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Expanding the functional human mitochondrial DNA database by the establishment of primate xenomitochondrial cybrids

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ABSTRACT **The nuclear and mitochondrial genomes coevolve to optimize approximately 100 different interactions necessary for an efficient ATP-generating system. This coevolution led to a species-specific compatibility between these genomes. We introduced mitochondrial DNA (mtDNA) from different primates into mtDNA-less human cells and selected for growth of cells with a functional oxidative phosphorylation system. mtDNA from common chimpanzee, pigmy chimpanzee, and gorilla were able to restore oxidative phosphorylation in the context of a human nuclear background, whereas mtDNA from orangutan, and species representative of Old-World monkeys, New-World monkeys, and lemurs were not. Oxygen consumption, a sensitive index of respiratory function, showed that mtDNA from chimpanzee, pigmy chimpanzee, and gorilla replaced the human mtDNA and restored respiration to essentially normal levels. Mitochondrial protein synthesis was also unaltered in successful "xenomitochondrial cybrids." The abrupt failure of mtDNA from primate species that diverged from humans as recently as 8–18 million years ago to functionally replace human mtDNA suggests the presence of one or a few mutations affecting critical nuclear–mitochondrial genome interactions between these species. These cellular systems provide a demonstration of intergenus mtDNA transfer, expand more than 20-fold the number of mtDNA polymorphisms that can be analyzed in a human nuclear background, and provide a novel model for the study of nuclear–mitochondrial interactions.**

The mitochondrial genome of vertebrates is extremely specialized, containing exclusively genes that are necessary for the synthesis of the catalytic components of the oxidative phosphorylation system. More than 95% of all proteins located in the mitochondrial compartments are encoded by the nuclear DNA, synthesized in cytoplasmic ribosomes and imported into mitochondria. These include factors that regulate mitochondrial DNA (mtDNA) gene expression such as mtDNA and RNA polymerases, mitochondrial transcription factors, RNA processing and modifying enzymes, transcription termination factors, mitochondrial ribosomal proteins, aminoacyl-tRNA synthetases, and translation factors (1, 2). All these factors have to recognize specific mtDNA (or mitochondrial RNA) sequences to perform their functions. In addition, an even larger number of nuclear DNA-coded factors have to interact with mtDNA-coded polypeptides for the correct assembly and function of the oxidative phosphorylation system. Overall, more than 100 nuclear-coded factors have to interact with mtDNA or its gene products in a sequence-specific manner. The mtDNA evolves at a faster rate than the nuclear DNA (3), but mtDNA mutations that affect critical interactions with nuclear DNA-coded factors would have to be balanced by compensatory changes in the corresponding nuclear gene. The concerted evolution of these two genomes has led to a interdependence of a large set of genes that in vertebrates was believed to be species specific (4–8).

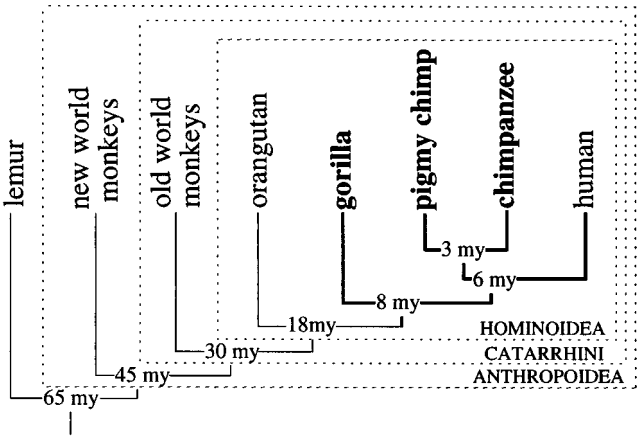

FIG. 1. Primate mtDNAs able to functionally replace human mtDNA. Numbers at the nodes correspond to estimated time in millions of years (my) of common ancestry of different primate evolutionary branches. Although there is controversy on distances and branching (10), we have adopted estimates based on recent molecular data which include nuclear-coded proteins and mtDNA (11–13). mtDNA from species in bold were able to functionally replace the human mtDNA. Dashed boxes represent the suborder Anthropoidea, the infraorder Catarrhini, and the superfamily Hominoidea.

Recently, Horai *et al.* (9) described the complete mtDNA sequence of four nonhuman apes: gorilla (*Gorilla gorilla*), common chimpanzee (*Pan troglodytes)*, pigmy chimpanzee (*Pan paniscus*), and orangutan (*Pongo pygmaeus*). Their study provided a very clear picture on the evolution of mtDNA in apes. Despite the relatively high number of sequence variations between different apes and human, these mitochondrial genomes are highly homologous (e.g., the "functional homology," when noncoding and synonymous mutations are not considered, between human and orangutan mtDNAs is 95%). The evolutionary distance between the different primate groups is a topic of great controversy, but we adopted the distance estimated from recent molecular data that includes the comparison of a number of proteins and DNA sequences (Fig. 1; refs. 10–13).

This work describes our attempt to restore oxidative phosphorylation function of a human cell line lacking mtDNA (ρ^0) by inserting mitochondria from species representing the major evolutionary related primate groups into its cytoplasm.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. Chimpanzee (*Pan troglodytes*) adenovirus 12–simian virus 40-transformed fibroblasts

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and African green monkey (*Cercopithecus aethiops*) simian virus 40-transformed kidney COS-7 cells were obtained from the American Type Culture Collection (ATCC CRL-1609 and ATCC CRL-1651, respectively) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml sodium pyruvate. Pigmy chimpanzee (*Pan paniscus*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), squirrel monkey (*Saimiri sciureus*), and lemur (*Lemur catta*) skin fibroblasts were obtained from the Coriell Institute for Medical Research Repository. Cells were cultured in high-glucose DMEM, supplemented with sodium pyruvate, as well as 15% (pigmy chimpanzee cultures only) or 20% FBS. Human 143B TK$^-$ cells were maintained in DMEM supplemented with 10% FBS and pyruvate. Its mtDNA-less derivative, 143B/206 ρ^0 (kindly provided by M. P. King, Columbia University), used as the nuclear donor in the cybrid fusions, was maintained in DMEM supplemented with 10% FBS, pyruvate, and 50 μg/ml uridine.

Cybrid Fusions. Individual fibroblast lines were plated at 5×10^5 cells per 35-mm^2 culture dish 1 day prior to cytoplast fusion. Enucleation of primate cells and fusion with 1.5×10^6 143B/206 ρ^0 was performed as described by King and Attardi (14). Fusion products were divided among 10 100-mm^2 culture dishes. The cybrids were maintained in selective DMEM containing 5% FBS, 5% dialyzed FBS, 100 μg/ml sodium pyruvate, and 100 μg/ml 5-bromo-2′-deoxyuridine. Cells were fed every 3 days with fresh selective medium. After approximately 14–18 days, 6–10 proliferating clones were isolated using cloning rings and were cultivated for stocks, DNA isolation, and functional assays (14).

DNA Analysis. For both parental and cybrid cell lines, confluent 75-cm^2 flasks were trypsinized and total DNA was isolated by digestion with 1.5 mg/ml proteinase K in 10 mM Tris·HCl, pH 7.4/10 mM NaCl/25 mM EDTA/1% SDS at 37°C overnight. The DNA was extracted with phenol/isoamyl alcohol, precipitated by isopropyl alcohol, dried, and resuspended at approximately 1 mg/ml in Tris/EDTA buffer. Nuclear DNA polymorphisms were tested by subjecting total DNA to PCR amplification using *Pfu* DNA polymerase (Stratagene) to amplify a region containing the myotonic dystrophy variable triplet repeat located on human chromosome 19 (15). The samples were denatured at 70°C for 2 min prior to electrophoresis through a 6% polyacrylamide gel. The gel was dried and exposed to an x-ray film. mtDNA polymorphisms were analyzed by digesting total DNA with *Pvu*II, electrophoresing through a 0.8% agarose gel, transferring to Zeta-Probe (Bio-Rad) membrane, and probing with a 9.1-kb mtDNA-specific probe. The probe was prepared by PCR amplification of total human DNA corresponding to positions 3305 and 12413 of the mitochondrial genome. The fragment was labeled with 5 μCi (1 μCi = 37 kBq) of [α-^{32}P]dCTP by the random primer method (Boehringer Mannheim).

Oxygen Consumption. Six cybrid clones from each fusion were grown to confluence in 75-cm^2 flasks. The cells were fed with fresh medium 1 day prior to testing. The flasks were trypsinized, cells were counted, and 2.5×10^6 cells were resuspended in 3 ml of DMEM lacking glucose but supplemented with 5% FBS and 100 μg/ml sodium pyruvate (14). Two samples were prepared from each flask and each cell line was tested three or more independent times for a minimum of six readings per cybrid clone. Oxygen consumption readings were performed as suggested by the manufacturer (Yellow Springs Instruments model 5300).

Mitochondrial Protein Synthesis. Mitochondrial protein synthesis was determined by pulse-labeling cell cultures in the presence of emetine as described by Chomyn (16). Cells were grown to confluence in 60-mm^2 dishes treated with 100 μg/ml emetine for 4 min, followed by pulse label with 300 μCi of [^{35}S]methionine/[^{35}S]cysteine (EXPRE^{35}S^{35}S; DuPont/NEN) for 30 min, then immediately harvested. Sample aliquots of 45 μg of total protein were resolved by electrophoresis on a 15–20% concave exponential gradient polyacrylamide gel (16). The gel was stained with Coomassie brilliant blue, fixed in a methanol/acetic acid/water solution (30%/10%/60%, vol/vol), and treated with Flouro-hance (Research Products International) prior to drying of the gel and exposure to an x-ray film at −80°C.

RESULTS

Following fusion between the human ρ^0 cells and primate cytoplasts, we found that common chimpanzee, pigmy chimpanzee, and gorilla mtDNA could functionally replace human mtDNA, generating transmitochondrial cell lines (hereafter referred to as xenomitochondrial cybrids) able to grow under selection for oxidative phosphorylation function (i.e., medium lacking uridine). Uridine-independent clones were not obtained from fusions between human ρ^0 cells and cytoplasts from lemur, species representing Old-World monkey, New-World monkey, or orangutan (Fig. 1). Most mtDNA donor cells (except common chimpanzee and green monkey) were primary fibroblasts which enucleated at identical efficiencies. All enucleated cells were inspected before fusions and all of them showed the typical morphology of cytoplasts. The number of cytoplasts per plate was also similar for the different cell lines. Successful fusions (with chimpanzee and gorilla cytoplasts) gave rise to approximately 200 independent clones, whereas fusions of the human ρ^0 cell with cytoplasts from less evolutionarily related primates did not produce a single clone, even after 45 days under selection. Unsuccessful fusions were repeated side-by-side with fusions using gorilla cytoplasts and, consistently, only fusion with gorilla cytoplasts yielded uridine-independent clones.

Although cell lines with very low respiratory activity can still grow in the absence of uridine (17–19), it was possible that some mtDNAs would restore cellular respiration to levels that would not allow for growth in a stringent selective medium. Therefore, we attempted to isolate xenomitochondrial cybrids from the fusion between the human ρ^0 cells and orangutan cytoplasts by reducing the stringency of the selection procedure. This was accomplished by feeding the fusion products every other day with a medium containing 10% nondialyzed FBS, as the low uridine levels in the serum reduce the dependence of the cell lines on oxidative phosphorylation for growth. Several colonies were isolated from this fusion, but because of irregular morphology and cells proliferating only immediately after being fed, it became clear that oxidative phosphorylation was not restored to any significant level. Southern blot analysis performed on DNA extracted from putative human–orangutan xenomitochondrial cybrids confirmed that the DNA from these cells did not contain mtDNA and therefore were not real cybrids, but rather human ρ^0 cells surviving on the little nutrients provided by the nondialyzed FBS contained in the media (not shown).

Six to ten proliferating clones from each productive fusion (of approximately 200 clones per fusion) with chimpanzees and gorilla cytoplasts were analyzed by Southern blotting to determine the origin of mtDNA and by PCR amplification to confirm the human origin of the nuclear DNA. Fig. 2 *Upper* shows the restriction pattern of mtDNA digested with *Pvu*II for 143B (human), primate parental fibroblast lines, and two representatives from each xenomitochondrial cybrid. As expected, the cybrids have the characteristic banding of their parental mtDNA, and not that of the human. The *Pvu*II digestion pattern was as predicted from the published mtDNA sequence for pigmy chimpanzee and gorilla, but common chimpanzee mtDNA had one polymorphic *Pvu*II site. To ensure that the parental cell lines were in fact from the primate species they were supposed to be, we PCR-amplified the promoter region from DNA extracted from the parental

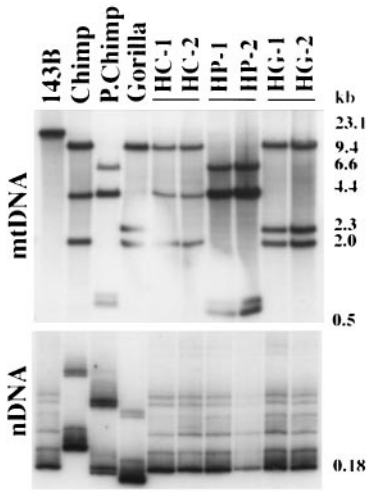

FIG. 2. Characterization of xenomitochondrial cybrids. Total DNA purified from xenomitochondrial cybrids and parental cell lines was digested with *Pvu*II and analyzed by Southern blotting using a human mtDNA-specific probe (*Upper*) and used as templates for a PCR using primers flanking the myotonic dystrophy polymorphic repeat (*Lower*). The radioactive PCR products were separated by 6% PAGE and exposed to an x-ray film. The figure shows that the xenomitochondrial cybrids having common chimpanzee (HC), pigmy chimpanzee (HP), and gorilla (HG) mtDNAs have a human nuclear background. 143B is a human osteosarcoma cell line. Positions of molecular weight markers are shown on the right.

common chimpanzee, pigmy chimpanzee, gorilla, orangutan, and green monkey cells. These PCR fragments were cloned into pCRII vector and sequenced. With the exception of a few polymorphic sites, the sequence analysis confirmed the identity of the parental lines as the correct primate species. A polymorphic marker on human chromosome 19 confirmed that the cybrids contained a human nuclear background (Fig. 2 *Lower*). The mtDNA levels were estimated to be similar for all cell lines on the basis of the intensity of the mtDNA hybridizing signal relative to the ethidium bromide staining of total DNA loaded in the gel.

Although the xenomitochondrial cybrids were able to survive in selective medium, their oxidative phosphorylation system could be seriously impaired. Cellular respiration was assessed by comparing oxygen consumption rates of the xenomitochondrial cybrids to the human 143B cell line. Fig. 3*A* demonstrates that the average oxygen consumption of all three xenomitochondrial cybrid groups was comparable to that of the 143B human cell line. The average oxygen consumption values for the xenomitochondrial cybrids for common chimpanzee, pigmy chimpanzee, and gorilla mtDNA were decreased, on average, by 20%, 34%, and 27%, respectively, compared with the parental human 143B line.

To ensure that the xenomitochondrial cybrids were producing all mitochondrially synthesized polypeptides at normal levels, cells were pulse-labeled with [^{35}S]methionine/[^{35}S]cysteine in the presence of emetine (an inhibitor of cytoplasmic protein synthesis). Mitochondrial polypeptides were resolved by electrophoresis in a 15–20% exponential gradient polyacrylamide gel and compared with an identically labeled human 143B cell line. All six representative xenomitochondrial cybrids contained comparable levels of characteristic bands representing mitochondrially synthesized proteins (Fig. 3*B*). We observed variable gel migration of many polypeptides in the xenomitochondrial cybrids, reflecting not size variations, but rather differences in amino acid composition. These same aberrant migrations were also observed in mitochondrial translation products of the parental primate cell lines (not shown).

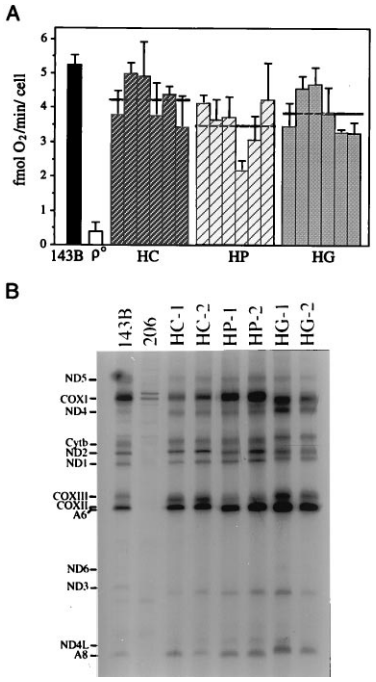

FIG. 3. Mitochondrial function in xenomitochondrial cybrids. (*A*) Oxygen consumption rates of six xenomitochondrial cybrids from each productive fusion and the human 143B and 143B/206 (ρ^0) cell lines. Oxygen consumption values were measured with a Clark-type electrode on 2.5×10^6 cells in 3 ml of medium as described (14, 19). Each xenomitochondrial cybrid and human cell line was tested a minimum of six times. Error bars represent the standard deviation of the individual cell lines. Horizontal bars represent the average consumption rate of the combined "same species" xenomitochondrial cell. (*B*) Autoradiogram of a concave exponential gradient (15–20%) gel representing the resolution of the mitochondrially synthesized proteins. Cells were pulse-labeled with [^{35}S]methionine/[^{35}S]cysteine for 30 min in the presence of emetine, a cytoplasmic protein synthesis inhibitor. Cells were solubilized in SDS and subjected to PAGE as described (16). Individual mitochondrially synthesized polypeptides are identified on the left. Xenomitochondrial cybrids: HC, human/common chimpanzee; HP, human/pigmy chimpanzee; HG, human/gorilla.

DISCUSSION

Stable intergenus mtDNA transfer has not been previously described, and only a few examples of interspecific mtDNA transfer exist. Niki and Matsuura (20) described repopulating a *Drosophila* species with the mtDNA from a closely related species. Beale and Knowles also succeeded in transferring mtDNA between closely related species of *Paramecium* (21). Mitochondrial DNA transfer by natural hybridization has been identified between species of hybridogenetic frogs (22) and between deer species which underwent successive gender-biased backcrosses (23). Interspecies somatic hybrids between mouse and hamster, mouse and rat, and mouse and human have been obtained and studied by different groups, but in these cases the maintenance of mitochondrial genomes (usually from only one species) was associated with the presence of a complete set of cognate chromosomes (4–8). Our results suggest that productive nuclear–mitochondrial DNA interactions can, in the case of primates, occur not only between different species but also between different genera, up to approximately 8–18 million years after species radiation. Mitochondrial genomes from species that diverged from humans 18 million years ago or longer were not able to functionally replace human mtDNA. It is worth noting that although there is controversy in the exact evolutionary distance between these primates, it is well accepted that humans are more closely related to gorilla and chimpanzees than they are to orangutan

Table 1. Sequence variation of mtDNA and mtDNA-encoded polypeptides in Hominoidea

Comparison	Nucleotide differences				Amino acid differences*			
	Promoters†	12S rRNA	16S rRNA	tRNAs	I	III	IV	V
European–Japanese	0	3	3	0	0	0	0	0
European–African	0	6	5	5	0	0	0	0
European–common chimp	2	35	81	59	102	22	19	16
European–pigmy chimp	2	30	95	57	99	24	17	15
European–gorilla	2	33	116	89	127	21	26	21
European–orangutan	4	81	164	155	265	38	54	54
Common chimp–pigmy chimp	2	11	37	31	60	15	5	12
Common chimp–gorilla	1	40	104	89	143	27	22	30
Common chimp–orangutan	6	87	157	153	285	41	53	59

The table, modified from Horai *et al*. (9), shows a comparison between human mtDNA (European, Japanese, and African) and nonhuman primate mtDNA (common chimpanzee, pigmy chimpanzee, gorilla, and orangutan). Data are derived from sequences deposited in GeneBank (9, 31).

*Amino acid substitutions that have been described as polymorphic in humans (32) were excluded from the analysis. Different subunits of the same oxidative phosphorylation complex (I–V) were pooled.

†Promoter regions (33) correspond to human mtDNA positions 396–411 (light-strand promoter) and 554–568 (major heavy-strand promoter).

(10, 11, 13). This abrupt change in compatibility suggests that one or a few sequence differences in the mtDNA from relatively distant primates would preclude the restoration of oxidative phosphorylation function and/or mtDNA maintenance. Previous work with transmitochondrial cybrids harboring pathogenic mtDNA mutations showed that transmitochondrial cybrids can grow in the absence of uridine (i.e., in selective medium) even when the functional level of oxidative phosphorylation is very low (17–19). Our initial expectation was that oxidative phosphorylation function would decrease gradually according to the evolutionary distance of the mtDNA donor species, and at a certain evolutionary distance, respiratory function would be so low that repopulation of human ρ^0 cells would not be possible. This scenario turned out not to be true, and the critical nuclear DNA–mtDNA interactions that have been affected in unsuccessful xenomitochondrial cybrids are not known. A potential candidate for a failed interaction would be primate promoter recognition by the nuclear-coded human mitochondrial RNA polymerase (2). Mitochondrial transcription is relatively simple, and to date, only one transcription factor (mtTFA) has been identified in mammalian mitochondria (24). Although mtTFA is highly promiscuous (25, 26), the mitochondrial RNA polymerase seems to be more specific to cognate promoters. The human mitochondrial RNA polymerase is not able to recognize murine or bovine promoters, but it can recognize the gorilla promoter (27). The lack of transcription from the L-strand promoter would preclude mtDNA replication, as the latter function depends on a mtDNA-coded RNA primer. This possibility is being investigated by *in vitro* transcription assays of different primate promoters. The alignment of the D-loop region of human, common chimpanzee, gorilla, and orangutan mtDNAs showed that the promoter regions and binding sites for mtTFA were highly conserved among these species. The conserved sequence box I (CSB I) also showed a high degree of conservation, but CSB II and CSB III were barely distinguishable in gorilla and orangutan D-loop sequences. The function of these evolutionary conserved sequences is obscure, but CSB II has been proposed to participate in the formation of an RNA replication primer (28). Although differences in the D-loop could explain our results, any of several other mtDNA polymorphisms in rRNA, tRNA, or protein coding genes could be responsible for the incompatibility with nuclear-encoded factors from another species. This model system may provide a new tool for approaching the pathogenesis of mitochondrial disorders associated with a severe depletion of mtDNA (29), as it may lead to the identification of critical interactions necessary for mtDNA maintenance.

Respiration of xenomitochondrial cybrids was slightly reduced compared with the parental human 143B cell line. However, human–human transmitochondrial cybrids also have a variable respiratory capacity, with some cell lines showing a much lower oxygen consumption than the ones observed with xenomitochondrial cybrids (14). None of the polymorphisms in chimpanzees or gorilla seems to affect cellular respiration significantly in a human nuclear background. However, we cannot rule out a mild effect of some of these polymorphisms in oxidative phosphorylation function. In fact, Villani and Attardi (30) recently showed that approximately 25% of cytochrome-*c* oxidase activity needed to be inhibited to affect oxygen consumption of 143B cells. Further detailed studies of individual oxidative phosphorylation complexes will be necessary to determine potential kinetic changes in enzymic activity. The normal mitochondrial protein synthesis did demonstrate unequivocally that polymorphisms in rRNA, tRNA, and promoter regions are functionally "neutral." The availability of xenomitochondrial cybrids harboring human nuclear DNA and mtDNA from common chimpanzee, pigmy chimpanzee, and gorilla allows us to expand the functional database of "human mtDNA" polymorphisms by more than 20-fold. Table 1 is modified and expanded from the work of Horai *et al.* (9) and shows the number of mtDNA polymorphisms between apes and humans. Only amino acid changes that have not been reported as polymorphic in humans (ref. 32, also available at http://www.gen.emory.edu/mitomap.html) were included in the table.

The high levels of polymorphisms, and the observed variability in electrophoretic migration between several mtDNA-encoded polypeptides in the cybrids, also provide a valuable set of markers for somatic cell experimentation. In addition, these cellular systems may also provide important clues to the nature of critical nuclear-mtDNA interactions necessary for oxidative phosphorylation function and mtDNA maintenance.

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