POLYMORPHIC SITES AND THE MECHANISM OF EVOLUTION IN HUMAN MITOCHONDRIAL DNA

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ABSTRACT

Twelve restriction enzymes were used to screen for the presence or absence of cleavage sites at 441 locations in the mitochondrial DNA of 112 humans from four continents. Cleavage maps were constructed by comparison of DNA fragment sizes with those expected from the published sequence for one human mtDNA. One hundred and sixty-three of the sites were polymorphic, i.e., present in some individuals but absent from others, 278 sites being invariant. These polymorphisms probably result from single base substitutions and occur in all functional regions of the genome.——In 77 cases, it was possible to specify the exact nature and location (within a restriction site) of the mutation responsible for the absence of a restriction site in a known human mtDNA sequence and its presence in another human mtDNA. Fifty-two of these 77 gain mutations occur in genes coding for proteins, 34 being silent and 18 causing amino acid replacements; moreover, nine of the replacements are radical.——Notable also is the anomalous ratio of transitions to transversions required to account for these 77 restriction site differences between the known human mtDNA sequences and other human mtDNAs. This ratio is lower for most groups of restriction sites than has been reported from sequence comparisons of limited parts of the mtDNA genome in closely related mammals, perhaps indicating a special functional role or sensitivity to mutagenesis for palindromic regions containing high levels of guanine and cytosine.——From the genomic distribution of the 163 polymorphic sites, it is inferred that the level of point mutational variability in tRNA and rRNA genes is nearly as high as in protein-coding genes but lower than in noncoding mtDNA. Thus, the functional constraints operating on components of the protein-synthetic apparatus may be lower for mitochondria than for other systems. Furthermore, the mitochondrial genes for tRNAs that recognize four codons are more variable than those recognizing only two codons.——Among the more variable of the human mitochondrial genes coding for proteins is that for subunit 2 of cytochrome oxidase; this polypeptide appears to have been evolving about five times faster in primates than in other mammals. Cytochrome c, a nuclearly encoded protein that interacts directly with the oxidase 2 subunit in electron transport, has also evolved faster in primates than in rodents or ungulates. This example, along with that for the mitochondrial rRNA genes and the nuclear genes coding for mitochondrial ribosomal proteins, provides evidence for coevolution between specific nuclear and mitochondrial genes.

COMPARISON of cleavage maps made by the double-digestion method has proved valuable for estimating the degree of divergence among the mitochondrial genomes of animals. It was with this method that BROWN, GEORGE

and WILSON (1979) detected the rapid divergence of mtDNA relative to nuclear DNA, a phenomenon confirmed by later sequencing studies (BROWN et al. 1982). A more accurate method of restriction mapping, termed the high-resolution method, becomes possible when one complete nucleotide sequence has been determined for a species and when the mean extent of divergence between individuals is <2%. With this method, one usually can map restriction sites to within ±2 base pairs (bp) (CANN, BROWN and WILSON 1982); the double-digestion method, by contrast, has an average error of ±200 bp (CANN, BROWN and WILSON 1982; CANN 1982).[1] Because of the availability of a complete sequence for one human mtDNA (ANDERSON et al. 1981), it is now possible, with the high-resolution mapping method, to examine the types and incidences of mutations at hundreds of sites throughout the mitochondrial genomes of numerous individuals in the human population (CANN, BROWN and WILSON 1982; CANN and WILSON 1983). A comparable opportunity exists for mouse and cow mtDNAs (see, for example, FERRIS et al. 1983b). This paper shows how such variability studies with human mtDNAs allow testing of hypotheses concerning the mechanisms of mtDNA evolution. A preliminary account of this work has appeared (CANN, BROWN and WILSON 1982), as has a description of the length-mutational differences among these mtDNAs (CANN and WILSON 1983).

MATERIALS AND METHODS

Human individuals and mtDNA preparation: MtDNA was highly purified from placenta, liver or tissue culture cells derived from 112 human beings as described by BROWN (1980), CANN (1982) and CANN and WILSON (1983). These individuals represent four broad geographic categories, as follows: 12 Australian aborigines, 19 people of African descent, 35 of Asian descent and 45 from Europe, North Africa or the Middle East; they included the 21 individuals studied by BROWN (1980). Two of the samples were from twins, and one was from a cell line (HeLa S3).

Restriction mapping: Each of the purified mtDNAs was digested with 12 restriction endonucleases: (a) AluI, (b) AvaII, (c) DdeI, (d) FnuDII, (e) HaeIII, (f) HhaI, (g) HinfI, (h) HpaI, (i) HpaII, (j) MboI, (k) RsaI and (l) TaqI. The enzymes were purchased from New England BioLabs or Bethesda Research Laboratories and used under conditions specified by the supplier. DNA fragments generated by endonuclease digestion were labeled at the ends with ^{32}P according to published descriptions (DROUIN and SYMONS 1979; BROWN 1980) and electrophoresed at room temperature into vertical gels containing 3.5% polyacrylamide (40 × 15 × 0.08 cm, 0.05 M Tris, 0.05 M boric acid and 0.001 M EDTA at pH 8.3) or 1.2% agarose (20 × 15 × 0.10 cm, 0.04 M Tris, 0.02 M sodium acetate and 0.02 M EDTA at pH 7.5). Autoradiography was done on vacuum-dried gels by standard methods (BROWN 1980; MANIATIS, FRITSCH and SAMBROOK 1982). The sizes of the fragments were estimated by comparison with cleavage fragments of phage DNAs (ϕX174 and PM2) and with those of the human mtDNA whose sequence is completely known (ANDERSON et al. 1981). By considering these fragment sizes in relation to those predicted by the known sequence, we constructed cleavage maps. The rationale and application of this mapping method has been presented before (CANN, BROWN and WILSON 1982; CANN and WILSON 1983; FERRIS et al. 1983a,b).

RESULTS

Locations of variable sites: Table 1 lists the 163 variable sites detected in our survey of 112 human mtDNAs and the functional regions in which these sites

[1] Although it has been suggested that side-by-side comparisons of fragments made in the same electrophoretic gel can improve the accuracy of mapping restriction sites (FERRIS, WILSON and BROWN 1981; FERRIS et al. 1981), we want to emphasize that this practice improves only the ordering of such sites and not the precision of their placement in the genome.

TABLE 1

Locations and nature of 163 variable restriction sites in 113 human mtDNAs

Location and nature of site		No. of variant individuals[c]	No. of inferred mutations[d]
Position and enzyme[a]	Base change[b]		
D loop			
8j	9, G-A	11	6
134 l	135, T-C	1	1
5′ Noncoding			
255f	257, A-G	1	1
259a	262, C-T	1	1
340j	340, C-G	1	1
12S RNA			
663e	663, A-G	3	2
707i	710, T-G		
or	or	1	1
712i	714, A-G		
719j	720, T-A	1	1
740j	740–3	4	3
1240a	1241, C-G	3	3
1403a	1404, A-G	17	8
1463e	1463–6	1	1
1484e	1484, G-C	34	19
Val tRNA			
1610a	1610–3	6	5
1637c	1637–41	3	3
1667c	1667–71	1	1
16S rRNA			
1715c	1715–9	2	2
1917a	1917–21	1	1
2208a	2208–11	1	1
2472a	2473, A-G	1	1
2635a	2635, G-A	1	1
2734a	2734–7	6	5
2758k	2758–61	7	3
2849k	2849–52	2	2
3090e	3092, T-C	C	C
Leu[UUR] tRNA			
3250g	3250, T-G	9	6
Urf 1			
3315e	3315–8	2	1
3391e	3394, T-C, R	3	3
3537a	3537–40	1	1

[a] Each variable site is referred to by the nucleotide position with which it starts in the light strand of the Cambridge sequence (ANDERSON *et al.* 1981) and by a single letter (a–l), which designates the enzyme recognizing that site. The correspondence between letter and enzyme is alphabetical (see MATERIALS AND METHODS for the alphabetical list of enzymes).

[b] The position of each base change is indicated, followed by the nature of the substitution (for site gains relative to the Cambridge sequence). In the cases of sites 16303k and 16310k, they are gains relative to the sequences published by GREENBERG NEWBOLD, and SUGINO. (1983) but losses relative to the Cambridge sequence. Finally, R and S refer to amino acid replacement and silent substitutions.

[c] C refers to the Cambridge nucleotide sequence (ANDERSON *et al.* 1981).

[d] Inferred by phylogenetic analysis using the tree topology shown in Figure 5 of CANN and WILSON (1983).

TABLE 1—*Continued*

Location and nature of site		No. of variant individuals[c]	No. of inferred mutations[d]
Position and enzyme[a]	Base change[b]		
3563g	3566, C-T, R	C	C
3592h	3593, C-T, S	12	4
3842e	3843, A-G, S	1	1
3849e	3849-52	2	2
3899l	3899-902	9	9
3930c	3930-4	C	C
4092g	4095, C-T, S	1	1
Met tRNA			
4411a	4411-4	4	4
Urf 2			
4631a	4631-4	1	1
4769a	4769-72	C	C
4793e	4793, A-G, S	2	2
5009k	5009, A-G, S	8	5
5176a	5176-9	6	4
5261e	5261-4	1	1
5269l	5269-72	1	1
5351f	5351, A-G, S	3	2
Trp tRNA			
5552c	5552-6	1	1
L strand origin			
5742i	5742-5	2	2
5754i	5755, G-C		
or	or	1	1
5756i	5756, G-C		
CO 1			
5978a	5978-81	1	1
5983g	5984, A-G $\Big\}$ S	1	1
5984b	5984, A-G		
5996a	5996-9	1	1
6022a	6022-5	1	1
6145g	6149, A-C, S	C	C
6211g	6211-5	6	2
6356c	6357, C-T, S	2	2
6425e	6425, G-T, S	2	1
6501i	6503, A-G, S	1	1
6871g	6871-5	2	2
6931g	6931-5	1	1
6957e	6957-60	2	2
7025a	7028, C-T, S	25	6
7055a	7055-8	3	1
7335l	7335-8	5	4
Ser[UCN] tRNA			
7458g	7458-62	C	C
7461l	7461-4	1	1
7474a	7474-7	1	1
CO 2			
7617f	7617, A-G, R	2	2
7750c	7750-4	11	5
7859j	7859-62	1	1

TABLE 1—Continued

Location and nature of site		No. of variant individuals[c]	No. of inferred mutations[d]
Position and enzyme[a]	Base change[b]		
7897k	7897–900	10	6
7912k	7912–5	3	2
7970g	7973, C-T, R	C	C
7986g	7986–90	7	5
8165e	8167, T-C, S	1	1
8249b	8251, G-T or A, S	1	1
8250e	8250–3	2	1
UrfA6L			
8391e	8391–4	1	1
ATPase 6			
8592j	8592–5	2	2
8723b	8724, A-G, S	1	1
8783g	8783–7	2	1
8877f	8877, G-T, R	1	1
8994e	8994–7	2	1
9009a	9009, C-A, S	2	1
9053f	9053–6	12	8
9070l	9072, A-G, S	3	2
9150j	9150, A-G, S	3	2
CO 3			
9266e	9266–9	1	1
9294e	9294–7	1	1
9380f	9380–3	C	C
9400f	9402, G-A, R	1	1
9509k	9509, T-G, R	C	C
9553e	9553–6	2	2
9714e	9716, T-C, S	1	1
9751l	9751–4	1	1
9859g	9860, C-A, R	1	1
Urf 3			
10066f	10066, T-G, R	1	1
10084l	10086, A-G, R	1	1
10352a	10355, T-C, S	C	C
10364e	10364–7	1	1
10394c	10398, A-G, R	47	18
Arg tRNA			
10413a	10415, T-C	5	5
Urf 4L			
10689e	10689–92	1	1
10694a	10697, C-T, S	1	1
10725e	10725–8	3	3
Urf 4			
10771j	10771, A-G, S	6	5
10806g	10810, T-C, S	8	1
10893l	10895, A-G, R	1	1
10933b	10933, C-G, S	2	2
11146c	11149, A-G, S	1	1
11161i	11164, A-G, S	2	1
11329e	11329, A-G, S	2	2
11350a	11350–3	2	2

Location and nature of site		No. of variant individuals[c]	No. of inferred mutations[d]
Position and enzyme[a]	Base change[b]		
11447k	11447–50	3	3
11922j	11922–5	1	1
Urf 5			
12406h	12406–11	3	3
12560a	12560–3	3	3
12795j	12796, C-A, R		
or	or	4	4
12798j	12798, C-G, S		
12925g	12928, C-T, R	1	1
13031g	13031–5	49	19
13051e	13051–4	5	3
13100i	13101, A-C, S	1	1
13103g	13103–7	1	1
13182j	13182, T-G, F	1	1
13208f	13208–11	6	5
13367b	13367–71	4	3
13404l	13404–7	3	2
13641l	13644, C-A, S	3	2
13702e	13702–5	4	2
14015a	14015–9	1	1
Urf 6			
14279j	14281, C-T, S	1	1
14322a	14324, T-C, R	1	1
14394c	14398, A-G, S	1	1
14439g	14442, T-A, R	C	C
14509a	14510, G-A, R	C	C
14608c	14608–12	1	1
Cyt b			
14749e	14750, A-G, R	1	1
14869j	14869–72	1	1
14956l	14956–9	2	1
15005g	15006, C-A, R	2	1
15172e	15172–5	1	1
15234g	15234–8	1	1
15235j	15235, A-G, S	1	1
15238c	15238–42	2	1
15250c	15250–4	13	7
15723g	15723–7	1	1
15883e	15883–6	4	3
Noncoding			
15885i	15887, T-G	1	1
Thr tRNA			
15925i	15925–8	3	3
Pro tRNA			
15996c	15996–16000 }	1	1
16000g	16000–4 }		
D loop			
16049k	16049–52	1	1
16065g	16065–9	2	1
16130k	16130–3	24	16

Location and nature of site		No. of variant individuals[c]	No. of inferred mutations[d]
Position and enzyme[a]	Base change[b]		
16208k	16208-11	6	4
16246g	16246, A-G	1	1
16303k	16304, T-C	12	4
16310k	16311, T-C	36	14
16373j	16375, C-T	1	1
16390b	16390-4	11	5
16398e	16399, A-G	8	7
16490g	16491, T-A	1	1
16517e	16519, T-C	39	17

occur; it does not include the 278 constant sites, since they can be inferred from the published sequence (ANDERSON et al. 1981). The approximate locations of many of these variable and constant sites were given by CANN, BROWN and WILSON (1982) and CANN and WILSON (1983), and an updated and revised illustration appears in Figure 1.

In only three cases does it appear that these polymorphisms are not independent events. Individual 71 has two overlapping site changes, the loss of the HinfI site (GAGTC) which starts at position 5983 and the gain of an AvaII site (GG$_{\text{T}}^{\text{A}}$CC) at position 5984 (see Table 1). One mutation, a A → G transition (GAGTCC → GGGTCC), is sufficient to cause the simultaneous loss of the HinfI site and gain of the AvaII site. Similarly, in individual 21 it is likely that a base substitution creating an AvaII site at 8251 has caused the HaeIII loss at 8250. The third possible case involves the loss of both a DdeI site and a HinfI site in individual 59, which could be the result of a base substitution at position 16000. Hence, 160 variable base-pair positions may account for the 163 variable restriction sites encountered in our survey.

With the exception of the glutamate and aspartate tRNA genes, all of the known mtDNA genes contained restriction sites for the endonucleases used here. Furthermore, the number of sites is highly correlated with the number of base pairs per gene (Figure 2).

As is apparent from Tables 2 and 3, all the major classes of functional regions of the human mitochondrial genome have been the focus of base substitution. Table 2 summarizes the variation for each major functional region in two ways: by giving, first, the percentage of the sites that vary and, second, the number of phylogenetically inferred mutations per site. The second method of estimation comes from a tree analysis that is based on the parsimony principle and on the assumption of strictly maternal transmission and clonal divergence of mtDNAs; the tree appears in CANN and WILSON (1983). According to these approaches, the noncoding regions are 1.4 to 2.8 times more variable than the protein-coding regions, which are slightly more variable, on average, than the tRNA and rRNA genes.

Table 3 gives more information about variability among the 13 regions coding for proteins. In three cases (i.e., Urfs 6, 4L and A6L) the variability
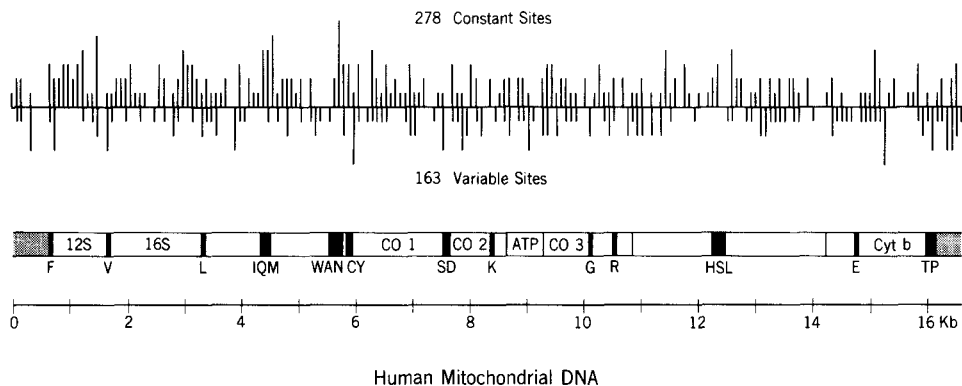
Human Mitochondrial DNA

FIGURE 1.—Locations of cleavage sites and functional regions in human mtDNA. The 16,569-bp circular genome sequenced by ANDERSON *et al.* (1981) is drawn in linear form. The major bar shows the regions of known function: 22 tRNA genes, each represented by a single letter and black shading, two rRNA genes (12S and 16S), and 13 genes coding for proteins, eight of which are unidentified and five of which are known, namely, three cytochrome oxidase subunits (CO 1, 2 and 3), one ATPase subunit (ATP) and cytochrome *b* (Cyt b). Dotted areas represent the large noncoding region, extending from 16,024 to 576 bp. The upper panel shows the locations of cleavage sites found in mtDNAs from 112 humans plus the Cambridge reference sequence with the aid of 12 restriction enzymes. Vertical lines *below* the horizontal line show the variable sites, *i.e.*, those present in some but not all of these mtDNAs. The vertical lines *above* the horizontal line show those sites present in all of the human mtDNAs examined. Height of the vertical lines is proportional to the number of sites found within an 80-bp segment. Preliminary versions of this figure have appeared in CANN, BROWN and WILSON (1982) and CANN and WILSON (1983).

estimates are based on too few sites to be reliable. Among the remaining ten cases, the most variable are Urf 3, Urf 5, subunit II of cytochrome oxidase and subunit 6 of ATPase.

Among tRNA genes, those recognizing four codons are significantly more variable than those that recognize only a pair of codons (Table 4). The apparent difference in variability between the two subclasses of tRNA recognizing a pair of codons—namely, codons ending with a purine (NNR) and codons ending with a pyrimidine (NNY)—is not statistically significant.

Analysis of site gains: The exact position and nature of those base substitutions that account for the presence of a restriction site in a given mtDNA and its absence in a known sequence can usually be inferred with considerable confidence (see Table 1). This is because the human mtDNAs being compared are so closely related that the number of substitutions per restriction site is very unlikely to be more than one.

Fifty-two of the 77 substituted positions leading to site gains occur in regions coding for proteins. Of these, 34 are due to silent substitutions (which do not result in amino acid substitutions), a proportion (65%) like that observed when the Urf 5 region of humans and their closest living relatives, the great apes (BROWN *et al.* 1982), were sequenced. Table 5 lists the 18 amino acid replacements inferred from the site gains. Six mitochondrially encoded proteins have apparently undergone multiple amino acid replacements, namely, Urfs 1, 3 and 6, subunits 2 and 3 of cytochrome oxidase and cytochrome *b*.
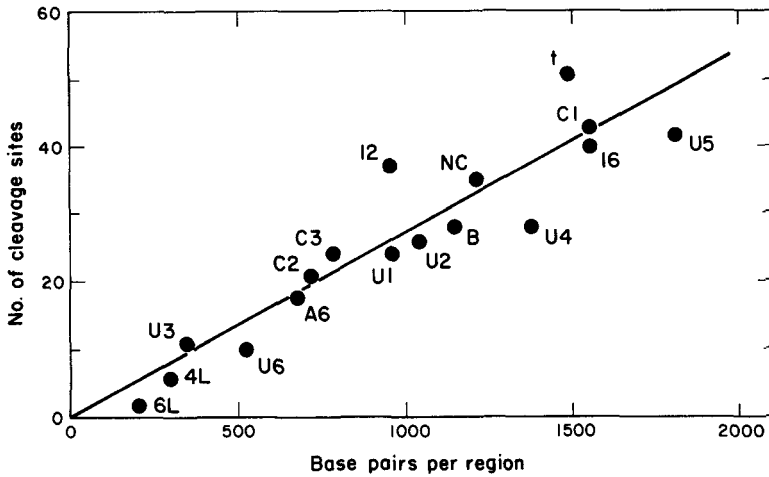
FIGURE 2.—Distribution of 441 cleavage sites among 17 regions for 113 human mtDNAs. The abbreviations are as follows: A for adenosine triphosphatase, B for cytochrome *b*, C for cytochrome oxidase, NC for noncoding region (including the D loop), t for all the transfer RNAs, U for unidentified reading frame, 12 for 12S ribosomal RNA, 16 for 16S ribosomal RNA; 4L and 6L also refer to unidentified reading frames. The lengths of these regions in base pairs are taken from ANDERSON *et al.* (1981).

TABLE 2

Summary of variability by function

Function	Base pairs	Variable sites (a)	Total sites (b)	Inferred changes (c)	Variability 100 a/b	100 c/b
Ribosomal RNA	2,511	17	77	54	22	70
Transfer RNA	1,490	13	50	33	26	66
Protein	11,354	113	279	245	41	88
Noncoding	1,214	20	35	86	57	246
Total	16,569	163	441	418	37	95

Nine of these replacements are radical, because they involve substantial changes in size, composition and polarity (GRANTHAM 1974).

The apparent ratio of transitions to transversions is 2.5:1 for gain mutations in human mtDNA, as shown in Table 6. This is significantly less than the ratio observed by sequence comparisons of an 896-bp segment among African hominoid mtDNAs (11:1, BROWN *et al.* 1982), the cytochrome oxidase II genes of two rat species (8:1, BROWN and SIMPSON 1982) and a 900-bp segment from the noncoding region from several humans (24:1, GREENBERG, NEWBOLD and SUGINO 1983). The proportion of transitions is strongly dependent on the frequency of occurrence of the restriction site (Table 7), and sites that are poorly represented in human mtDNA appear to have a particularly low ratio of transitions to transversions (1.5:1).

TABLE 3

Site variability related to function for protein-coding regions

Region	Base pairs	Variable sites (a)	Total sites (b)	Inferred muta-tions (c)	Variability 100 a/b	Variability 100 c/b	Rank based on sites[a]	Rank based on inferred mutations[a]
Many sites (>10)								
Urf 3	346	5	11	22	45	200	3	1
Urf 5	1811	15	39	51	38	131	5	2
CO 2	718	10	21	25	48	119	2	3
ATPase 6	678	9	18	19	50	106	1	4
Urf 1	957	10	24	24	42	100	4	5
Cyt b	1144	11	28	19	39	68	5	6
Urf 4	1374	10	28	19	36	68	7	6
CO 1	1552	16	43	27	37	63	6	8
Urf 2	1041	8	26	17	31	65	8	7
CO 3	783	9	24	10	38	42	5	9
Few sites (≤10)								
Urf 6	522	6	10	6	60	60	n	n
Urf 4L	293	3	6	5	50	83	n	n
Urf A6L	205	1	2	1	50	50	n	n

[a] n means not ranked because the number of sites is so small.

DISCUSSION

Ours is the most comprehensive comparative study available for complete mitochondrial genomes that are extremely closely related. This provides an opportunity to conduct an examination of the susceptibility of various parts of this genome to genetic change that is relatively free from the problem of multiple substitutions at the same nucleotide position. By contrast, previous comparisons of whole mitochondrial genomes at the sequence level (ANDERSON *et al.* 1981, 1982; BIBB *et al.* 1981) were subject to the multiple-hit problem. Indeed, according to multiple hit calculations (BROWN *et al.* 1982; MIYATA *et al.* 1982), the average silent position has experienced an estimated three substitutions since the divergence of cow, mouse and human mtDNA. In the present study, we have compared genomes for which the average number of silent substitutions is less than 0.03 per silent base pair. We suggest, therefore, that the pattern of sequence divergence inferred from our comparative study of human mtDNA may have several implications for understanding the forces that drive evolutionary change in mtDNA.

To explain the high rate of both point and length mutational divergence in mammalian mtDNA, as compared with the rates in nuclear DNA, one can invoke a general factor that elevates the rate by five- to tenfold at all positions in the mitochondrial genome (CANN and WILSON 1983). Two classes of explanation exist for this general elevation of the evolutionary rate in mtDNA. One appeals to an enhanced mutation pressure that perhaps results from poor fidelity or a lack of editing in mtDNA replication, deficient repair, rapid DNA

TABLE 4

Variability in transfer RNAs

Transfer RNA	Codon	Variable sites	Constant sites	Total sites
4 Codons				
Leu	CUN	0	3	3
Val	GUN	3	1	4
Ser	UCN	3	1	4
Pro	CCN	2	1	3
Thr	ACN	1	0	1
Ala	GCN	0	3	3
Arg	CGN	1	2	3
Gly	GGN	0	2	2
Sum		10	13	23
2 Codons				
Leu	UUR	1	1	2
Met	AUR	1	3	4
Gln	CAR	0	4	4
Lys	AAR	0	2	2
Glu	GAR	0	0	0
Trp	UGR	1	0	1
Sum	(NNR)	3	10	13
2 Codons				
Phe	UUY	0	1	1
Ile	AUY	0	1	1
Tyr	UAY	0	2	2
His	CAY	0	1	1
Asn	AAY	0	4	4
Asp	GAY	0	0	0
Cys	UGY	0	4	4
Ser	AGY	0	1	1
Sum	(NNY)	0	14	14

turnover or high oxidant levels (BROWN, GEORGE and WILSON 1979; BROWN *et al.* 1982; BROWN, 1981, 1983). The other appeals to the possibility of a relative lack of a recombinational mechanism by which natural selection could eliminate mildly deleterious mutations that are linked to an advantageous mutation (*cf*, CANN and WILSON 1983).[2] Although fungal mtDNAs recombine, there is no convincing evidence that recombination occurs in mammalian mtDNA except perhaps in the displacement loop (OLIVO *et al.* 1983).

If general factors (*e.g.*, enhanced mutation pressure and lack of recombination) were entirely responsible for the high rate of mtDNA divergence, one would expect the tRNA and rRNA genes of mtDNA to evolve only five to

[2] There is an important difference between our recombinational explanation (for the low rate of nuclear DNA evolution relative to the mtDNA rate) and the one offered by BROWN *et al.* (1984) who suggested that recombination might slow down the rate of accumulation of neutral mutations. We suspect that recombination can have no effect on the neutral evolutionary rate because the DNA sequence incorporated by recombination is as likely as that removed by recombination to harbor neutral mutations (see also TAKAHATA and SLATKIN 1983).

TABLE 5

Radical amino acid replacements in human mtDNAs as indicated by restriction analysis

Protein	Location in bp	Nature of replacement	
		Amino acids	Radicalness[a]
Urf 1	3391	Tyr-His	+
	3563	Thr-Ile	+
Urf 3	10066	Phe-Cys	+
	10084	Asn-Asp	−
	10394	Thr-Ala	−
Urf 4	10893	Asn-Asp	−
Urf 5	12925	Pro-Ser	+
Urf 6	14322	Asn-Ser	−
	14439	Tyr-Asn	+
	14509	Val-Ala	−
ATPase 6	8877	Phe-Leu	−
Cytochrome oxidase 2	7617	Asp-Gly	+
	7970	Ser-Pro	+
Cytochrome oxidase 3	9400	Ala-Thr	−
	9509	Leu-Phe	−
	9859	Cys-Trp	+
Cytochrome *b*	14749	Thr-Ala	−
	15005	Ala-Asp	+

[a] Degree of chemical difference based on differences in size, composition and polarity; the plus symbols refer to differences greater than 73, as reported in Table 2 of GRANTHAM (1974).

TABLE 6

Transitions and transversions inferred from site gains

Region (and position in codon)	Total gains		% transversions	
	Observed	Inferred[a]	Observed	Inferred[a]
Protein (1)	9	29	0	0
Protein (2)	6	7	33	43
Protein (3)	37	63	34	28
Protein (all)	52	99	28	21
tRNAs and rRNAs	11	48	41	61
Noncoding	14	57	29	7
All	77	204	30	26

[a] Inferred by phylogenetic analysis.

TABLE 7

Base substitutions responsible for site gains in human mtDNA

Enzyme	Sequence	No. of sites[a] Obs/Exp	Gain mutations Transitions	Gain mutations Transversions
Well-represented sites				
AluI	AGCT	63/52	10	2
DdeI	CTNAG	71/52	4	0
HaeIII	GGCC	52/28	11	2
Subtotal		186/132	25	4
Poorly represented sites				
AvaII	GGTCC, GGACC	8/16	2.5	1.5
FnuDII	CGCG	6/28	0	0
HhaI	GCGC	17/28	4	2
HinfI	GANTC	36/52	7	6
HpaII	CCGG	23/28	2.5	3.5
MboI	GATC	23/52	6	4
RsaI	GTAC	34/52	3	1
TaqI	TCGA	29/52	4	1
Subtotal		176/308	29	19

[a] Obs refers to the observed number of sites in Cambridge sequence (ANDERSON *et al.* 1981). Exp refers to the expected number of sites calculated from the base composition of the light strand (24.7% T, 31.2% C, 30.9% A and 13.1% G) of this same mtDNA. The total number of sites observed in this survey of 112 humans (plus the Cambridge sequence) is as follows for each enzyme: 76 (*AluI*), 76 (*DdeI*), 63 (*HaeIII*), 12 (*AvaII*), 6 (*FnuDII*), 23 (*HhaI*), 48 (*HinfI*), 29 (*HpaII*), 33 (*MboI*), 37 (*RsaI*), 34 (*TaqI*). χ^2 for the two classes of sites is 6.55, $P < 0.02$.

ten times faster than their nuclear counterparts. Preliminary studies, however, suggested a much higher rate than expected for these mitochondrial genes (BROWN *et al.* 1982; CANN, BROWN and WILSON 1982), and the divergence pattern documented here for humans and by FERRIS *et al.* (1983b) for mice confirms and extends this result. It is appropriate, therefore, to discuss in detail the possibility that an additional factor, *e.g.*, relaxed constraints on the translation apparatus (BROWN *et al.* 1982; CANN, BROWN and WILSON 1982; BROWN 1983), must be invoked to explain the especially high rate of mitochondrial tRNA and rRNA evolution.

Rate of evolution of the translation apparatus

The translation apparatus of a small genome, such as animal mtDNA, which encodes only 13 polypeptides (ANDERSON *et al.* 1981), would be expected to function under more relaxed constraints than would a system that translates thousands of kinds of mRNA (BORST and GRIVELL 1981; JUKES 1981; CANN, BROWN and WILSON 1982; BROWN 1983). The constraints could be further relaxed in mitochondria, in which nearly every gene may be represented by many protein products (CHING and ATTARDI 1982; OLIVER, GREENBERG and WALLACE 1983; MARIOTTINI *et al.* 1983); in bacteria and nuclei, however,

there are probably some genes that are represented by only one or a few protein molecules per cell (McILWAIN 1946). If a mutation were to raise the frequency of a particular error, such as the incorporation of threonine at codons specifying leucine, the functional consequences could be lethal for a complex system, in which there are sure to be some proteins whose function will be severely impaired by misincorporation of even one threonine per poly-peptide chain. But it is quite possible, in a small system, that there are no positions in any of the few kinds of proteins synthesized where function would be lost if leucine were replaced by threonine.

There is direct experimental evidence that mutations in two of the three classes of components in the translation apparatus can affect the accuracy, speed and efficiency of translation. We refer to bacterial mutations in tRNAs and ribosomal proteins (GORINO 1974; KURLAND 1979; YARUS 1979, 1982). Comparative structural studies of the interactions between tRNAs and codons emphasize the possible role of tRNAs in translational fidelity (LAUBUDA and PÖRSCHKE 1983). In addition, there is circumstantial evidence from compara-tive structural studies suggesting that rRNA also has a role in translational proofreading (THOMPSON and HEARST 1983). The fact that two of the rRNA regions implicated in proofreading are expanded in rRNA specified by the nucleus and absent from mitochondrial rRNA (THOMPSON and HEARST 1983; BRIMACOMBE, MALY and ZWEIB 1983) is noteworthy. It can, therefore, be predicted that mutations in any of the three classes of components in the mitochondrial translation apparatus can affect the accuracy, speed and effi-ciency of translation. If such mutations are more likely to be tolerated in machinery that makes fewer kinds of proteins, one can further predict that the rate of evolutionary change in all three classes of components will be correspondingly higher.

Ribosomal RNA genes: This prediction fits with our finding that the rRNA genes of mtDNA are nearly as variable as the noncoding and protein-coding regions of mtDNA. Yet, as is well known from the early work of SINCLAIR and BROWN (1971) and PALLERONI *et al.* (1973), the rRNA genes in nuclei and bacteria are remarkably conservative, a phenomenon that has been useful in defining phylogenetic relationships among different kingdoms of organisms (FOX *et al.* 1980). We calculate that nuclear rRNA gene divergence in verte-brates may be 40 times slower than for an average segment of single copy nuclear DNA. Consider the following case. The rRNA genes of two frog species, *Xenopus laevis* and *Hyla chrysoscelis*, differ by about 1.4% in base se-quence (TOIVONEN *et al.* 1983). Their divergence time is at least 150 million years (WALLACE, MAXSON and WILSON 1971; BISBEE *et al.* 1977), from which we estimate a divergence rate of less than 0.01% per million years. [We have made a very similar estimate from comparing the complete nucleotide se-quences published for the nuclear 18S rRNA genes of Xenopus and Rattus by TORCZYNSKI, BOLLON and FUKE (1983).] In contrast, the rate of single-copy DNA divergence for the best studied pair of frog species, *X. laevis* and *X. borealis*, is about 0.4% per million years, this estimate coming from a ΔT_m value of 12° and an assumed divergence time of about 30 million years (GALAU *et al.* 1976).

Transfer RNA genes: Our survey of restriction site variability in 20 tRNA genes supports the inference, made by Brown *et al*. (1982) from interspecific sequence comparisons of three tRNA genes, that mitochondrial tRNA sequences diverge at least 100 times faster than nuclear tRNA sequences. During vertebrate evolution, the average rate of nuclear tRNA divergence has been about 100 times slower than single-copy nuclear DNA. By contrast, in human (Table 2) and mouse mtDNAs (Ferris *et al*. 1983b), the mean rate of tRNA divergence approaches the average rate of divergence for the mitochondrial genome.

Ribosomal proteins: Electrophoretic results suggest that mitochondrial ribosomal proteins evolve nearly ten times faster than do the proteins of the conventional ribosomes in the cell sap (Matthews, Hessler and O'Brien 1978). Most of these mitochondrial proteins (>70) must be encoded by nuclear genes, and, thus, their fast evolution is unlikely to be due to enhanced mutation pressure or lack of recombination; it is more likely to be the consequence of an elevated probability of fixation of mutations, which is consistent with either relaxed constraints on the translation apparatus or positive selection for compensating mutations that would permit the rRNA-ribosomal protein complexes to maintain function.

Contextual constraints in mitochondrial mRNAs: As Lipman and Wilbur (1983) pointed out, the hypothesis of relaxed constraints on the translation apparatus could have the consequence of relaxed constraints at silent sites in mitochondrial mRNAs. They have shown that the bases permitted at such sites are most nearly random for mitochondrial mRNA, bacterial mRNAs being intermediate and vertebrates being the least random in this respect. It is well known from studies of prokaryotic systems that a codon's context, *i.e.*, the nature of its neighboring bases, has an effect on the accuracy of codon translation (Gorini 1974; Yarus 1979; Gavrilova, Perminova and Spirin 1981). If this is also the case for animal mitochondria, the high rate of silent substitution in mitochondrial genes coding for proteins may well be due, in part, to relaxed constraints on the translation apparatus. Nevertheless, we suggest that this effect would be small because nuclear pseudogenes, which presumably lack contextual constraints, evolve only slightly faster than the silent rate for functional nuclear genes and much more slowly than the silent rate for mitochondrial genes (Brown *et al*. 1982; Miyata *et al*. 1982).

Relaxed contextual constraints might also be expected to raise the acceptability of amino acid replacements by a small factor. Our evidence that six mitochondrially encoded proteins have incurred multiple amino acid replacements during diversification of the human gene pool is interesting in this regard, especially when considered in relation to the proportion of radical replacements. This proportion is slightly higher for human mitochondrial proteins than for fibrinopeptides, lysozymes, globins, carbonic anhydrases and insulins (R. L. Cann and A. C. Wilson, unpublished calculations).

Thus, the high rate of evolutionary change in mammalian mtDNA is probably the result of a blending of two forces. One could be increased mutation pressure and/or the lack of recombination, both of which would enhance the frequency of change at all positions. The other could be relaxed translational

constraints, which would have a major effect on components of the translation machinery and a minor effect on the mitochondrial genes coding for proteins.

Coevolution of cytochrome oxidase II and cytochrome c

The fact that subunit II of cytochrome oxidase is highly variable among humans (Table 3) is indicative of a recent change in the tempo of evolution in one region of the mitochondrial genome. Low-resolution mapping suggests that this gene has also been highly variable in apes (FERRIS, WILSON and BROWN 1981; FERRIS *et al.* 1981) but conservative in rodents (BROWN and SIMPSON 1981). High-resolution mapping (FERRIS *et al.* 1983b) and heteroduplex mapping (CHRISTIANSEN and CHRISTIANSEN 1983) confirm that rodents have undergone relatively little evolutionary change in this region.

Reinforcement for this view comes from phylogenetic analysis of the sequences published for the cytochrome oxidase II region of human, mouse, rat and cow, as shown and referenced in Figure 3. This figure shows the number of amino acid substitutions, inferred by parsimony analysis, fixed along the lineages leading from a common ancestor to the present day species. Since divergence, 57 substitutions are estimated to have accumulated along the primate lineage but only eight to 14 on the other mammalian lineages. This deviation from an expectation of the molecular clock hypothesis (WILSON, CARLSON and WHITE 1977) was recognized by BROWN and SIMPSON (1982) and BROWN *et al.* (1983), and the analysis in Figure 3 emphasizes its magnitude.

Only one other mitochondrial gene seems to exhibit faster evolution in primates than in rodents. For cytochrome *b*, the ratio of amino acid replace-
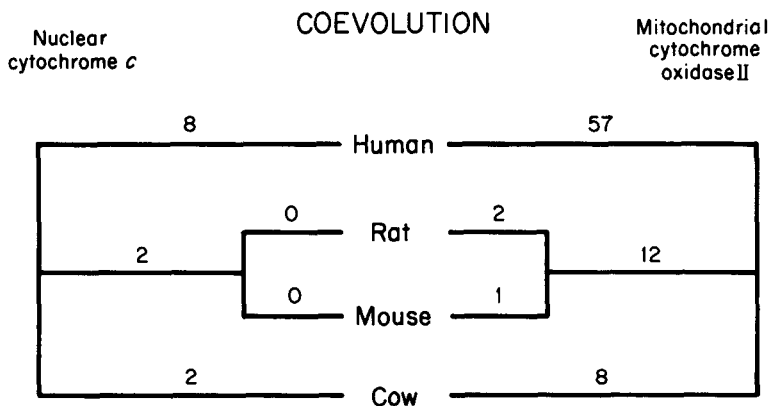

FIGURE 3.—Phylogenetic distribution of amino acid replacements in cytochrome *c* and subunit II of cytochrome oxidase. The apportioning of base substitutions causing amino acid differences was based on the parsimony principle, using directly determined amino acid sequences of cytochromes *c* (DAYHOFF 1972, CARLSON *et al.* 1977) and nucleotide sequences for the cytochrome oxidase II gene (ANDERSON *et al.* 1981, 1982; BIBB *et al.* 1981; BROWN and SIMPSON 1982). We chose not to apportion such substitutions whenever two or more equally parsimonious solutions were available at a given position. Seven of the 87 substitutions needed to account for the amino acid differences among the four oxidase subunits were in this ambiguous category and do not appear in the figure.

ments in the primate lineage to that in the nonprimate lineages is about 2, a smaller value than is observed for subunit 2 of cytochrome oxidase.

Also evident from Figure 3 is a strong suggestion for coevolution between specific nuclear and mitochondrial genes. Cytochrome c, the protein that receives electrons indirectly from cytochrome b and passes them directly to the cytochrome oxidase II subunit in vivo, has long been known to have undergone faster evolution in primates than in rodents (CARLSON et al. 1977).

In addition, cytochrome c and cytochrome oxidase have undergone complementary functional changes away from the ancestral mammalian state in the lineage leading to higher primates. In tests with purified components, the cytochrome c and the cytochrome oxidase of higher primates react well with each other but poorly with cytochrome oxidase and cytochrome c from nonprimates (OSHEROFF et al. 1983). This is the first probable instance of coevolution between nuclear and cytoplasmic genes whose products interact directly and in which the coevolution is evident at the levels of both function and primary structure.

There is another case for coevolution between nuclear and mitochondrial genes. This involves the mitochondrial rRNA genes and the nuclear genes coding for proteins in the mitochondrial ribosomes. The apparently high rate for these proteins, relative to that for the proteins in conventional ribosomes (MATTHEWS, HESSLER and O'BRIEN 1978), could be due to coevolution with the rapidly evolving rRNA genes in mtDNA. However, functional tests to complement these evolutionary rate comparisons have not been performed. We also emphasize the need to document the apparent rates of ribosomal protein evolution by primary structural analysis, since the differences observed in two-dimensional gels (MATTHEWS, HESSLER and O'BRIEN 1978) could be the result of posttranslational modifications rather than amino acid replacements.

An additional distinct feature of the cytochrome example, as compared with the ribosomal example, is that the factor responsible for the accelerated evolution in the primate lineage appears to be unique to the mitochondria of higher primates rather than to animal mitochondria in general. This elevation cannot be ascribed to a general factor (e.g., increased mutation pressure), because only one gene is strongly affected. The implication is that the accelerated evolution of cytochrome oxidase subunit 2 must be explained in terms of the relaxation of a functional constraint specific to this gene.

Transitions and transversions at restriction sites

The low ratio of transitions to transversions inferred from restriction site gains (Table 7) suggests either that the human population has experienced an unusual type of evolution or that restriction sites are unusual in this respect. The restriction sites surveyed here have atypical base sequences; in addition to containing a high level of G and C, these sites are palindromic and, therefore, have the same base composition on the two strands of DNA. Typical vertebrate mitochondrial sequences, by contrast, differ from restriction sites by exhibiting strand bias and having a relatively low content of G and C

(BROWN 1983). The facts that most types of restriction sites are underrepresented in human mtDNA, even after correction has been made for its base composition and strand bias (see Table 7), and that their distribution is non-random (ADAMS and ROTHMAN 1982) may be considered reasons for expecting them to exhibit unusual evolutionary behavior.

The sequencing of numerous segments of mitochondrial coding regions from a few human individuals will allow a choice between these alternative explanations for the low ratio of transitions to transversions observed at most restriction sites in human mtDNA. Such studies have already been done on two regions of human mtDNA (GREENBERG, NEWBOLD and SUGINO 1983; OLIVER, GREENBERG and WALLACE 1983), and preliminary sequencing observations at Berkeley (R. L. CANN, unpublished results) on four additional regions hint strongly that it is the restriction sites that are unusual as regards the ratio of transitions to transversions. High-resolution mapping comparisons of mouse mtDNAs are also suggestive of a low ratio of transitions to transversions at many restriction sites (FERRIS *et al.* 1983b). The possibility of a special functional role or sensitivity to mutagenesis for certain palindromic regions containing high levels of guanine and cytosine, therefore, deserves to be explored.

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