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Three Patterns of Mitochondrial DNA Nucleotide Divergence in the Meadow Vole, *Microtus pennsylvanicus*

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Summary. The DNA sequence was determined for the cytochrome c oxidase II (COII), $tRNA^{Lys}$, and ATPase 8 genes from the mitochondrial genome of the meadow vole, *Microtus pennsylvanicus*. When compared to other rodents, three different patterns of evolutionary divergence were found. Nucleotide variation in $tRNA^{Lys}$ is concentrated in the TΨC loop. Nucleotide variation in the COII gene in three genera of rodents (*Microtus, Mus, Rattus*) consists predominantly of transitions in the third base positions of codons. The predicted amino acid sequence in highly conserved (>92% similarity). Analysis of the ATPase 8 gene among four genera (*Microtus, Cricetulus, Mus, Rattus*) revealed more detectable transversions than transitions, many fixed first and second position mutations, and considerable amino acid divergence. The rate of nucleotide substitution at nonsynonymous sites in the ATPase 8 gene is 10 times the rate in the COII gene. In contrast, the estimated absolute mutation rate as determined by analysis of nucleotide substitutions at fourfold degenerate sites probably is the same for the two genes. The primary sequences of the ATPase 8 and COII peptides are constrained differently, but each peptide is conserved in terms of predicted secondary-level configuration.

Key words: Mitochondrial DNA evolution — $tRNA^{Lys}$ — Cytochrome c oxidase II — ATPase 8 — Rodentia — Divergence rates — Peptides

Introduction

The mitochondrial genome (mtDNA) has attracted considerable attention because of its utility to investigations of molecular evolution and phylogeny. In large measure this is the result of the relatively small size of the genome (about 16.2 kb), its mode of maternal inheritance, and, perhaps most importantly, the fact that it appears to evolve rapidly (Brown 1985; Vawter and Brown 1986). As more and more DNA sequence data from various species have become available, it has become possible to begin to determine the patterns (tempo and mode) of evolutionary divergence in some of the genes (e.g., Brown et al. 1982; DeSalle et al. 1986, 1987) and some of the peptides that are encoded in mtDNA (Chomyn et al. 1986; Irwin et al. 1991). Consequently, new concepts about molecular evolution of mtDNA are emerging and are being rapidly incorporated into techniques for constructing phylogenies and interpreting relationships (reviewed by Hillis and Moritz 1990; Simon 1991).

Arvicolid rodents originated about 5×10^6 years ago and rapidly diverged into numerous lineages (Chaline and Graf 1988). One genus, *Microtus,* has attracted our attention because of its rapid speciation in North America (Zakrzewski 1985). One of the species, the meadow vole, *Microtus pennsylvanicus,* has divergent mtDNAs (Plante et al. 1989; Pumo et al., unpublished). In preparing mtDNA restriction maps for this species, we used Southern blot hybridization to determine the gene content of the restriction fragments. This approach revealed that a 553-bp *Hind* III fragment of the *Microtus* mtDNA failed to hybridize in a consistent way to laboratory mouse (*Mus musculus*) mtDNA even under low stringency conditions. The results of the Southern blot experiments suggested to us that DNA sequence in this region of the *Microtus* genome might be more divergent than in other regions. Subsequent cloning and DNA sequencing revealed that this frag-

ment contained a portion of the $tRNA^{Lys}$ gene, the ATPase 8 gene, and a portion of ATPase 6.

It is noteworthy that Thomas and Beckenbach (1986) had a similar experience to ours when they used DNA hybridization to map mtDNA restriction fragments from another microtine, *Microtus townsendii*. In their investigation, a 700-bp *Hin*d III fragment that mapped to the region of the ND5–ND6 genes failed to hybridize to any portion of the mouse mtDNA genome, and it was suggested that the 700-bp fragment might be nonhomologous to mouse mtDNA (Thomas and Beckenbach 1986). These observations seem to suggest that some regions of the microtine genome might be unusually divergent from that of mice. This in turn indicates that there is considerable intragenomic variation in evolutionary rates. The nature and extent of this variation is important when comparing taxa and constructing phylogenies.

We present DNA sequence for two peptide-coding genes, cytochrome c oxidase II (COII), and ATPase 8 (referred to as URF A6L by Breen et al. 1986) and the $tRNA^{Lys}$ gene in the meadow vole, *M. pennsylvanicus*. These sequences illustrate three very different patterns of nucleotide divergence. These different patterns provide new evidence on the possible extent of intragenomic variation and illustrate that mtDNA genes are not all constrained in the same manner.

Materials and Methods

Animals. Specimens of the meadow vole, *M. pennsylvanicus* (Rodentia: Microtine), were collected on Long Island, NY. This species is geographically widespread in North America; it is a common inhabitant of old field habitats.

Cloning. Mitochondrial DNA was isolated from the P1 lineage of *M. pennsylvanicus* (Wright et al. 1983; Pumo et al. 1988) and restriction-digested with *Hin*d III. The digest yielded five fragments that were ligated into the pGEM-7Zf(+) plasmid (Promega). Competent DH5α cells (GIBCO BRL) were used as recipients for the recombinant plasmids. Following ligation and transformation, the cells were plated on "blue" Luria–Bertani agar plates containing 0.5% IPTG, 40 μg/ml X-gal, and 100 μg/ml ampicillin. The plates were incubated overnight at 37°C, and white colonies were picked the following day according to the manufacturer's instructions (GIBCO BRL).

DNA Isolation. The plasmids were isolated from transformed bacterial cells using a "mini prep" procedure (Maniatis et al. 1982) involving digestion of bacteria with lysozyme, treatment with alkaline ionic detergent, and phenol–chloroform extraction. The DNA was digested with *Hin*d III and electrophoresed on an agarose gel to verify the presence of the desired *Microtus* inserts.

To prepare DNA of sequencing quality, the clones containing the 2075-bp and 553-bp fragments (p4437-1 and p4435-5c, respectively) were grown overnight at 37°C and purified by passage through an ion-exchange resin, Qiagen Tip-20, according to the manufacturer's instructions (Qiagen). DNA samples from two 5-ml bacterial cultures were combined for each DNA sequencing reaction with each primer.

DNA Sequencing. Purified double-stranded DNA was alkaline-denatured using 0.2 M NaOH at 37°C for 30 min. The mixture was neutralized by adding four volumes of 5 M ammonium acetate (pH 7.5) and the DNA was precipitated with four volumes of 100% ethanol (−70°C, 5 min). The pellet was washed with 70% ethanol and resuspended in 7 μl of sterile distilled water. The DNA was sequenced using [^{35}S]dATP (1000 Ci/mM, 10 mCi/ml) and T7 DNA polymerase. All sequencing reactions were carried out using Sequenase (United States Biochemical) and the manufacturer's directions. An aliquot of 1 μM of primer was used per reaction. The DNA was electrophoresed on an 8% polyacrylamide wedge gel (0.4–1.2 mm). The SP6 and T7 promoter primers were used for sequencing the first 200–250 bp into the cloned fragments. For COII, specific oligonucleotide primers were then constructed for the "look and leap" approach (Barnes 1987). One oligonucleotide, 5′-GTCATTGGTTGGAATCATCG, complemented the coding strand from bp 359 to 381; the second oligonucleotide, 5′-ACGATTATAAGTGTATGGTCGTGAA, complemented bp 68–93.

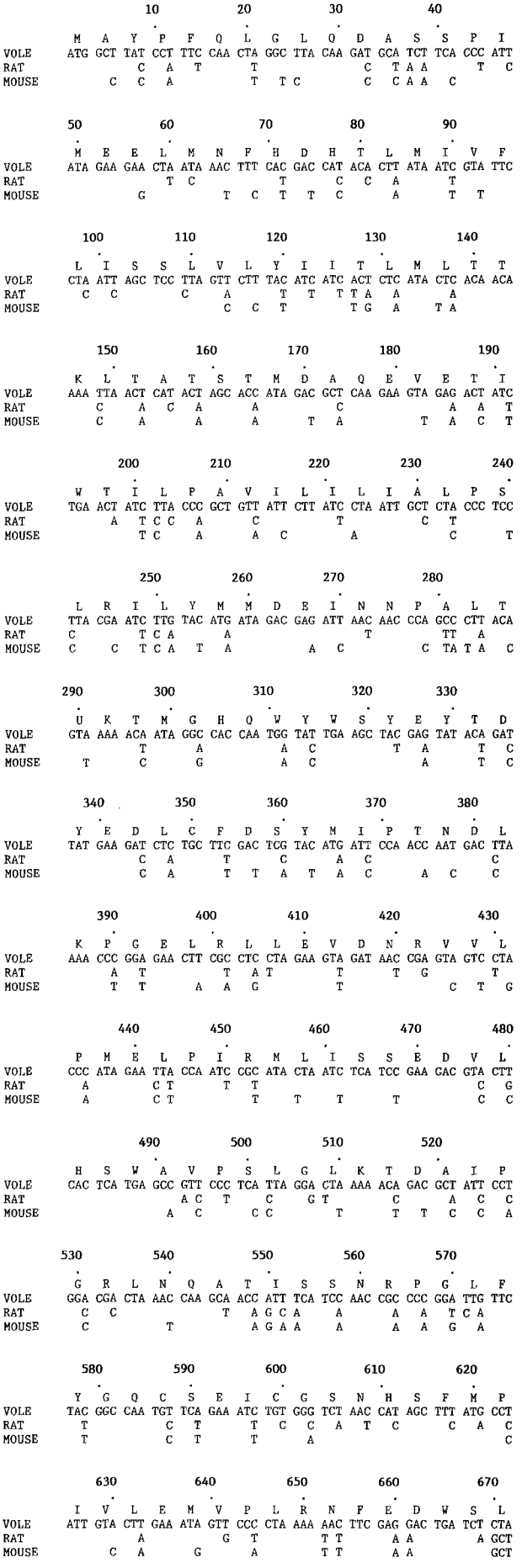
Sequence Analysis. IBI-Pustell sequence analysis software (version 2.03) was used to translate and align sequence data and to predict polypeptide structural features. The human mitochondrial genetic code (Anderson et al. 1981) was used to translate nucleotide sequence data. Synonymous and nonsynonymous nucleotide substitution rates were estimated using the methods of Li et al. (1985) with software kindly sent to us by Dr. Wen-Hsiung Li, University of Texas.

Results and Discussion

Nucleotide Divergence in $tRNA^{Lys}$

The nucleotide sequence difference for the $tRNA^{Lys}$ gene is 14% in pairwise comparisons among meadow vole, mouse, and rat (Fig. 1). This percentage is close to the corresponding percentages for the COII gene, but greater than the percentages for the ATPase 8 gene. These results do not conflict with previous observations that tRNA genes in the mitochondrial genome evolve more slowly than do most of the protein-coding genes (Brown et al. 1982; Thomas et al. 1989). The ratio of transitions to transversions in $tRNA^{Lys}$ is 1:3 for the mouse/meadow vole, 7:4 for the mouse/rat comparisons, and 9:3 for the rat/meadow vole comparison. The meadow vole $tRNA^{Lys}$ gene generates a tRNA that has a base pair mismatch in the acceptor stem (U is opposite G, Fig. 2). G–U mismatches in the acceptor stem are the most common type of mismatch in mitochondrial tRNAs (Cantatore and Saccone 1987). In the mouse there is C–G pair in that position. The rat $tRNA^{Lys}$ also has a mismatch—although it is different from that in the meadow vole (A is opposite C).

In addition to the constraint on mitochondrial tRNA sequence that results from the role of tRNA in translation, some tRNAs also are constrained because of their RNA-processing function (Battey and



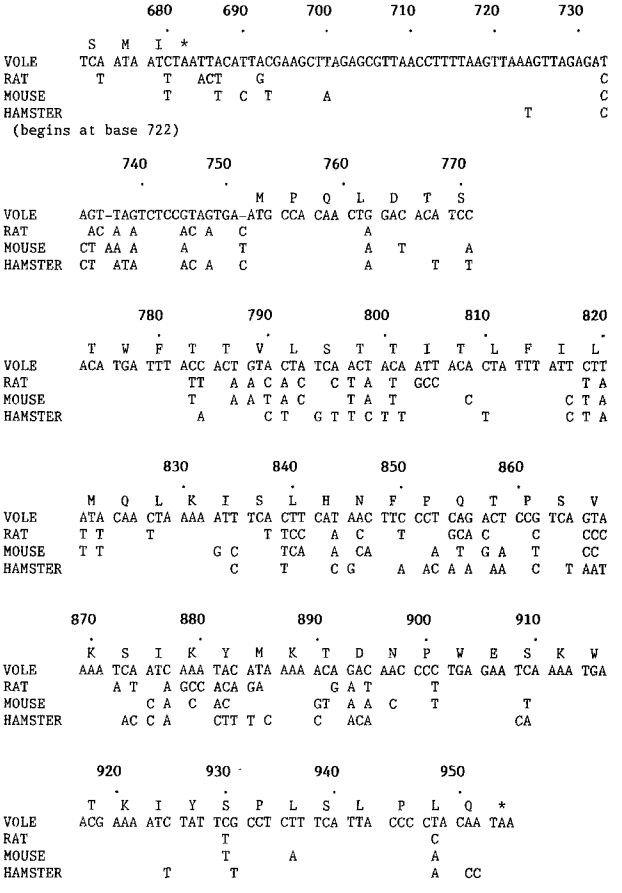


Fig. 1. Nucleotide sequence of the COII (1–683 bp), $tRNA^{Lys}$ (684–751 bp), and ATPase 8 (752–955 bp) genes of the meadow vole, *Microtus pennsylvanicus*, (P1 mtDNA lineage). The sequence corresponds to the L-strand (5′ → 3′). The predicted amino acid sequence for *M. pennsylvanicus* (one letter code) is shown above the corresponding codons. Nucleotide positions that vary between this vole sequence and other available rodent sequences are shown below the vole sequence. Regions of identity between the vole and other rodent sequences are blank.

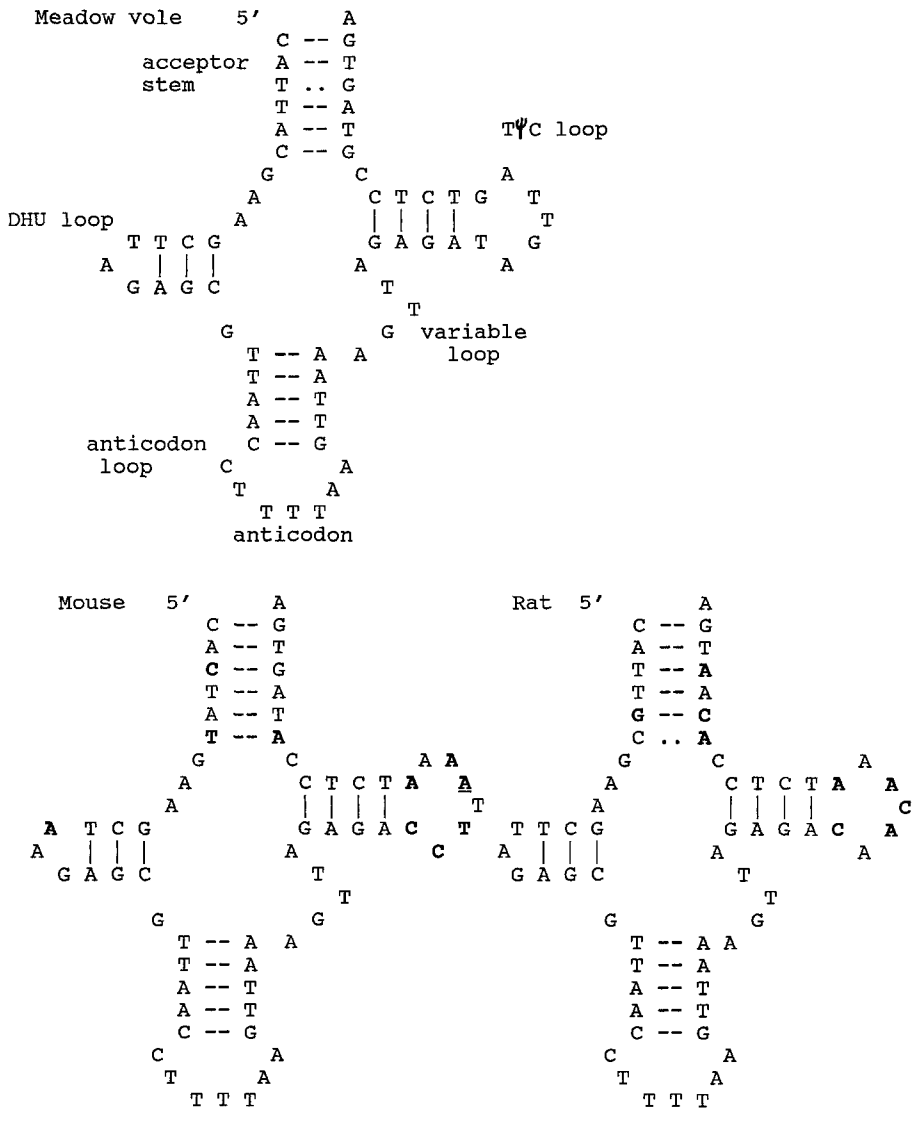


Fig. 2. The nucleotide sequence for the $tRNA^{Lys}$ gene of *Microtus pennsylvanicus* shown in the putative folding pattern for tRNA. The $tRNA^{Lys}$ genes in mouse and rat are included for comparison. Boldface letters indicate that the mouse or rat base sequence at that position differs from the vole sequence. An underlined letter represents an additional base relative to the vole sequence. Dots are located in positions where the opposing bases are not complementary.

Clayton 1980; Ojala et al. 1981). The $tRNA^{Lys}$ gene probably is subject to such processing constraints because it is the only tRNA gene between the COII and ATPase 8 peptide-coding genes (Ojala et al. 1981).

The COII and ATPase 8 Genes

Cytochrome c oxidase (EC 1.9.3.1) is the final enzyme in the electron transport chain; it catalyzes the transfer of electrons from cytochrome c to molecular oxygen (Bisson et al. 1982; Hatefi 1985). COII is the smallest of the three cytochrome c oxidase subunits that are encoded in mitochondrial DNA. COII has a high-affinity binding site for cytochrome c and appears to be a ligand for a copper molecule required for enzyme function (Bisson et al. 1982).

Aspects of the molecular evolution of the COII gene have been investigated previously, in part because this gene has proven useful to molecular systematics. Restriction site analysis (Ferris et al. 1983; Cann et al. 1984) and nucleotide sequence comparisons (Andeson et al. 1981, 1982; Bibb et al. 1981; Brown and Simpson, 1982) have been interpreted by some (Cann et al. 1984; Ramharack and Deeley 1987) to mean that there is a higher rate of predicted amino acid substitution in the COII gene in primate mtDNA than there is in rodents or other mammals. To some extent, these ideas might need to be revised because they are based on comparisons with DNA sequence from human beings, which until recently (Easteal, 1991) was thought to evolve at a different rate than in other primates.

Cann et al. (1984) and Ramharack and Deeley (1987) compared the COII and nuclear cytochrome c genes in primates and concluded that both the mitochondrial and nuclear genes demonstrated fairly high rates of amino acid replacement in primates. Cann et al. (1984) thought that these genes may have coevolved as a result of their related function. Rizzuto et al. (1988) examined and three nuclear-encoded subunits (VI, Va, and Vb) for cytochrome c

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CO II
                10         20         30         40         50         60
                .          .          .          .          .          .
VOLE     MAYPFQLGLQ DASSPIMEEL MNFHDHTLMI VFLISSLVLY IITLMLTTKL THTSTMDAQE
MOUSE                T                                      S
RAT                  T         T                            S

                70         80         90        100        110        120
                .          .          .          .          .          .
VOLE     VETIWTILPA VILILIALPS LRILYMMDEI NNPALTVKTM GHQWYWSYEY TDYEDLCFDS
MOUSE                    M                    V
RAT                                           V

               130        140        150        160        170        180
                .          .          .          .          .          .
VOLE     YMIPTNDLKP GELRLLEVDN RVVLPMELPI RMLISSEDVL HSWAVPSLGL KTDAIPGRLN
MOUSE
RAT               L                                        PI

               190        200        210        220
                .          .          .          .
VOLE     QATISSNRPG LFYGQCSEIC GSNHSFMPIV LEMVPLKNFE DWSLSMI
MOUSE       VT                                 Y   N  A
RAT         VT   L                    L       Y   N  A

ATPASE 8
                10         20         30         40         50         60
                .          .          .          .          .          .
VOLE     MPQLDTSTWF TTVLSTTITL FILMQLKISL HNFPQTPSVK SIKYMKTDNP WESKWTKIYS
HAMSTER               ASS            F  DLHKK  N  YL LF PT     Q
RAT               I II SMA      F     S QT  AP  P  TMATE  N           L
MOUSE             I II SM       F   V S QT  LA  P   LTT  VKT    L      L

VOLE     PLSLPLQ
HAMSTER       QP
RAT           P
MOUSE     H   Q
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Fig. 3. Derived amino acid sequence (one letter code) for two peptide-coding genes of *Microtus pennsylvanicus*: cytochrome c oxidase II CoII; ATPase 8. Corresponding amino acid sequence is shown from mouse, rat, and hamster (ATPase 8 only for the latter). Blanks indicate sequence identity to the vole sequence; amino acids that differ from the vole sequence are shown in the figure.

oxidase in cow and human. They found that the deduced amino acid sequence similarity for subunits VI, Vb, and Va is 82%, 85%, and 95%, respectively. Thus, the Va subunit is the most conserved of the cytochrome c oxidase subunits for which data are available.

ATPase 8 is one of two mitochondrially encoded subunits for the proton–ATP synthase complex. This complex couples the electrochemical potential generated by the respiratory chain enymes to the synthesis of ATP (for reviews see Amzel and Pederson 1983; Hatefi 1985). ATPase 8 sometimes is referred to as A6L because there is only about 20% similarity between the mammalian nucleotide sequence and the yeast ATPase 8 sequence (Macreadie et al. 1983; Breen et al. 1986; Walker et al. 1991). Although the nucleotide similarity is low between yeast and mammals, several characteristics are shared between the predicted polypeptides, including four identical residues at the amino terminus and a similar asymmetric charge distribution (Macreadie et al. 1983; Breen et al. 1986). In contrast to the similar levels of nucleotide and amino sequence divergence observed between subunits of COII encoded in the mitochondria and nucleus, the mitochondrial subunits of the H^+-ATPase are much less conserved between cow and rat at the nucleotide and amino acid sequence levels than is a subunit encoded in nuclear DNA (Chomyn and Tsai Lai 1989). Finally, comparisons of published mtDNA sequences from COII and ATPase 8 (e.g., human vs mouse vs cow) clearly suggest that the mode and tempo of evolution differs bewteeen the COII and ATPase 8 protein-coding genes (i.e., Anderson et al. 1981, 1982; Bibb et al. 1981).

Mode and Tempo of Evolution

The DNA and predicted amino acid sequences for the COII and ATPase 8 genes from *M. pennsylvanicus* are presented in Figs. 1 and 3. For comparison, the nucleotide differences between the meadow vole sequence and the published sequences for mouse, *Mus musculus* (Bibb et al. 1981), rat, *Rattus norvegicus* (Gadaleta et al. 1989), and Chinese hamster, *Cricetulus griseus* (Breen et al. 1986) are included, where available. The meadow vole mtDNA sequence is the same as other published sequences in that both peptide-coding genes use the strong initiator, ATG. The coding strand of *M. pennsylvanicus,* like other sequenced vertebrates, exhibits a strong nucleotide bias against G. The relative nucleotide frequency of meadow vole sequence presented in Fig. 1 is the same as mouse, rat, and hamster.

The results of pairwise comparisons (among ro-

Table 1. Summary of DNA sequence and amino acid comparisons for the COII and ATPase 8 mitochondrial genes in rodents

Species	COII				ATPase 8			
	% ND	% TS	% AAD	K_A (SE)	% ND	% TS	% AAD	K_A (SE)
Vole : rat	19	57	7	0.021 (±0.006)	25	46	31	0.215 (±0.043)
Vole : mouse	20	57	4	0.019 (±0.006)	23	38	34	0.225 (±0.044)
Rat : mouse	15	59	4	0.006 (±0.003)	20	49	21	0.130 (±0.032)
Vole : hamster[a]	—	—	—	—	25	37	28	0.187 (±0.039)
Hamster : rat	—	—	—	—	33	39	39	0.321 (±0.055)
Hamster : mouse	—	—	—	—	30	31	39	0.292 (±0.052)

Abbreviations are: ND, nucleotide difference; TS, transitions; AAD, amino acid difference; K_A, mean and standard error (SE) of nonsynonymous substitutions per nonsynonymous site (Li et al. 1985)

[a] DNA sequence data are not available for the hamster COII gene

dents) of nucleotide sequences and predicted amino acid primary sequences are presented in Table 1. Table 1 provides the uncorrected values for nucleotide difference and the percentage of transitions found in direct comparisons of DNA sequences for the COII and ATPase 8 genes. The data presented in Table 1 suggest that the two genes exhibit strikingly different patterns, or modes, of evolution, even though the "raw" values for nucleotide difference only vary by approximately 5% (15–20% vs 20–25% among meadow vole, mouse, and rat). For example, in comparisons of the COII gene, more than half of the detectable base pair differences are transitions (A↔G, T↔C) (Table 1) and more than 82% of these are located in third positions in the codons. Consequently, the predicted amino acid sequence for the COII peptide in meadow vole, mouse, and rat varies by only 4–7% (Table 1). By way of contrast, in the comparisons of ATPase 8 genes more than half of the detectable differences are transversions (A↔T, G↔C, A↔C, T↔G). Moreover in pairwise comparisons that include the Chinese hamster, 46–56% of the base pair mutations are located in first and second positions of codons. The high number of transversions and the intracodon locations of these mutations in the ATPase 8 gene results in the prediction that the encoded peptide will exhibit highly divergent primary structure within the rodents. Indeed, whereas the COII peptide is predicted to have at least 93% amino acid similarity among meadow vole, mouse, and rat, the ATPase 8 peptide is predicted to have only 66–69% amino acid similarity when meadow voles are compared to rats or mice.

Although interspecific comparisons of nucleotide sequences will provide minimum numbers of transitions and transversions, the actual numbers of mutations that would have occurred would undoubtedly be greater than the number obtained by base-by-base sequence comparison. The algorithm of Li et al. (1985) provides a method for estimating the mean number (and standard error) of nonsynonymous substitutions per nonsynonymous site (K_A). The K_A value is corrected for back mutations (multiple hits). On this basis of comparison, approximately 10 times as many nucleotide substitutions have occurred per nonsynonymous site in the ATPase 8 gene as compared to the COII gene (Table 1). This 10-fold difference between the genes is in keeping with the apparent difference in amino acid substitution (Table 1).

The occurrence of numerous first and second position mutations in the ATPase 8 DNA sequence shows that a higher fraction of nucleotide positions is free to vary in this gene as compared to COII. It has been stated that protein-coding genes should exhibit differing rates of divergence if differing fractions of the nucleotide positions are free to vary (Fitch and Markowitz 1970; Takahata 1987; Palumbi 1989). When sequence divergence values for the COII and ATPase 8 genes are plotted, the ATPase 8 gene exhibits both a higher rate of overall divergence and a higher saturation level than does the COII gene (Fig. 4a). Based on our comparison of the two genes, the divergence rate of the ATPase 8 gene is about 25% greater than is that of the COII gene (Fig. 4a). Our analysis of rodent COII and ATPase 8 genes thus appears to support the hypothesis that differences in the fraction of nucleotides free to vary results in different divergence rates.

Other factors might contribute to the mode and tempo of evolution of the ATPase 8 gene. In the sequenced rodent ATPase 8 genes, more than 50% of the detectable or scored mutations are transversions (Table 1). Previous analyses have implied that in mammalian mtDNA detectable transversions would be expected to outnumber transitions at perhaps 60 million years (Myr) of divergence time, which is well after saturation (Brown et al. 1982). One would, therefore, expect to detect more transitions than transversions, especially in intraordinal comparisons. In a comparison of mouse/rat ATPase 8 mtDNA sequence, 55% of the detectable mutations are transversions (Table 1) and, therefore, within murine rodents the transversions can outnumber transitions well before 50–60 Myr of divergence time (Fig. 4a).

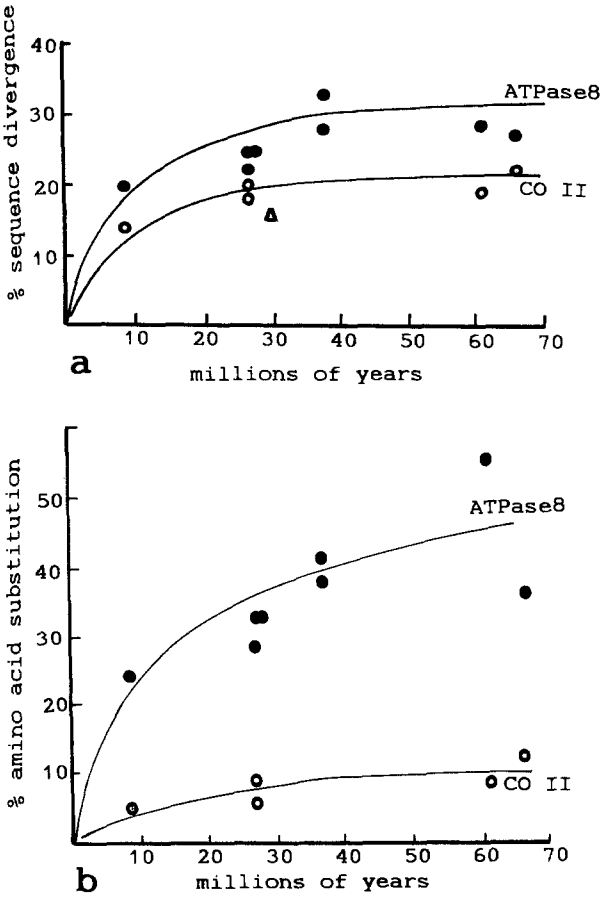

Fig. 4. **a** Percent minimum nucleotide divergence for the COII and ATPase 8 genes. The triangle represents the human/monkey comparison discussed in the text. **b** Amino acid substitution rate for COII and ATPase 8 peptides. Note that amino acid substitution does not appear to saturate in ATPase 8, whereas it does in COII. The divergence of murid rodents from cricetids (26 Myr ago) predates the origin of the Arvicolidae: 36 Myr ago, *Cricetulus/Rattus* and *Mus*; 60 Myr ago, primates/rodents; 65 Myr ago, ancestral ungulates/rodents. The exact divergence times may be debated, but alternative dates would not alter the proportionate difference between the two genes.

Table 2. Numbers of transitions (TS) and transversions (TV) in each of the codon positions, over time, for the COII and ATPase 8 peptide-coding genes

		Divergence time (Myr ago)			
		10	26	36	65
		First position			
COII	TS	10	16	—	13
	TV	1	4	—	16
ATPase 8	TS	6	7	9	8
	TV	4	8	7	11
		Second position			
COII	TS	1	3	—	2
	TV	0	0	—	3
ATPase 8	TS	4	7	0	6
	TV	4	4	9	9
		Third position			
COII	TS	50	58	—	63
	TV	40	53	—	52
ATPase 8	TS	10	6	10	12
	TV	13	14	17	17

The comparisons used are: 10 Myr ago, *Rattus* vs *Mus*; 26 Myr ago, *Microtus* vs *Rattus*; 36 Myr ago, *Microtus* vs *Cricetulus*; 65 Myr ago, *Microtus* vs *Bos*. DNA sequence data are not available for hamster COII gene

When the COII gene is analyzed by codon position, the pattern that emerges is very similar to that documented for the cytochrome *b* gene (Irwin et al. 1991). The ATPase 8 gene offers a striking contrast to the COII and cytochrome *b* genes in this type of analysis. In the ATPase 8 gene there is no indication of a strong bias toward transitions at any of the three codon positions (Table 2). Moreover, mutations in the third position outnumber mutations at the first position by $<1.5\times$, whereas in COII the third position mutations exceed those at the first position by about $5\times$.

Several explanations might be hypothesized for the phenomena described here. One possibility is that there is a lack of bias toward transitions or transversions in the rodent ATPase 8 gene. Although this would be unique in comparison to other studied mtDNA genes in mammals, the possibility cannot be discounted. Moreover, there are data from studies of *Drosophila* species that suggest either the lack of a bias or, possibly, a slight bias toward transversions in the ATPase 8 gene (Wolstenholme and Clary 1985). Another possibility, which also would be unique in mammalian mtDNA, is that the mutation rate is substantially higher in the ATPase 8 gene than it is in other genes within the same genome. As the result of an exceptional mutation rate in this gene, transversions would be accumulated at a faster than predicted rate. Indeed, if one extrapolates from the graph presented in Brown et al. [1982 (Fig. 4)], the mutation rate in the ATPase 8 gene might be as much as twofold greater than in the COII gene. In comparison to the ND4 and ND5 genes, the number of transversions in the ATPase 8 gene in meadow voles as compared to mice would place the divergence of their respective genes at greater than 65 Myr ago. The COII gene in these two rodents, by way of contrast, can be plotted to about 35 Myr ago, which is approximately the beginning of the Oligocene when rodents first appear in the fossil record (Romer 1966). Some evidence that might support the hypothesis that the ATPase 8 gene has an exceptional mutation rate comes from the fact that in most of the studied rodents the number of third position mutations in ATPase 8 sequence is lower than expected. A very high mutation rate in the ATPase 8 gene would allow a high rate of multiple hits that in turn would have the effect

Table 3. Mean number of transitional (A_i) and transversional (B_i) mutations per site based on the observed proportion of transitional and transversional differences for nondegenerate (i = 0), twofold degenerate (i = 2), and fourfold degenerate (i = 4) sites for the COII and ATPase 8 peptide-coding genes

		Divergence time (Myr ago)			
		10	26	36	65
COII	A_0	0.005 (±0.003)	0.014 (±0.006)	—	0.014 (±0.006)
	B_0	0.001 (±0.002)	0.011 (±0.005)	—	0.044 (±0.011)
	K_0	0.006 (±0.004)	0.025 (±0.008)	—	0.058 (±0.012)
ATPase 8	A_0	0.082 (±0.029)	0.127 (±0.039)	0.053 (±0.025)	0.118 (±0.040)
	B_0	0.060 (±0.023)	0.111 (±0.033)	0.143 (±0.039)	0.170 (±0.045)
	K_0	0.142 (±0.037)	0.238 (±0.050)	0.196 (±0.044)	0.288 (±0.057)
COII	A_2	0.444 (±0.093)	0.771 (±0.191)	—	0.774 (±0.193)
	B_2	0.004 (±0.005)	0.004 (±0.005)	—	0.044 (±0.018)
	K_2	0.448 (±0.093)	0.774 (±0.191)	—	0.818 (±0.194)
ATPase 8	A_2	0.218 (±0.094)	0.244 (±0.107)	0.330 (±0.135)	0.238 (±0.110)
	B_2	0.051 (±0.037)	0.072 (±0.046)	0.100 (±0.054)	0.214 (±0.088)
	K_2	0.269 (±0.101)	0.316 (±0.116)	0.430 (±0.144)	0.452 (±0.135)
COII	A_4	0.255 (±0.121)	*[a]	—	0.269 (±0.307)
		P = 17.5[b]	P = 15.0		P = 19.0
	B_4	0.675 (±0.179)	*	—	1.213 (±0.557)
		Q = 40.0	Q = 52.0		Q = 46.7
	K_4	0.930 (±0.167)	*	—	1.483 (±0.380)
ATPase 8	A_4	0.144 (±0.181)	0.409 (±0.243)	0.199 (±0.272)	0.831 (±0.565)
		P = 4.0	P = 6.9	P = 4.7	P = 9.4
	B_4	0.693 (±0.342)	0.457 (±0.199)	0.857 (±0.490)	0.310 (±0.143)
		Q = 12.0	Q = 9.9	Q = 12.7	Q = 6.9
	K_4	0.837 (±0.282)	0.866 (±0.286)	1.055 (±0.384)	1.140 (±0.578)

K_i is the mean number of substitutions ($A_i + B_i$) per site. The comparisons used are: 10 Myr ago, *Rattus* vs *Mus*; 26 Myr ago, *Microtus* vs *Rattus*; 36 Myr ago, *Microtus* vs *Cricetulus*; 65 Myr ago, *Microtus* vs *Bos*. The calculations result from analyzing nucleotide sequence according to the method of Li et al. (1985). Numbers in parentheses are standard errors. DNA sequence data are not available for hamster COII gene

[a] Cannot be determined with currently available algorithms

[b] P is the proportion of transitional differences; Q is the proportion of transversional differences

of obscuring the number of third position mutations that have occurred.

In order to learn more about the differences between COII and ATPase 8, we applied the algorithms of Li et al. (1985) to our data set. These methods provide one means of testing for transition/transversion bias and estimating absolute mutation rates. They have the advantages of accounting for multiple hits and can be adjusted for features that are unique to mtDNA (Li, personal communication). The output (Table 3) includes mean number of transitional and transversional mutations per site determined independently for nondegenerate (nonsynonymous), and twofold and fourfold degenerate (synonymous) sites.

At nondegenerate sites, both transitions and transversions will result in amino acid substitutions. Consequently, comparisons of such sites can be used to detect any possible bias toward transitions or transversions. In the absence of bias, any nucleotide is equally likely to replace any other and the mean number of transversional mutations per site would be equal to twice the mean number of transitional mutations ($B_0 = 2A_0$). For example, the base T can be changed to C, a transition, or A or G, both transversions. We tested the hypothesis that $B_0 = 2A_0$ for the data presented in Table 3 and found that no transition or transversion bias existed for the COII gene in the comparisons between vole and rat or cow. There was also no bias for the ATPase 8 gene in the comparisons of vole to hamster or cow. However, there was a transition bias for both genes in the rat versus mouse comparison and for the ATPase 8 gene in the vole versus rat comparison.

The twofold degenerate sites in mtDNA are interesting because at these sites transitions are silent, whereas tranversions always result in amino acid substitutions. Here again the two genes are very different. In intraordinal comparisons of the COII gene, transitions are approximately 100 times more common than transversions at the twofold degenerate sites (Table 3). By way of contrast, transitions are only four times more common than transversions when the ATPase 8 gene is compared intraordinally. In some of the comparisons not shown in Table 3 (mouse/vole, rat/hamster), transitions do

not outnumber transversions at twofold degenerate sites in ATPase 8. These data imply that at least some of the differences between these two peptide-coding genes might be accounted for by differences in their encoded peptides. The COII gene data clearly suggest that the COII peptide is relatively intolerant of amino acid substitutions. Negative selection that constrains the primary sequence of a peptide could result in an artificially high ratio of transitions to transversions at twofold degenerate sites in the gene. The ATPase 8 peptide, on the other hand, either is relatively tolerant of residue substitutions, or, alternatively, has diverged rapidly due to positive selection.

Our data show that the nucleotide sequence of the ATPase 8 gene has diverged more rapidly than has the sequence of the COII gene (Fig. 4). One possibility is that the absolute mutation rate in ATPase 8 is greater than in COII. Although no mechanism has been proposed, there has been some general discussion of "hotspots" in mammalian mtDNA. In particular there has been some interest in the ATPase 8 and ATPase 6 genes because the Kearns–Sayre Syndrome (KSS) in human mtDNA usually involves a deletion–recombination phenomenon located within this stretch of DNA (Shoffner et al. 1989; Schon et al. 1989). In theory, analysis of fourfold degenerate sites in the COII and ATPase 8 DNA sequences should provide one means of testing for a difference in absolute mutation rates between the two genes.

As can be seen in Table 3, the algorithms of Li et al. (1985) produce only limited results when applied to fourfold degenerate sites. The algorithms are designed with the assumption that $Q = 2P$ (where P and Q are the proportion of transitions and transversions, respectively) at fourfold degenerate sites. Under this assumption, any nucleotide is equally likely to replace any other. Moreover, the algorithms will not produce satisfactory results if the number of mutations per site is near saturation ($P + Q = 75$) (Li, personal communication). The difficulties in applying an algorithm with such assumptions are exemplified by comparison of the COII gene in rats and voles (Table 3). In this example, the estimated mean number of transitions per fourfold degenerate site (A_4) is a negative number and cannot be used to calculate K_4 (the mean number of substitutions per site), which is the estimated absolute mutation rate. In this particular comparison, $P + Q = 67$ and is close to saturation. Other comparisons also fail to produce usable results because $Q \gg 2P$ (Table 3). This high proportion of transversions compared to transitions at synonymous sites supports the idea that there might be a bias toward transversions in both the COII and ATPase 8 genes.

As a result of difficulties with application of the algorithms to fourfold degenerate sites, conclusions about absolute mutations rates are tentative at best. However, data from the rat/mouse comparison, which may be valid, indicate that absolute mutation rate does not differ significantly in the two genes. This means that the notable differences in rates of nucleotide sequence divergence and amino acid substitution between the COII and ATPase 8 genes (Fig. 4) are most likely the result of factors other than a different absolute mutation rate.

Finally, we also can ask whether or not there is any indication of rate differences associated with particular species. Based on our interpretation of the DNA sequence data, it appears that the rate of divergence in the COII mtDNA sequence is about the same in rodents and primates (Fig. 4). In this regard, we differ from the interpretation of Ramharack and Deeley (1987). These authors argued that the COII gene has evolved substantially faster in primates than it has in rodents. This difference in opinion results from selection of different dates to mark the time of divergence of *Rattus* and *Mus*. Ramharack and Deeley (1987) selected 35 Myr ago as an appropriate divergence time for the two genera and for comparison used a date of about 30 Myr ago to mark the divergence of Old World monkeys and great apes. Disagreement about the data that best represent the divergence time for *Mus* and *Rattus* is widespread; published dates range, remarkably, from about 8 Myr ago to 35 Myr ago (e.g., Sarich 1985; Minghetti et al. 1985; Wilson et al. 1987; Beintema et al. 1988). The older dates are based on interpretation of molecular evidence, whereas the more recent dates come from the fossil record (Wilson et al. 1987). We followed the evidence summarized in Catzeflis et al. (1987) in using 8–11 Myr ago for the *Mus/Rattus* divergence and approximately 26 Myr ago for the murid/arvicolid divergence.

The Role of Constraints in the Evolution of the Rodent COII and ATPase 8 Genes

In addition to differences in measured rate of nucleotide sequence divergence, it is clear that the rates of amino acid substitution also differ between ATPase 8 and COII (Fig. 4b). Indeed, in rodents the rate of amino acid substitution in the ATPase 8 peptide is nearly five times greater than in the COII peptide (Fig. 4b). Moreover, at least some of the substitutions would have required at least two nucleotide substitutions. For instance, at position 17 (Fig. 3) rats and mice have methionine, hamsters a serine, and vole a threonine. As one would expect, the saturation level for amino acid substitution also is far greater and far more variable in the ATPase 8 peptide than in the COII peptide (Fig. 4b).

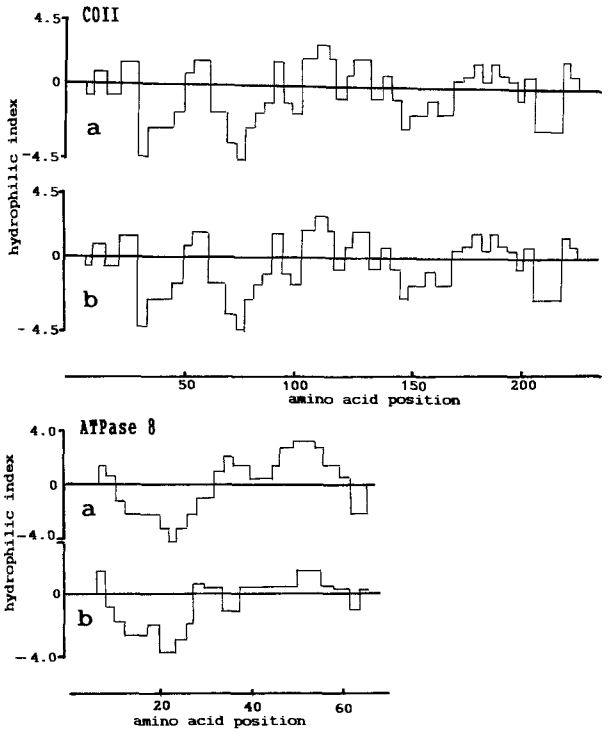

Fig. 5. Hydropathy plots for the predicted amino acid sequence for COII and ATPase 8. Note the extreme conservation of the COII peptide and the overall conservation exhibited by the ATPase 8 peptide. Plots for vole **a** and mouse **b** are shown.

In pairwise comparisons of the COII peptide only 4–7% of the amino acids have been substituted, whereas in the ATPase 8 peptide up to 40% of the amino acids are predicted to differ (Table 1). Hayasaka et al. (1988) found up to 40% amino acid substitution in a portion of the ND5 peptide in primates and they concluded that the corresponding portion of the ND5 mtDNA gene was under "relaxed" constraints. Judging from the high level of amino acid substitutions, the rodent ATPase 8 peptide is at least constrained differently, if not in a more relaxed way, than is the COII gene. It is well established that some proteins are highly tolerant of amino acid substitutions (Bowie et al. 1990). Viewed from the perspective of the peptides, it thus appears that the ATPase 8 peptide might be far more tolerant of variation in its primary sequence than is the COII peptide. It might be hypothesized that the rapid DNA sequence divergence in the rodent ATPase 8 gene is indicative of noteworthy divergence in the gene product itself. In that case, one might expect that the large amount of amino acid sequence divergence might have conferred conformational divergence in the peptide.

In order to test some of the possible interpretations of the DNA sequence data, we used the Chou and Fasman (1978), Garnier et al. (1978), and Kyte and Doolittle (1982) methods to predict secondary structure (turns, β-sheets, and α-helices), hydrophobic, and hydrophilic features of the ATPase 8 and COII peptides in rodents for which sequence data are available (Fig. 5). Some recent studies have indicated that these methods produce a fairly accurate comparative picture of protein structure (Fasman and Gilbert 1990; Jähnig 1990). At the same time, these methods do not provide the ideal level of precision regarding subtle, but potentially important, conformational features of proteins (Greer 1990).

As would be expected from the DNA sequences and predicted primary structures, the COII peptide in three genera of rodents is virtually identical in terms of predicted secondary structure and other features (Fig. 5). In this peptide the primary structure is highly constrained and, therefore, only a few amino acid substitutions are tolerated (Fig. 4b). The ATPase 8 peptide serves as an interesting, almost paradoxical, comparison to COII. Within the limitations of the analysis used by us, the ATPase 8 peptide appears to be well conserved in terms of secondary structure. The hydrophobic/hydrophilic features are at least similar even though portions of its primary structure are relatively unconstrained (Figs. 1 and 5) and its corresponding DNA sequence evolves very rapidly. The similarity in configuration suggests that the intergeneric divergence in primary sequence is the result of stochastic events rather than the result of an adaptive process.

In the ATPase 8 peptide, most of the amino acid substitutions are clustered in two regions (Fig. 3). The first is a hydrophobic region that probably is structured in β-sheets (Figs. 3 and 5). Hydrophobic regions in membrane proteins generally are oriented toward the inner face of the molecule and often are tolerant of numerous amino acid substitutions (Bowie et al. 1990). The second variable region is more unusual because it is predicted to be hydrophilic and located on the outer surface of the protein; this region consists of a sequence of approximately 26 amino acids (39% of the total) and, in the meadow vole/mouse comparison, is the location of 61% of the amino acid substitutions. The total number of substitutions in this region (meadow vole compared to hamster, rat, and mouse) is divided equally between hydrophilic (polar) and hydrophobic (nonpolar) amino acids. Although surface hydrophilic regions in membrane proteins sometimes are associated with transmembrane channels constructed with α-helices (Farber and Petsko 1990), the ATPase 8 peptide is not predicted to have an α-helix in this region (Fig. 5).

In terms of the general pattern of protein evolution, the ATPase 8 peptide differs from both the COII peptide and from the cytochrome *b* peptide, which was investigated comparatively by Irwin et al. (1991). The cytochrome *b* peptide can tolerate more amino acid substitutions in its primary structure than can the COII peptide and, therefore, is less conservative (or constrained) in that way (Irwin et

al. 1991). On the other hand, the primary structure of the cytochrome *b* peptide is far more conserved than is that of the ATPase 8 peptide. Furthermore, in the cytochrome *b* peptide the outer surface of the molecule is more conserved than is the transmembrane or inner surface (Irwin et al. 1991).

In terms of constraints, it is apparent that mitochondrial protein-coding genes are widely variable. It also is clear that constraints can be focused on totally different aspects of different peptides. Consequently, it is difficult to predict the evolutionary boundaries that are set by natural selection. In one instance (COII) the primary structure of a peptide is directly constrained and the DNA sequence thus exhibits few mutations other than those that are in third positions. In another instance (ATPase 8) it seems that the overall three-dimensional configuration and secondary structure of the peptide can be conserved with relatively little constraint on portions of the primary structure. Chomyn et al. (1986) came to a similar conclusion based on their investigations of mitochondrial genes that encode some of the protein subunits that form part of the hydrophobic shell of the NADH–ubiquinone oxidoreductase complex. In the latter example it appears that the appropriate fit of the protein subunit into the intramembrane complex can be attained even though the primary sequence varies widely among species for which data are available (Chomyn et al. 1986).

Different levels of constraint thus are determined by (1) the functional interaction of these peptide subunits with other subunits in their respective complexes, (2) interaction of the subunit peptides with other components of the inner mitochondrial membrane or matrix, and (3) as yet undefined rules that govern peptide configurations. Additionally, it also is clear that different levels of constraint can have considerable impact on the mode and tempo of DNA sequence evolution. Rapid rates of DNA sequence evolution do not necessarily signal that meaningful divergence has taken place in a peptide, or that gene products are unconserved, or that selection is greater in one instance than in another. Instead, differences in evolutionary rates at the mtDNA sequence level are more likely to be indicative of constraints operating at different levels.

Note added in proof: The DNA sequence reported herein was submitted to the EMBL Data Library, accession number X60285.

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