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Evolution

Extensive polymorphism in the mitochondrial DNA of apes

(cleavage map/ribosomal gene/ape population/human evolution)

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ABSTRACT **Ape species are 2–10 times more variable than the human species with respect to the nucleotide sequence of mtDNA, even though ape populations have been smaller than the human population for at least 10,000 years. This finding was made by comparing purified mtDNAs from 27 individuals with the aid of 25 restriction endonucleases; for an additional 59 individuals, comparisons were made with fewer enzymes by using the blot hybridization method. The amount of intraspecific sequence divergence was greatest between orangutans of Borneo and Sumatra. Among common chimpanzees, a large component of the variation is due to two highly distinct forms of mtDNA that may reflect a major geographic subdivision. The least amount of sequence variation occurred among lowland gorillas, which exhibit only twice as much sequence variation as humans. The large intraspecific differences among apes, together with the geological and protein evidence, leads us to propose that each ape species is the remnant of an ancient and widespread population that became subdivided geographically and reduced in size and range, perhaps by hominid competition. The low variation among human mtDNAs is consistent with geological evidence that the human species is young. The distribution of site changes within the mitochondrial genome was also examined. Comparison of closely related mtDNAs shows that the ribosomal RNA genes have diverged more slowly than the rest of the genome.**

The human species has an unusually low level of genetic variation in mtDNA. Two humans picked at random are expected to possess mtDNAs that differ by only 0.36% in nucleotide sequence (1). By contrast, the level of intraspecific variation reported for other mammals is 3–30 times higher (2–4).

The research described below was aimed at finding out whether the low variation in mtDNA is unique to our species or shared by our closest relatives, the great apes. By using many restriction endonucleases, which provide a fast method of estimating the amount of difference in nucleotide sequence among mtDNAs (5), we have obtained estimates of intraspecific variation in chimpanzees, gorillas, and orangutans. These estimates contrast with those for humans and shed light on the evolutionary history of ape and human populations. Our intraspecific comparisons also provide a perspective on the susceptibility of the region coding for rRNA to evolutionary change.

MATERIALS AND METHODS

Tissues and Cell Lines. mtDNA was purified (5) from 5 orangutans (*Pongo pygmaeus*), 10 common chimpanzees (*Pan troglodytes*), 2 pygmy chimpanzees (*Pan paniscus*), and 4 gorillas (*Gorilla gorilla*). Records show no immediate kinship among these individuals. The six human mtDNAs analyzed correspond to individuals 3, 6, 9, 10, 15, and 21 of Brown (1). Total cellular DNA was prepared from one additional pygmy chimpanzee and 59 additional common chimpanzees. The tissues, blood samples, and cell lines used were supplied by zoos and primate research centers.

Restriction Endonucleases and Electrophoresis. Nineteen restriction endonucleases (New England BioLabs) were used to digest purified mtDNAs. Digestions were performed according to the supplier's directions. Fragments were radioactively labelled, subjected to electrophoresis, and detected as described by Brown (1). The smallest routinely scored fragment was 150 base pairs (bp) in 1.2% agarose and 60 bp in 3.5% acrylamide gels.

Cleavage Maps and Fragment Patterns. The location of restriction sites in mtDNA was determined for 19 endonucleases, using as a reference the published map for a single representative of each species (6). Six other endonucleases made too many fragments to be mapped conveniently. For these, the fragment patterns were determined for individual chimpanzees and gorillas as Brown (1) has done for humans.

Blot Hybridization. Total cellular DNA from blood samples was prepared as described in Zimmer *et al.* (7). A radioactive hybridization probe (specific activity, 10^8 cpm of $^{32}P/\mu g$ DNA) was made by nick translation of 50 ng of purified pygmy chimpanzee mtDNA (8). The mtDNA fragments present in a restriction endonuclease digest of 2–7 μg of cellular DNA were detected by hybridization with a labelled probe (10^7 cpm per filter) after the fragments had been separated by electrophoresis in 0.8% agarose gels and transferred to nitrocellulose filters (7).

Estimation of Sequence Divergence from Cleavage Maps. The percentage divergence in base sequence among mtDNAs was estimated from comparison of cleavage maps by using equation 15 of Nei and Li (9). An assumption of this method is that the cleavage site differences are due to base substitution. Empirical evidence justifying this assumption has been presented (1, 5).

RESULTS

Orangutans. Fig. 1 shows the mtDNA cleavage maps for 29 variable sites in five orangutans. Although differing from one another at many sites, the maps are identical in length and in the arrangement of 33 invariant sites. The biggest differences are between Bornean and Sumatran orangutans, which are considered to be distinct subspecies (10). The most similar mtDNAs come from the same island.

A tree relating the mtDNA maps was constructed (Fig. 2). All Sumatran orangutans cluster together, as do those from Borneo. The mean difference in nucleotide sequence between

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Abbreviation: bp, base pair(s).

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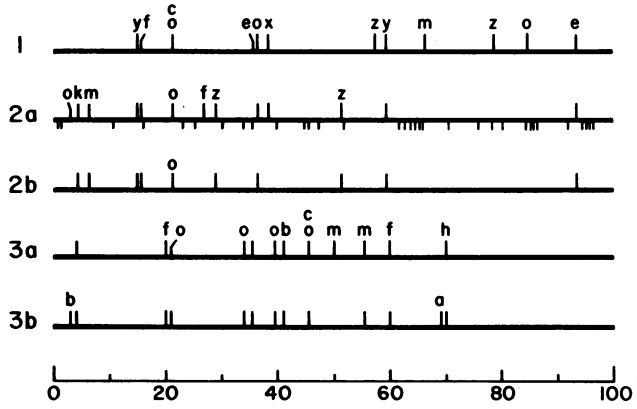

FIG. 1. Location of 29 variable restriction sites in mtDNAs of three Sumatran (maps 1, 2a, and 2b) and two Bornean (maps 3a and 3b) orangutans. Vertical lines represent variable cleavage sites. a, *Eco*RI; b, *Hin*dIII; c, *Hpa* I; d, *Bgl* II; e, *Xba* I; f, *Bam*HI; g, *Pst* I; h, *Pvu* II; i, *Sal* I; j, *Sac* I; k, *Kpn* I; l, *Xho* I; m, *Ava* I; n, *Sma* I; o, *Hin*cII; w, *Bst*EII; x, *Bcl* I; y, *Bgl* I; z, *Fnu*DII. Vertical lines that lack letters are homologous in position to one on a map above. The scale is in map units with the origin of replication at 0 and the direction of replication to the right. The 33 invariant sites appear below map 2a (cf. ref. 6).

the Bornean and Sumatran branches of the tree is estimated from the maps to be 5%.

Chimpanzees. *Maps.* Chimpanzee mtDNA exhibits a lower degree of variability than orangutan mtDNA. The 31 variable positions in the maps of 13 individual mtDNAs are shown in Fig. 3. From the fraction of sites in common, we estimated the percentage difference in nucleotide sequence for all possible pairs of these 13 individuals (Table 1). The biggest difference (3.7%) is between common and pygmy chimpanzees. Among all common chimpanzees, the mean pairwise difference is 1.3% and among all pygmy chimpanzees it is 1.0%.

An evolutionary tree for the mtDNA maps (Fig. 4) shows the existence of three major types of mtDNA in chimpanzees. One type is characteristic of pygmy chimpanzees and the other two occur exclusively in the common chimpanzees. The sequence difference between the latter two types is 2.0%.

Fragments. Ten of the common chimpanzee mtDNAs and one of the pygmy chimpanzee mtDNAs that had been analyzed by cleavage mapping were digested with six restriction enzymes that recognize four or five base sites. The average number of fragments detected electrophoretically was 140 per mtDNA—i.e., with this method, we were able to detect ≈140 restriction sites

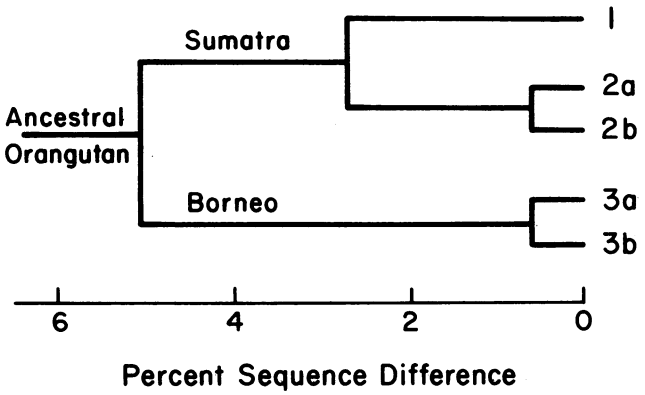

FIG. 2. Evolutionary tree for the mtDNAs of five orangutans. This tree was obtained by the parsimony method (6). The percent sequence differences between branches of the tree are calculated from the maps by using equation 15 of Nei and Li (9).

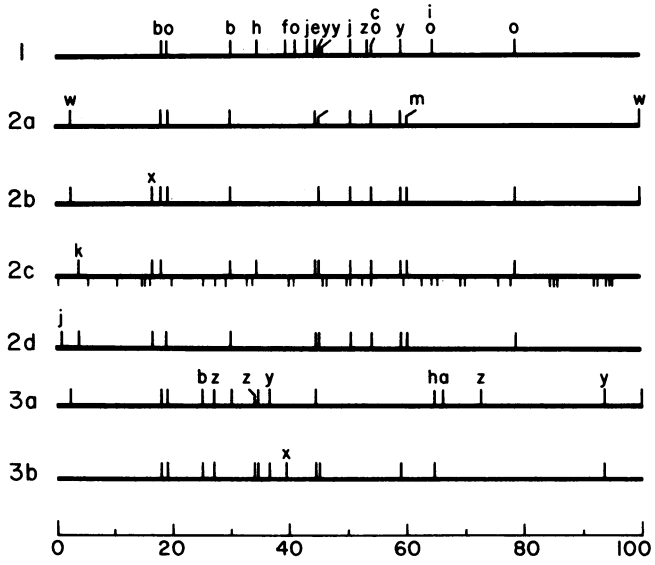

FIG. 3. Locations of 31 variable restriction sites in mtDNAs of 10 common chimpanzees [map 1 (individuals 4, 5, and 8), map 2a (9), map 2b (individuals 1 and 6), map 2c (individuals 2, 3, and 10), and map 2d (individual 7)] and three pygmy chimpanzees [map 3a (individuals 1 and 3) and map 3b (individual 2)]. Restriction sites are as in the legend to Fig. 1. The mtDNA of pygmy chimpanzee 3 was mapped by the blot hybridization method (see ref. 6). The 36 invariant sites appear below map 2c (see ref. 6). *Bam*HI sites occur at 15, 20, and 40 map units.

in addition to those mapped. As is evident from the fragment patterns listed in Table 2, this method has great sensitivity. Several of the individuals having identical cleavage maps for six base-recognition sites were readily differentiated on the basis of fragment patterns produced by digestion with enzymes recognizing four base sites (e.g., individuals 4, 5, and 8, Table 2). Furthermore, the pygmy chimpanzee shares no mtDNA fragment patterns with common chimpanzees and the two major types found within the common chimpanzees also share no patterns. Thus, the fragment-pattern comparisons confirm and extend the inferences based on the mapping approach.

Screening by the blot hybridization method. We screened mtDNA from 59 additional common chimpanzees by examining *Bam*HI digests of cellular DNA with the blot hybridization method. This enzyme was chosen because it distinguished readily between the two major types of common chimpanzee mtDNA, as is evident from the maps in Fig. 3. In chimpanzees exhibiting type 1 mtDNA, *Bam*HI produced fragments of 12,400, 3,200, and 870 bp; in those with type 2, a site loss has resulted in the fusion of the two largest fragments into a 15,600-bp fragment. According to this test, 10 mtDNAs were of type 1, 48 were of type 2, and one had a pattern that can be derived from type 1 by the gain of a *Bam*HI site within the 3200-bp fragment. Our typing of the 59 common chimpanzee mtDNAs was confirmed by a similar, but more limited, study in which *Sac*

Table 1. Comparison of mtDNA maps among chimpanzees

	Percent sequence difference					
Map no.	1	2a	2b	2c	2d	3a
2a	2.1					
2b	2.3	0.5				
2c	1.7	1.3	1.1			
2d	2.3	1.3	1.1	0.7		
3a	4.3	3.0	3.6	4.1	4.5	
3b	3.5	2.9	3.6	3.2	3.6	1.5

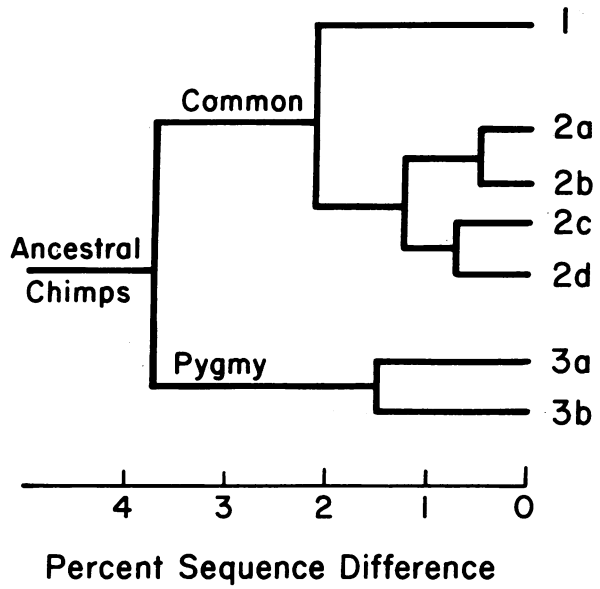

FIG. 4. Evolutionary tree for the seven types of chimpanzee mtDNA shown in Fig. 3. The tree was obtained by the parsimony method (6). The percent sequence differences between branches of the tree are taken from Table 1. Gorilla mtDNA was used to root the tree.

I digests of cellular DNA were analyzed with a mtDNA probe (B. Chapman, unpublished results).

Table 3 gives the distribution of the two types of mtDNA among 49 chimpanzees that have been tentatively classified as to subspecies on the basis of external morphology. Type 1 mtDNA occurs almost exclusively in the eastern subspecies (*P.t. schweinfurthi*) and type 2 mtDNA predominates in the western subspecies (*P.t. verus*).

Gorillas. Gorillas were the least variable for mtDNA of the great apes. The mtDNAs from four lowland gorillas were variable at five positions (Fig. 5). The mean pairwise sequence difference was 0.55% and the two most divergent branches in the gorilla tree differ by 0.9%. Digests of gorilla mtDNA with *Hpa* II, *Mbo* I, *Hinf* I, *Mbo* II, *Taq* I, and *Ava* II, which provided ≈140 additional sites, showed no differences between individuals 1 and 4, which also had identical cleavage maps.

Humans. Humans were the least variable of the hominoids for mtDNA. Only five positions were variable in a sample comprised of two Blacks, two Whites, and two Asians. No variation beyond that reported by Brown (1) was observed when four additional restriction endonucleases were used (*Bgl* I, *Fnu*DII, *Bcl* I, and *Bst*EII). The mean pairwise difference, 0.30%, was calculated from maps based on the 19 endonucleases used for the ape species. This value is in reasonable agreement with the value, 0.36%, found in studies using much larger sample sizes and comparing many more cleavage sites (ref. 1; R. L. Cann, personal communication.).

Table 2. Cleavage patterns for restriction endonucleases recognizing four- and five-base sites in mtDNA from 10 common chimpanzees

Map no.*	Individual	Fragment pattern					
		Hpa II	*Mbo* I	*Taq* I	*Hinf*I	*Mbo* II	*Ava* II
1	4	A	A	A	A	A	A
1	5	B	B	A	A	A	A
1	8	A	B	A	A	A	A
2a	9	C	C	B	B	B	B
2b	1	C	D	C	B	C	C
2b	6	C	D	D	B	C	C
2c	2, 3 and 10	D	E	E	C	C	C
2d	7	E	F	F	D	D	C

Fragment patterns were obtained from mtDNAs by digestion with restriction enzyme and then labeling the fragments at the ends, separating them electrophoretically, and autoradiographing them.
* See Fig. 3.

Table 3. Correspondence between subspecies designation and type of mtDNA in common chimpanzees at the Holloman Primate Center

Subspecies	Number of individuals		Correspondence, %	Geographic region*
	Type 1	Type 2		
verus	1	29	97	A
troglodytes	0	3	100	B
schweinfurthi	8	6	57	C
koolakamba	1†	1	50	D

Subspecies designations are based on external morphology (11). One of the identifications is inconsistent with records concerning geographic origin—although identified as *schweinfurthi*, records indicate that this chimpanzee came from Sierra Leone (region A); consistent with this geographic origin, its mtDNA is of type 2. In addition, some of the other specimens designated as *schweinfurthi* probably do not belong to this subspecies (see ref. 18).
* Region in which the subspecies is supposed to occur (11): A, West African countries; B, region from eastern Nigeria to Congo Brazzaville; C, Central and East African countries; D, southern Congo, Brazzaville, and southern Gabon.
† *Bam*HI fragment pattern is derived from type 1 by a single site gain.

Distribution of Site Changes. Besides giving information about genetic variability among individuals, our mtDNA comparisons extend knowledge of the distribution of sites at which variation has occurred within this genome. It is expected that comparisons of closely related mtDNAs will provide the most sensitive way of detecting differences in rates of evolutionary change between different regions. Only 1 of the 51 site changes observed in intraspecific comparisons of hominoid mtDNAs that differ by less than 3% has occurred in the ribosomal region [i.e., at 81–97 map units (6)]. If the changes were distributed at random, six would be expected in this region. The deviation from expectation is statistically significant.

DISCUSSION

Ape Species May Be Old. The big differences among ape individuals shown by mtDNA comparisons can be reconciled with the view that ape species are older than the human species. A modern ape species typically consists of fewer than 10,000 individuals distributed over a small geographic area. The ex-

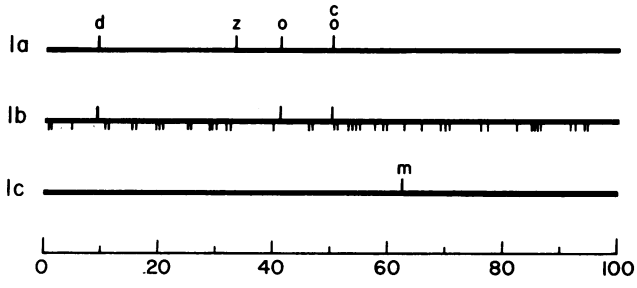

FIG. 5. Locations of five variable restriction sites in the mtDNAs of four lowland gorillas. Restriction sites are as in the legend to Fig. 1. Two individuals, 1 and 4, had identical maps (1a); the other two individuals, 2 and 3, had maps 1b and 1c, respectively. The 45 invariant sites are shown below map 1b (see ref. 6).

istence of highly divergent mtDNA lineages within a species prompts us to think of it as the remnant of a formerly widespread and large population that became subdivided hundreds of thousands or even millions of years ago by geographic barriers and, perhaps, by hominid competition.

From mtDNA map comparisons, we can calculate the divergence time (t) of two populations (X and Y) by using Eq. 1:

$$t = 0.5\,[\delta_{xy} - 0.5\,(\delta_x + \delta_y)] \qquad [1]$$

where δ_{xy} is the mean percent sequence divergence between the two populations and δ_x (or δ_y) is the mean percent sequence divergence for all possible pairs of individuals within population X (or Y). This equation is based on equation 25 of Nei and Li (9) and the assumption that mtDNA evolution in apes has occurred at the rate of 2 substitutions per 100 bp per million years. This is the approximate rate inferred from studies of other mammals (5).

The divergence time of the Bornean and Sumatran orangutans is estimated to be at least 1.5 million years from the maps, and this estimate is consistent with geological and protein evidence. Although now confined to two islands, orangutans occurred widely in the Pleistocene from China to Indonesia (10). Their wide distribution would have been facilitated by the frequent existence of land bridges between the Asian mainland and the islands of Indonesia during the past 2 million years (12). Fossil evidence for the presence of hominids in Java 1.9 million years ago (13) raises the possibility that they reduced the range of orangutans and confined them to Borneo and Sumatra. Contact between the two populations of orangutans may also have been hindered by the presence of large rivers in the Sunda land mass between Borneo and Sumatra (14). The idea of an ancient divergence time for these populations is also supported by protein evidence (15).

The mtDNA sequence difference between common and pygmy chimpanzees is also suggestive of an ancient divergence time. The estimated time, based on the map comparisons, is 1.3 million years ago. This agrees with geological evidence that the Zaire River, which separates the two species, has maintained its course for the past 1.5 million years (16). This time is consistent with protein evidence (15, 17).

Among common chimpanzees, the geographic range of the two major types (1 and 2) of mtDNA remains to be determined rigorously. Our studies were conducted using captive chimpanzees whose geographic origins are, in most cases, uncertain (see Table 3). As is evident from the careful electrophoretic study of polymorphic proteins by Goodman and Tashian (18), authentic eastern representatives of the common chimpanzee differ markedly from western representatives. The differences in allelic frequencies are suggestive of an ancient separation between these populations, and we calculate a divergence time of 1 million years between the two types of mtDNA. It is also evident from their study that the morphological classification of captive chimpanzees may sometimes have been inaccurate.[§] Hence, a mtDNA study of chimpanzees in the wild will be required to ascertain the geographical distribution of the two types of mtDNA.

Table 4. Site variability in two regions of mtDNA

Region	Changes per site: Closely related mtDNAs	Changes per site: Distantly related mtDNAs
Ribosomal RNA	0.08	1.31
Nonribosomal	0.45	1.09
Whole genome	0.41	1.11

Changes per site were calculated by dividing the number of site changes (inferred phylogenetically) by the number of positions surveyed (13 for intraspecific and interspecific comparisons in the ribosomal RNA region and 110 for intraspecific and 119 for interspecific comparisons in the nonribosomal regions). Interspecific results are from Ref. 6. Closely related mtDNAs are those differing by <3% in nucleotide sequence.

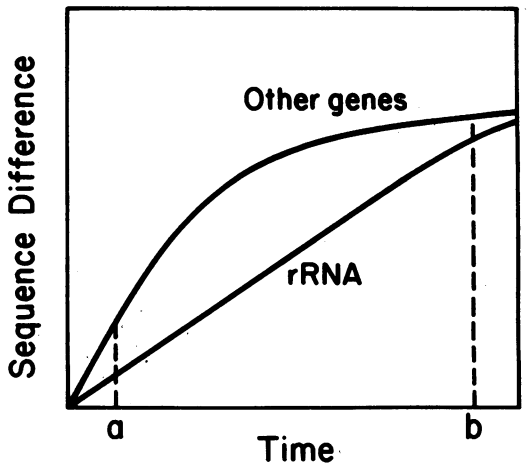

FIG. 6. Suggested dependence of sequence divergence on time of divergence for ribosomal RNA genes and other genes of mtDNA.

Gorillas were the least variable of the apes for mtDNA sequences. We attribute this to the sampling of only one subspecies, the lowland gorilla, which occupies a small geographic area (21). Protein divergence between lowland and mountain gorillas indicates a divergence time as old as that between the two species of chimpanzees (17). So, we expect the mtDNA divergence between the two gorilla subspecies to be large. If this is true, then every great ape species has a level of mtDNA diversity exceeding that in the human species by a factor of three or more.

The Human Species Is Young. The low level of mtDNA variation in humans fits with geological evidence that our species is a young one. Fossil studies indicate that the transformation of *Homo erectus* into *H. sapiens* was in progress 200,000 years ago and may not have been completed until within the past 100,000 years (22–24). Thus, we conclude, tentatively, that mtDNA diversity is related to species ape in hominoids.[¶]

Implications for Primate Management. Our demonstration of large genetic differences within ape species points to the need for renewed attention to the problem of managing breeding colonies of both captive and wild apes.

A rational approach to the preservation of the genetic diver-

[§] There is further evidence for a great deal of nuclear gene polymorphism among common chimpanzees. Morphologically defined eastern and western subspecies showed significant differences at four out of six blood group loci (19). A high level of globin gene polymorphism also exists in chimpanzees (ref. 7; unpublished data). There are other reports, however, indicating a low level of protein polymorphism in chimpanzees (15, 20). In our opinion, there is need for a more rigorous and extensive comparison of the level of nuclear polymorphism in chimpanzees and other hominoids.

[¶] Another factor could contribute to the low level of mtDNA variability among humans. Tree analysis of both map and nucleotide sequence data shows that the lineage leading to human mtDNA is slightly shorter than those leading to chimpanzee and gorilla mtDNA (unpublished). Such an effect can be explained by back mutation, parallelism, or an evolutionary slowdown in the hominid lineage. A slowdown, if in effect during the period of human diversification, would contribute to the low level of variability in the human population. Evidence that this contribution is unlikely to be large will be presented elsewhere.

sity present in apes will require molecular screening of wild populations. Additional screening of captive populations will also be important, to avoid inadvertent mixing of genetically very distinct lineages of apes.

Slow Divergence of Ribosomal DNA. Comparison of the distribution of site changes in mtDNA of closely and distantly related organisms gives a perspective on the rate at which ribosomal RNA genes diverge. Our intraspecific comparisons in hominoids suggest that the rate of divergence in ribosomal RNA genes is several times lower than that in the rest of the mitochondrial genome. A similar observation has been made with rodents (ref. 2; unpublished) and by extensive studies of variation among humans (R. L. Cann, personal communication).

The intraspecific observations appear to contrast with previous observations based on interspecific comparisons, which indicate that the extent of divergence in the ribosomal RNA genes is about the same as for other regions of the mitochondrial genome (Table 4; refs. 5 and 25). It may be possible to reconcile the two observations with the aid of Fig. 6, which draws attention to the nonlinear dependence of sequence difference on time of divergence for the mitochondrial genome as a whole (5). This nonlinear dependence is probably due to the existence of two classes of sites (i.e., silent and replacement) in the sequences coding for proteins. These sequences, which account for >65% of the genome (6), have experienced a high rate of substitution at silent sites (not causing amino acid substitutions) and a low rate at replacement sites (causing amino acid substitutions) (E. M. Prager, personal communication). Our suggestion is that ribosomal genes diverge at a rather steady rate that is intermediate between the silent and replacement rates. When closely related mtDNAs are examined (Fig. 6, time a), the ribosomal genes appear less divergent than the rest of the genome. By contrast, when distantly related mtDNAs are compared (Fig. 6, time b), the ribosomal genes seem to be about as divergent as the rest of the genome.

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