furthermore, sequencing of 3,000 bp of protein-coding mitochondrial cDNAs from an Australian marsupial (*Protorus tridactylus*) has failed to show any other sites of RNA editing (M. DÖRNER, personal communication). Thus, RNA editing seems to be restricted to this single position.

RNA editing has been described in plant mitochondria (HIESEL *et al.* 1989; GUALBERTO *et al.* 1989; COVELLO and GRAY 1989) and chloroplasts (HOCH *et al.* 1991) and in the mitochondria of trypanosomes (SIMPSON and SHAW 1989). The most likely mechanism by which the editing of the opossum $tRNA^{Asp}$ occurs is deamination of the cytosine residue. Current work is aimed at elucidating how this mechanism evolved. It could potentially have been achieved by the recruitment of a gene involved in deamination of cytidine residues in nuclear transcripts (HODGES *et al.* 1991).

Ribosomal RNA genes: As in other vertebrates, the genes for the *12S* and *16S rRNA* genes are separated by the $tRNA^{Val}$ gene and are bounded on the other sides by the genes for $tRNA^{Phe}$ and $tRNA^{Leu(UUR)}$, respectively. The inferred secondary structure of the *12S rRNA* gene of opossum and the cow (ANDERSON *et al.* 1982) was found to be conserved.

Protein-coding genes: As in other vertebrates, the opossum mitochondrial genome contains 13 protein-coding genes, 12 of which are encoded on the H-strand so that the L strand gives the sense reading frames. The codon usage in opossum is similar to that of other vertebrates (not shown). Eleven genes are inferred to use TAA as a translational termination codon. Of these, 6 have incomplete termination codons, which are presum-

TABLE 1

Mean base composition for the mitochondrial protein-coding genes of seven mammalian species

Codon position	A	G	C	T
1st	32.1 ± 0.7	20.7 ± 0.8	24.4 ± 1.6	22.8 ± 1.5
2nd	19.5 ± 0.2	12.2 ± 0.1	26.2 ± 0.7	42.1 ± 0.6
3rd	42.4 ± 3.4	5.0 ± 1.6	31.2 ± 7.0	21.4 ± 5.1

Mean base compositions (%) for the 13 protein-coding genes and standard deviations are given for each of the three codon positions.

TABLE 2

Expected numbers of sequence differences at saturation and observed numbers of differences among seven mammalian taxa

Codon position	Transitions		Transversions	
	Expected	Observed	Expected	Observed
1st	909	284–416	1855	194–591
2nd	999	110–292	1610	58–235
3rd	654	760–924	1855	751–1188

The expected numbers of transitions and transversions at saturation were calculated using the base composition in Table 1 and the method of HASEGAWA *et al.* (1985). In addition, the highest and lowest observed numbers of transitional and transversional differences among the seven taxa are given.

ably formed by polyadenylation of the transcript. Two genes (*ATPase 8* and *ND6*) use TAG as termination codons, whereas the AGG and AGA termination codons used in humans (ANDERSON *et al.* 1981) and frog (ROE *et al.* 1985) are not utilized in opossum. In eight cases, ATG is used as translational initiation codons, whereas ATA is used in four cases and ATT in one.

Evolutionary relationships among placental mammals: To elucidate the relationship among placental mammals, we used sequences from six complete mitochondrial genomes: rat, mouse, human, cow, whale and seal. Gaps had to be introduced at a few positions to align the protein-coding genes, especially at the 3′-ends of genes. Positions with gaps and areas where the alignment was ambiguous were excluded from subsequent analyses. The *ND6* gene, encoded on the L strand, differs significantly in its base composition from the other genes and was therefore excluded from the analyses when not otherwise stated. Similarily, tRNA, rRNA and non-coding sequences were excluded to avoid ambigous alignments and allow for a coherent maximum likelihood analysis.

Base composition: Base composition of nucleotide sequences is known to vary among taxa (*e.g.*, SUEOKA 1988) and this may obscure phylogenetic information. Table 1 shows the mean base composition for the seven mammalian species for each of the three codon positions. Most changes at third codon positions are silent, and this position demonstrates the highest level of compositional bias (MUTO and OSAWA 1987) as reflected by the higher standard deviation at this codon position. As for other mitochondrial genomes (GADELATA *et al.* 1989), guanosine residues (G) are underrepresented on the L strand in the opossum, an observation most apparent at the third codon positions, where the average G content is 5%. Furthermore, the adenosine content of third positions and the thymine content of second positions are high (42%).

To elucidate whether the nucleotide compositions of the protein-coding genes of the different taxa differ, we tested if the nucleotide distribution is homogeneous among the placental taxa by a chi-square test (VON HAESELER *et al.* 1993). Although this test suffers from the drawback that the sequences are not independent, it can be used as a rough guide to detect differences in base composition. The base compositions of the second codon positions are homogenous ($P = 0.996$) as are those of the first codon positions ($P = 0.073$). For the first positions, the base composition of the opossum differs significantly from that of the placentals. However, since this affects only the outgroup in the phylogenetic analysis, it is not expected to cause incorrect tree topologies to be inferred. The base composition at third codon positions is highly non-homogeneous among the placentals and is thus unsuitable for phylogenetic reconstruction. When the distribution of purines and pyrimidines at third codon positions is investigated, the inhomogeneity remains. However, this is due to a biased composition of purines and pyrimidines in the human mitochondrial genome. When the human sequence was removed from the analysis, the remaining taxa were homogeneous with respect to their purine-pyrimidine composition at third codon positions. Thus, since only one taxon has a base composition that differs from the other taxa, this is not expected to affect the estimation of the tree topology but only the inferred lengths of branches. Transversions at third codon positions can therefore be used to infer the branching order among placental lineages.

Multiple substitutions: In animal mitochondria, transitions predominate over transversions by a factor of at least 10 (BROWN *et al.* 1982; DESALLE *et al.* 1987). Furthermore, in the genetic code of mammalian mitochondria, transitions at third codon positions do not result in amino acid replacements. As a consequence, the third codon positions evolve several times faster than first and second codon positions, where the majority of changes cause amino acid replacements. Thus, transitions at third codon positions are expected to be particularly prone to losing phylogenetic information due to multiple hit phenomena.

We computed the expected pairwise differences at saturation using the base compositions given in Table 1 and the procedure of HASEGAWA *et al.* (1985). Table 2 gives the expected numbers of transitions and transversions at saturation for the three codon positions. When the observed transitional and transversional differences are compared to the numbers expected at saturation, we found that all

observations at first and second codon positions are well below the expected saturation level. At third codon positions, the transitional differences between all taxa compared are above the expected saturation level whereas for transversions at third codon positions, no value exceeds the saturation level. Thus, transitions at third codon positions are heavily affected by multiple substitutions and should be avoided in phylogenetic analyses of these taxa.

To investigate why the observed numbers of transitional differences at third codon positions are higher than the expected saturation level (Table 2), we calculated how the observed transitions are expected to vary as a function of observed transversions after divergence of two taxa assuming various transition-transversion ratios. Figure 4 shows how the relative number of observed transitions first increases and subsequently decreases as transversions erase the record of previous transitions (BROWN *et al.* 1982). Irrespective of the transition to transversion ratio, the amounts of observed substitutions converge to an equilibrium point when the sequences have been saturated with substitutions. For first and second codon positions, the numbers of transitions and transversions that are observed from the data are far from the saturation point both with respect to transitions and transversions. In contrast, at third codon positions, the numbers of transitions lie above the saturation point whereas transversions have not yet reached saturation. That the observed numbers of transitional sequence differences can exceed the numbers at saturation agrees with a study of mitochondrial sequences for ungulates where sequence differences were plotted against paleontologically inferred divergence times (IRWIN *et al.* 1991). Thus, the transitions at third positions of codons are saturated for all divergences in the phylogeny whereas transversional changes at third codon positions are well suited for phylogenetic inference.

First positions of leucine codons are similar to third codon positions in that they may experience silent transitions (TTR to CTR) (IRWIN *et al.* 1991). Consequently, they are likely to be saturated for deeper divergences among placentals. Therefore, transitions at first positions of leucine codons were excluded from subsequent analyses except where indicated.

Tree reconstruction: Due to their different base compositions, first and second codon positions were independently used to construct phylogenetic trees using the maximum likelihood procedure (FELSENSTEIN 1981). Figure 5 shows the best tree derived from the second codon positions. Maximum parsimony (SWOFFORD 1990) and neighbor-joining (SAITOU and NEI 1987) yielded trees of identical topology. Bootstrap analyses (FELSENSTEIN 1985) for maximum parsimony and neighbor-joining independently showed that all internal branches were seen in more than 95% of 1000 bootstrap replications. Identical results were obtained for first codon positions and for transversions at third codon positions.

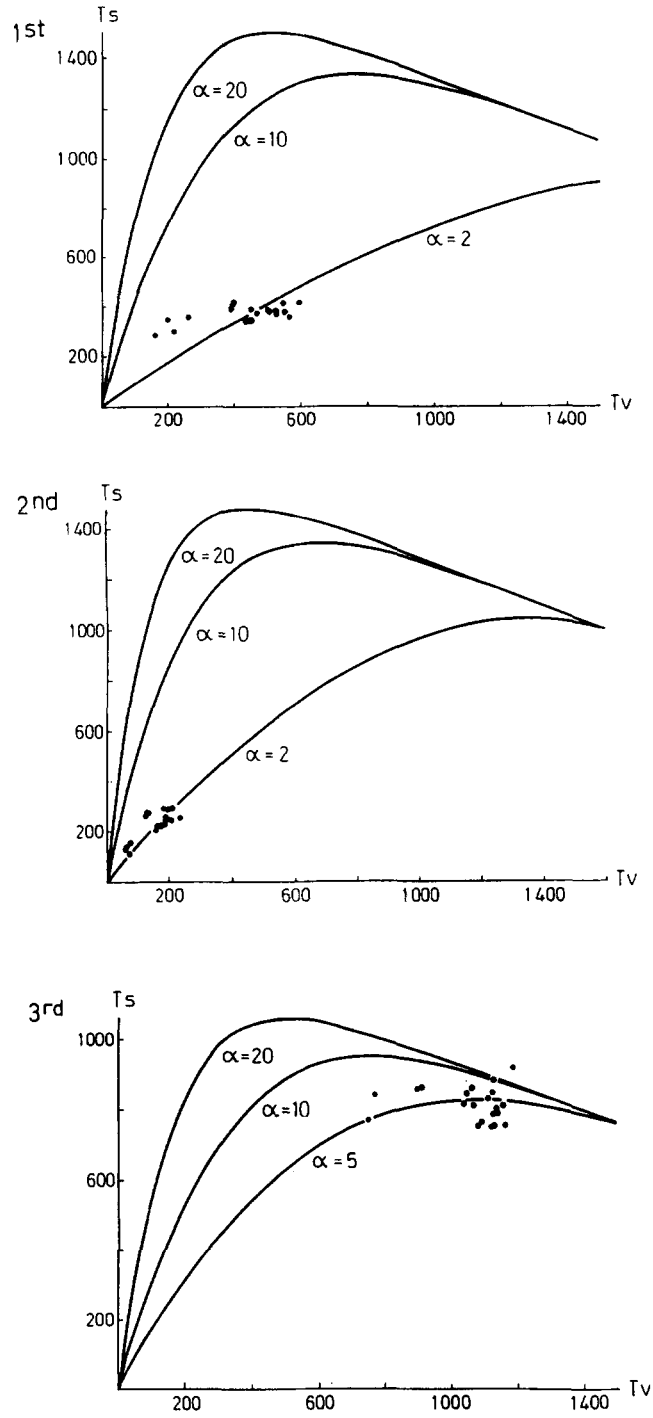


FIGURE 4.—Plot of expected observed transversions (Tv) *vs.* observed transitions (Ts) assuming various transition–transversion biases (α) calculated according to HASEGAWA *et al.* (1985) and using the the observed base compositions given in Table 1. Dots denote the transitional and transversional differences as observed from the data. The apparent transition bias for first and second codon positions of around 2 may be underestimated due to invariable positions in the sequences which cause the expected numbers of changes to be overestimated.

To evaluate also other possible tree topologies, the taxa were reduced to six by constraining the topologies to those where the mouse and rat are monophyletic.

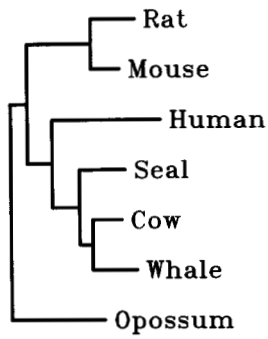


FIGURE 5.—Maximum likelihood tree relating the mitochondrial genomes of six placental mammmals and one marsupial mammal to each other. The topology is based on all differences at second codon positions of all protein-coding genes except *ND6*. Trees based on differences causing amino acid replacements at first codon positions and on transversions at third codon positions have identical topologies.

This allowed all possible 105 trees to be evaluated. The likelihoods for the trees based on first and second codon positions which had identical topologies were added and the best tree (Figure 5) was tested against all other topologies according to KISHINO and HASEGAWA (1989). Only one tree was not significantly worse than the tree in Figure 5. This tree differs from the best tree in having the seal as the sister taxon of the cow. The monophyly of ungulates, which is supported by other lines of evidence (PROTHERO *et al.* 1988), is thus the least well supported relationship in this data set.

To further evaluate the robustness of the tree topology to assumptions about the mode of evolution and the monophyly of placentals, we included all nucleotide differences oberved at first, second and third codon positions, the gene for ND6 as well as the homologous sequences of chicken and frog in the analysis. Several maximum likelihood computations with varying values of the transition/transversion ratio and varying substitution ratios of first and second codon positions relative to the third codon positions were performed. In all cases, the same tree topology as in Figure 5 was found (Figure 6), demonstrating the astounding robustness of the result.

Placental phylogeny: The analyses above establish with a high degree of confidence that of the species analyzed here, primates and ungulates are sister taxa and that rodents represent an early divergence among placentals. This is in sharp contrast to the view of placental evolution which regards primates and rodents as sister taxa to the exclusion of ungulates (ROMER 1966; KIELAN-JAWOROWSKA *et al.* 1979; YOUNG 1981; LI *et al.* 1987) but agrees with the opinion of some morphologists (MCKENNA 1975; SZALAY 1977) as well as trees obtained from nuclear-encoded protein genes (EASTEAL 1988, 1990, 1992; LI *et al.* 1990). Thus, both organellar and nuclear molecular data agree in the establishment of rodents as an outgroup to primates and ungulates.

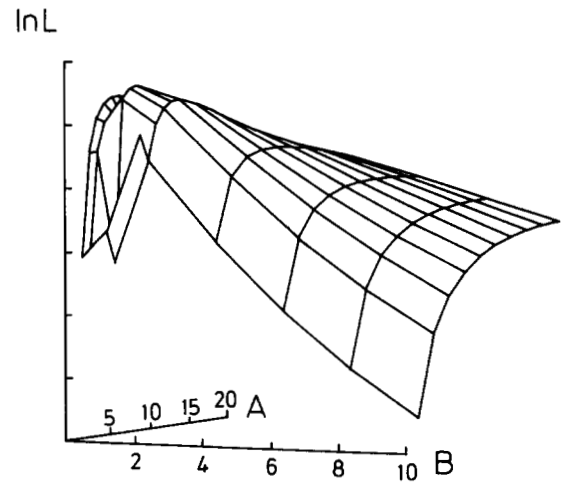


FIGURE 6.—Approximate maximum likelihood surface for different transition/transversion ratios (A) and different ratios of substitution rates for first and second *vs.* third codon positions (B) using all differences at all positions of 13 protein-coding genes from the species used in Figure 5 as well as the frog and the chicken sequences. The maximum value of the surface is in the neighborhood of (1, 5).

In the tree emerging from the mitochondrial data, the seal groups significantly with the cow and the whale thus associating carnivores and ungulates in a monophyletic group. In his classification of mammals, SIMPSON (1945) suggested that carnivores and ungulates were monophyletic and joined them in the group Ferungulata, which, however, did not include whales. The mitochondrial data confirm the view that carnivores and ungulates share a common ancestor and thus that Ferungulata represents a natural taxon to which cetaceans should be added.

Evolutionary rates of protein coding genes: The elucidation of the phylogeny of placentals has important consequences for the understanding of molecular evolution. Using artiodactyls as an outgroup to primates and rodents, it has been argued that the rate of molecular evolution is twice as fast on the lineage to rodents (WU and LI 1985). However, since artiodactyls rather than rodents appear to be the sister taxon of primates, the acceleration on the lineage to rodents may be absent (EASTEAL 1988; GU and LI 1992) or much less pronounced.

To test whether the rate of evolution is similar in various placental lineages, we investigated if the molecular evolution of the 13 protein-coding genes conforms to a molecular clock model. Due to differences in nucleotide composition third positions were excluded from the analyses. Maximum likelihood estimates were computed for the tree structure in Figure 5 under the assumption of identical evolutionary rates in all parts of the tree (clock assumption) as well as without such an assumption. A likelihood ratio test was applied to test whether the non-constrained tree resulted in a significantly better fit to the data. Table 3 shows that the clock assumption cannot be rejected in either first or second codon

TABLE 3

Clocktest for mitochondrial genes

Gene	Codon position 1	Codon position 2
ATP6	10.53	17.54*
ATP8	14.14	2.27
CO1	16.22*	18.90*
CO2	24.09*	35.07*
CO3	10.80	11.05
CYTb	15.23*	24.22*
ND1	3.10	13.04
ND2	1.18	9.25
ND3	11.41	5.88
ND4	16.00*	4.62
ND4L	4.49	11.51
ND5	7.51	8.65
ND6	6.86	7.89

Likelihood ratio statistic for the first and second positions of 13 protein-coding genes. The analysis is based on the tree in Figure 5. Asterisks denote significant deviations from the clock assumption. The genes in boldface are clock-like for both first and second codon positions. The test is based on a chi square distribution with 5 degrees of freedom and a significance level of $\alpha = 0.01$.

positions for eight of the 13 genes. Thus, for the majority of mitochondrial genes a clock assumption is valid. For three of the genes, the clock is rejected for first and second positions. Most strongly, this is the case for the *cytochrome oxidase 2* gene which shows a clear acceleration on the lineage leading to humans. This is in agreement with a previous observation that this gene evolved at an increased tempo during early primate evolution (RAMHARACK and DEELEY 1987). The genes for *cytochrome b* and *cytochrome oxidase 1* also fail to conform to a clock model at first as well as second positions. The former gene has been shown to be accelerated in primates (MA *et al.* 1993; IRWIN and ARNASON 1994) and at least for first codon positions, this is seen also in the current data.

Divergence dates: The eight genes that conform to a clock model within placental lineages (Table 3) were used to estimate the approximate times of divergence of the placental radiation. As a calibration point the divergence between placental and marsupial mammals was used. The paleontological record shows that by the early late Cretaceous, approximately 95 million years ago, the placental and marsupial lineages were well separated (CIFELLI and EATON 1987). Based on this, a date of 130 million years for the marsupial-placental divergence is commonly assumed (CARROLL 1988) and agrees with a date derived from comparisons of globin sequences (AIR *et al.* 1971). When this date is used as a calibration point, the average dates given in Table 4 are obtained. It is worth noting, that even if the calibration date would be shown to be wrong, the relative timing of the divergences would remain the same.

These divergence dates are in general agreement with paleontological data as well as previous molecular investigations with the exception of the divergence between the mouse and rat lineages. Paleontological data

TABLE 4

Tentative dates for divergences among placental lineages

Lineage divergence	Date
Rodents, ferungulates and primates	114 ± 15
Primates, ferungulates	93 ± 12
Carnivores, artiodactyles	55 ± 15
Cow, whale	41 ± 10
Mouse, rat	35 ± 17

Approximate dates of divergence were calculated using 1st and 2nd codon positions of each of the genes shown to evolve in a clock like manner in Table 3. As a calibration point, 130 million years for the divergence between placental and marsupial mammals was used. Means of the dates and one standard deviation are given.

suggest that the mouse and rat lineages diverged 8–12 million years ago (JAEGER *et al.* 1986). A study using quantitative immunological comparisons of albumin have arrived at dates between 20 and 35 million years ago (WILSON *et al.* 1977; SARICH 1985) and studies (*e.g.*, BROWNELL 1983) based on DNA/DNA hybridizations have arrived at a date of 17–25 million years ago. The date inferred from the tree analyses above remains approximately the same when the calculations are based on observed transversions at first and second codon positions. Thus, the divergence between mouse and rat may be substantially older than the current interpretation of the fossil record indicates. In particular, the fossil taxon *Antemus* which is generally thought to represent a common lineage leading to mice and rats (CATZEFLIS *et al.* 1992), may belong to one or the other of these lineages or possibly to neither. Interestingly, the morphological characters used to define the murine group and this fossil have recently been suggested to have evolved convergently among muroid rodents (CHEVRET *et al.* 1993).

It is noteworthy that the times of divergence of the rodent, primate, carnivore and ungulate lineages range from 41 to 114 million years ago. This is in contrast to the traditional view of placental evolution, which assumes a rapid radiation following a presumed catastrophic event causing the extinction of dinosaurs at the end of the Cretaceous. The fact that the mitochondrial sequence data are able to resolve the divergence of the placental groups as well as the tentative dates of these divergences may indicate that the evolution of early placentals was not bush-like but rather might have taken place during several tens of millions of years prior to the Cretaceous-Tertiary boundary.

We thank F. ALTHAUS for artwork, R. CROZIER, M. HASEGAWA, D. IRWIN, A. MEYER, A. SIDOW and especially E. PRAGER for comments on the manuscript and the DFG and Genzentrum München for financial support.

LITERATURE CITED

ADACHI, J., Y. CAO and M. HASEGAWA, 1993 Tempo and mode of mitochondrial DNA evolution in vertebrates at the amino acid sequence level: rapid evolution in warm-blooded vertebrates. J. Mol. Evol. **36:** 270–281.

AIR, G. M., E. O. P. THOMPSON, B. J. RICHARDSON and G. B. SHARMAN, 1971 Amino-acid sequences of Kangaroo myoglobin and hemoglobin and the date of marsupial-eutherian divergence. Nature **229:** 391–394.

ANDERSON S., A. T. BANKIER, B. G. BARRELL, M. H. L. DE BRUIJN, A. R. COULSON *et al.*, 1981 Sequence and organization of the human mitochondrial genome. Nature **290:** 457–465.

ANDERSON S., M. H. L. DE BRUIJN, A. R. COULSON, I. C. EPERON, F. SANGER *et al.*, 1982 Complete sequence of the bovine mitochondrial DNA. Conserved features of the mammalian mitochondrial genome. J. Mol. Biol. **156:** 683–717.

ARNASON, E., and D. M. RAND, 1992 Heteroplasmy of short tandem repeats in mitochondrial DNA of Atlantic cod, *Gadus morhua*. Genetics **132:** 211–220.

ARNASON, U. and E. JOHNSSON, 1992 The complete mitochondrial DNA sequence of the harbor seal, *Phoca vitulina*. J. Mol. Evol. **34:** 493–505.

ARNASON U., A. GULLBERG and B. WIDEGREN, 1991 The complete nucleotide sequence of the mitochondrial DNA of the fin whale, *Baleanoptera physalus*. J. Mol. Evol. **33:** 556–568.

BIBB, M. J., R. A. VAN ETTEN, C. T. WRIGHT, M. W. WALBERG and D. A. CLAYTON, 1981 Sequence and gene organization of the mouse mitochondrial DNA. Cell **26:** 167–180.

BENNETT, J. L., and D. A. CLAYTON, 1990 Efficient site-specific cleavage by RNase MRP requires interaction with two evolutionary conserved mitochondrial RNA sequences. Mol. Cell. Biol. **10:** 2191–2201.

BROWN, J. R., A. T BECKENBACH and M. J. SMITH, 1992 Mitochondrial DNA length variation and heteroplasmy in populations of White Stugeon (*Acipenser transmontanus*). Genetics **132:** 221–228.

BROWN, W. M., E. M. PRAGER, A. WANG and A. C. WILSON, 1982 Mitochondrial DNA sequences of primates: tempo and mode of evolution. J. Mol. Evol. **18:** 225–239.

BROWNELL, E., 1983 DNA/DNA hybridization studies of muroid rodents: symmetry and rates of molecular evolution. Evolution **37:** 1034–1051.

BULMER, M., K. H. WOLFE and P. M. SHARP, 1991 Synonymous nucleotide substitution rates in mammalian genes: Implications for the molecular clock and the relationship of mammalian orders. Proc. Natl. Acad. Sci. USA **88:** 5974–5978.

CABOT, E. L., and A. T. BECKENBACH, 1989 Simultaneous editing of multiple nucleic acid and protein sequences with ESEE. Comput. Appl. Biosci. **5:** 233–234.

CARROLL, R. L., 1988 *Vertebrate Paleontology and Evolution*. W. H. Freeman, New York.

CATZEFLIS, F. M., J.-P. AGUILAR and J.-J. JAEGER, 1992 Muroid rodents: phylogeny and evolution. Trends Ecol. Evol. **7:** 122–126.

CHEVRET, P., C. DENYS, J.-J. JAEGER, J. MICHAUX and F. M. CATZEFLIS, 1993 Molecular evidence that the spiny mouse (*Acomys*) is more closely related to gerbils (Gerbillinae) than to true mice (Murinae). Proc. Natl. Acad. Sci. USA **90:** 3433–3436.

CIFELLI, R. L., and J. G. EATON, 1987 Marsupial from the earliest Late Cretaceous of western US. Nature **325:** 520–522.

COVELLO, P. S., and M. W GRAY, 1989 RNA editing in plant mitochondria. Nature **341:** 662–666.

DESALLE, R., T. FREEDMAN, E. M. PRAGER and A. C. WILSON, 1987 Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila*. J. Mol. Evol. **26:** 157–164.

DESJARDINS, P., and R. MORAIS, 1990 Sequence and gene organization of the chicken mitochondrial gene. A novel gene order in higher vertebrates. J. Mol. Biol. **212:** 599–634.

EASTEAL, S., 1988 Rate constancy of globin gene evolution in placental mammals. Proc. Natl. Acad. Sci. USA **85:** 7622–7626.

EASTEAL, S., 1990 The pattern of mammalian evolution and the relative rate of molecular evolution. Genetics **124:** 165–173.

EASTEAL, S., 1992 A mammalian molecular clock? BioEssays **14:** 415–419.

FELSENSTEIN, J., 1981 Evolutionary trees fron DNA sequences: a maximum likelihood approach. J. Mol. Evol. **17:** 368–376.

FELSENSTEIN, J., 1985 Confidence limits in phylogenesis: an approach using the bootstrap. Evolution **39:** 783–791.

FELSENSTEIN, J., 1989 Phylogenetic Inference Programs (PHYLIP), University of Washington, Seattle, and University Herbarium, University of California, Berkeley.

GADALETA, G., G. PEPE, G. DE CANDIA, C. QUAGLIARIELLO, E.SBISÁ and C. SACCONE, 1989 The complete nucleotide sequence of the *Rattus norvegicus* mitochondrial genome: cryptic signals revealed by comparative analysis between vertebrates. J. Mol. Evol. **28:** 497–516.

GU, X., and W.-H. LI, 1992 Higher rates of amino acid substitutions in rodents than in humans. Mol. Phyl. Evol. **1:** 211–214.

GUALBERTO, J. M., L. LAMATTINA, G. BONNARD, J.-H. WEIL and J.-M. GRIENENBERGER, 1989 RNA editing in wheat mitochondria results in the conservation of protein sequences. Nature **341:** 660–662.

HASEGAWA, M., and H. KISHINO, 1989 Heterogeneity of tempo and mode of mitochondrial DNA evolution among mammalian orders. Jpn. J. Genet. **64:** 243–258.

HASEGAWA, M., H. KISHINO and T. YANO, 1985 Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. **22:** 160–174.

HAUSWIRTH, W. W., M. J. VAN DE WALLE, P. J. LAIPIS and P. D. OLIVO, 1984 Heterogeneous mitochondrial DNA D-loop sequences in bovine tissue. Cell **37:** 1001–1007.

HIESEL, R., B. WISSINGER, W. SCHUSTER and A. BRENNICKE, 1989 RNA editing in plant mitochondria. Science **246:** 1632–1634.

HOCH, B., R. M. MAIER, K. APPEL, G. L. IGLOI and H. KÖSSEL, 1991 Editing of a chloroplast mRNA by creation of an initiation codon. Nature **353:** 178–180.

HODGES, P. E., N. NAVARATNAM, J. C. GREEVE and J. SCOTT, 1991 Site-specific creation of uridine from cytidine in apolipoprotein B mRNA editing. Nucleic Acids Res. **19:** 1197–1201.

IRWIN, D. M., and U. ARNASON, 1994 Cytochrome *b* gene of marine mammals: phylogeny and evolution. Mol. Biol. Evol. (in press).

IRWIN, D. M., T. D. KOCHER and A. C. WILSON, 1991 Evolution of the cytochrome *b* gene of mammals. J. Mol. Evol. **32:** 128–144.

JAEGER J.-J., H. TONG and C. DENYS, 1986 Age de la divergence Mus-Rattus: comparaison des données paléontologiques et moléculaires. C. R. Acad. Sci. **14:** 917–922.

JANKE, A., and S. PÄÄBO, 1993 Editing of a tRNA anticodon in marsupial mitochondria changes its codon recognition. Nucleic Acids Res. **21:** 1523–1525.

KIELAN-JAWOROWSKA Z., T. M. BOWN and J. A. LILLEGRAVEN, 1979 Eutheria, pp. 221–259 in *Mesozoic Mammals, the First Two-thirds of Mammalian History*, edited by J. A. Lillegraven, Z. Kielan-Jaworowska and W. A. CLEMENS. University of California Press, Berkeley.

KISHINO, H., and M. HASEGAWA, 1989 Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J. Mol. Evol. **29:** 170–179.

LI W.-H., M. TANIMURA, P. M. SHARP, 1987 An evaluation of the molecular clock hypothesis using mammalian DNA sequences. J. Mol. Evol.**25:** 330–342.

LI, W.-H., M. GOUY, P. M. SHARP, C. O'HUIGIN and Y.-W. YANG, 1990 Molecular phylogeny of rodentia, lagomorpha, primates, artodactyla, and carnivora and molecular clocks. Proc. Natl. Acad. Sci. USA **87:** 6703–6707.

LANSMAN, R. A., R. O. SHADE, J. F. SHAPIRA and J. C. AVISE, 1981 The use of restriction endonucleases to measure mitochondrial DNA sequence relatedness in natural populations. III. Techniques and potential applications. J. Mol. Evol. **17:** 214–226.

MA, D.-P., A. ZHARKIKH, D. GRAUR, J. L. VANDEBERG and W.-H. LI, 1993 Structure and evolution of opossum, guinea pig, and porcupine cytochrome *b* genes. J. Mol. Evol. **36:** 327–334.

MCKENNA, M. C. 1975 pp. 21–46 in *Phylogeny of the Primates*, edited by W. P. LUCKETT and F. S SZALAY. Plenum Press, New York.

MIGNOTTE, F., M. GUERIDE, A.-M. CHAMPAGNE and J.-C. MOUNOLOU, 1990 Direct repeats in the non-coding region of rabbit mitochondrial DNA. Eur. J. Biochem. **194:** 561–571.

MUTO, A., and S. OSAWA, 1987 The guanine and cytosine content of genomic DNA and bacterial evolution. Proc. Natl. Acad. Sci. USA **84:** 166–169.

NOVACEK, M. J., 1992 Mammalian phylogeny: shaking the tree. Nature **356:** 121–125.

PÄÄBO, S., W. K. THOMAS, K. M. WHITFIELD, Y. KUMAZAWA and A. C. WILSON, 1991 Rearrangements of mitochondrial transfer RNA genes in marsupials. J. Mol. Evol. **33:** 426–430.

PROTHERO, D. R., E. M. MANNING and M. FISCHER, 1988 The phylogeny of the ungulates, pp. 201–234 in *The Phylogeny and Classification of the Tetrapodes, Vol. 2: Mammals*, edited by M. J. BENTON. Clarendon Press, Oxford, England.

RAMHARACK, R., and R. G. DEELEY, 1987 Structure and evolution of primate cytochrome *c* oxidase subunit II gene. J. Biol. Chem. **262:** 14014–14021.

ROE, B. A., D.-P. MA, R. K. WILSON and J. F.-H. WONG, 1985 The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. J. Biol. Chem. **260:** 9759–9774.

ROMER, A. S., 1966 *Vertebrate Palaeontology*, Ed. 3. University of Chicago Press, Chicago.

SACCONE, C., G. PESOLE and E. SBISÁ, 1991 The main regulatory region of mammalian mitochondrial DNA: structure-function model and evolutionary pattern. J. Mol. Evol. **33:** 83–91.

SAITOU, N., and M. NEI, 1987 The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:** 406–425.

SARICH, V. M., 1985 pp. 423–452 in *Evolutionary Relationships among Rodents, Multidisciplinary Analysis.* Plenum Press, New York.

SIMPSON, G. G. 1945 The principles of classification and a classification of mammals. Bull. Am. Mus. Nat. Hist. **85:** 1–350

SIMPSON, L., and J. SHAW, 1989 RNA editing and the mitochondrial cryptogenes of kinetoplastid protozoa. Cell **57:** 355–366.

SOLINAC, M., M. MONNEROT and J.-C. MOUNOLOU, 1983 Mitochondrial DNA heteroplasmy in Drosophila mauritiana. Proc. Natl. Acad. Sci. USA **80:** 6942–6946.

SOLINAC, M., M. MONNEROT and J.-C. MOUNOLOU, 1986 Conserted evolution of sequence repeats in *Drosophila* mitochondrial DNA. J. Mol. Evol. **24:** 53–60.

SUEOKA, N., 1988 Directional mutation pressure and neutral molecular evolution. Proc. Natl. Acad. Sci. USA **85:** 2653–2657.

SWOFFORD, D. L., 1990 PAUP: phylogenetic analysis using parsimony, version 3.0g. Illinois Natural History Survey, Champaign, Ill.

SZALAY, F. S., 1977 pp. 315–374 in *Major Patterns in Vertebrate Evolution*, edited by M. K. HECHT, P. C. GOODY and B. M. HECHT. Plenum Press, New York.

VON HAESELER, A., A. JANKE and S. PÄÄBO, 1993 Molecular phylogenetics. Verh. Dtsch. Zool. Ges. **86:** 119–129.

WALBERG, M. W., and D. A. CLAYTON, 1981 Sequence and properties of the human KB cell and mouse D-loop regions of mitochondrial DNA. Nucleic Acids Res. **9:** 5411–5421.

WILKINSON, G. S., and A. M. CHAPMAN, 1991 Length and sequence variation in evening bat D-loop mtDNA. Genetics **128:** 607–617.

WILSON, A. C., S. S. CARLSON and T. J. WHITE, 1977 Biochemical evolution. Annu. Rev. Biochem. **46:** 573–639.

WU, C.-I., and W.-H. LI, 1985 Evidence for higher rates of nucleotide substitution in rodents than in man. Proc. Natl. Acad. Sci. USA **82:** 1741–1745.

YOKOGAWA, T., Y. WATANABE, Y. KUMAZAWA, T. UEDA, I. HIRAO *et al.*, 1991 A novel cloverleaf structure found in mammalian mitochondrial $tRNA^{Ser}$ (UCN). Nucleic Acids Res. **19:** 6101–6105.

YOUNG, J. Z., 1981 *The Life of Vertebrates*, Ed. 3. Clarendon Press, Oxford, England.

Communicating editor: M. LYNCH