Fast Adaptive Coevolution of Nuclear and Mitochondrial Subunits of ATP Synthetase in Orangutan

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Nuclear and mitochondrial genomes have to work in concert to generate a functional oxidative phosphorylation (OXPHOS) system. We have previously shown that we could restore partial OXPHOS function when chimpanzee or gorilla mitochondrial DNA (mtDNA) were introduced into human cells lacking mtDNA. However, we were unable to maintain orangutan mitochondrial DNA in a human cell. We have now produced chimpanzee, gorilla, orangutan, and baboon cells lacking mtDNA and attempted to introduce mtDNA from different apes into them. Surprisingly, we were able to maintain human mtDNA in an orangutan nuclear background, even though these cells showed severe OXPHOS abnormalities, including a complete absence of assembled ATP synthetase. Phylogenetic analysis of complex V mtDNA-encoded subunits showed that they are among the most evolutionarily divergent components of the mitochondrial genome between orangutan and the other apes. Our studies showed that adaptive coevolution of nuclear and mitochondrial components in apes can be fast and accelerate in recent branches of anthropoid primates.

Introduction

The mitochondrial DNA (mtDNA) encodes for 13 polypeptides that interact with a large number of nuclear-encoded polypeptides to form a functional oxidative phosphorylation (OXPHOS) system. The nuclear genome also provides the machinery to synthesize and to assemble mtDNA-coded proteins with the nuclear-coded components of OXPHOS complexes I, III, IV, and V. Because of these close interactions, the mitochondrial and nuclear genes associated with OXPHOS undergo adaptive coevolution. Analysis of the substitution rate in protein-encoding mitochondrial genes showed that although the rate of synonym substitution is uniform, the rate of nonsynonym changes differs considerably among such genes, depending on functional constraints. Adaptive coevolution of OXPHOS components has been reviewed in details recently (Grossman et al. 2001).

Adaptive coevolution of OXPHOS components has been proposed to have an important role in the adaptation to larger brains (Goldberg et al. 2003) or to colder climates (Ruiu-Pesini et al. 2004). Natural selection will favor evolutionary coadaptation of interacting proteins that maintain or improve physiological functions. One example of the biological importance of intergenic coadaptation is the evidence that mtDNA interaction with the nuclear genome modifies cognition in mice (Roubertoux et al. 2003).

To study the functional importance of mtDNA and nuclear DNA coevolution, several mammalian models have been generated by transferring mtDNA of different species to a human or murine nuclear environments. Human xenomitochondrial cybrids were made by fusing human cells devoid of mtDNA (termed p0 cells) with enucleated cells (cytoplasts) from different species. Human cells harboring mtDNA from chimpanzee and gorilla were viable and had a functional OXPHOS (Kenyon and Moraes 1997). These human xenomitochondrial cybrids had a specific partial defect in complex I because of nuclear-mitochondrial incompatibilities (Barrientos, Kenyon, and Moraes 1998). Orangutan mtDNA, which diverged earlier than chimpanzee and gorilla from humans was not able to functionally replace human mtDNA (Kenyon and Moraes 1997). Mus musculus domesticus cybrids with several species of mouse mtDNA or rat (Rattus norvegicus) mtDNA have been described (Dey, Barrientos, and Moraes 2000; McKenzie et al. 2003). Mouse xenomitochondrial cybrids harboring rat mtDNA had a defect in oxidative phosphorylation, with reduced activities of multiple OXPHOS complexes (Dey, Barrientos, and Moraes 2000; McKenzie and Trounce 2000; McKenzie et al. 2003).

In this study, we have developed several novel primate p0 lines and attempted to repopulate them with mtDNA from various other primate species. The results were surprising, indicating that there is some directionality in specific nuclear-mitochondrial interactions. These studies also unveiled a fast adaptive coevolution of complex V subunits in orangutan.

Material and Methods

Cell Lines and Culture Conditions

Primary fibroblasts from pygmy chimpanzee (Pan paniscus), gorilla (Gorilla gorilla), and orangutan (Pongo pygmaeus) were obtained from the Coriell Institute for Medical Research Repository. Primary fibroblasts from rhesus macaque (Macaca mulatta) and baboon (Papio hamadryas) fibroblasts were obtained from surgical discarded skin material. Human fibroblast (TEX) was a skin-derived primary fibroblast cell line. All primary cells were infected with a retrovirus containing hTERT (human telomerase) gene and a retrovirus containing the adenovirus E6/E7 genes to extend their life span in culture (Lochmuller, Johns, and Shoubridge 1999).

Cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 µg/ml sodium pyruvate, and 50 µg/ml of uridine. Galactose medium contained 5.5 mM galactose, in no-glucose Dulbecco’s modified Eagle’s medium supplemented with 5% dialyzed fetal bovine serum and 100 µg/ml sodium
pyruvate. Selective medium without uridine was prepared by supplementing high-glucose Dulbecco’s modified Eagle’s medium with 10% dialyzed fetal bovine serum and 100 µg/ml sodium pyruvate.

Generation of Primate ρ0 Cell Lines

To generate derivative ρ0 cell lines (devoid of mtDNA), chimpanzee fibroblasts were grown in the presence of 150 ng/ml ethidium bromide for 4 months. Gorilla and baboon fibroblasts were grown for 3 months in the same conditions. Orangutan and rhesus macaque cell lines were grown in the presence of 500 ng/ml ethidium bromide for 3 and 4 months, respectively. Total depletion of mtDNA after treatment was assessed by Southern blot and the inability of the cell lines to grow in media lacking uridine (Moraes, Dey, and Barrientos 2001) or where glucose was replaced by galactose (Robinson et al. 1992).

Cybrid Fusions

To generate xenomitochondrial cybrids, mitochondrial donor cell lines were chemically enucleated by actinomycin D treatment and fused with the correspondent ρ0 cell line using polyethylene glycol (PEG) as described (Moraes, Dey, and Barrientos 2001). Briefly, 0.3 × 106 cells were plated in 35 mm dishes, treated with actinomycin D for 15 hours (Human TEX, 0.5 µg/ml; orangutan, 1 µg/ml; rhesus macaque 1.5 µg/ml), and fused with ρ0 cells with PEG 1,500 (60 s). Fused cells were grown 24 hours in complete medium and then trypsinized and plated at low density on 10 mm dishes with selective medium (without uridine). Individual clones were isolated by the cloning ring method.

Southern Blots

The mitochondrial DNA haplotype of xenomitochondrial cell lines was determined by Southern blots. Total DNA was digested with PvuII in the cases of chimpanzee/human and gorilla/human cybrids and the respective parental cell lines. MtDNA was detected in the blots using a probe corresponding to human mtDNA positions 14747 to 15000. In the case of orangutan/human cybrids, total DNA was digested with KpnI, and mtDNA was detected in the blots using a probe corresponding to human mtDNA positions 4120 to 4530. The probe was labeled with 5 µCi of [α-32P]dCTP by the random primer method (Roche, Burlington, NC).

Nuclear Markers

Human microsatellite markers have been used for analysis of genetic variation in apes (Coote and Bruford 1996). Nuclear markers were obtained from Research Genetics/Invitrogen (Carlsbad, Calif.). The loci with polymorphic tetranucleotide repeats used were: D3S2427, D14S587, D17S1290, D9S934, and DXS6797-F. Total DNA was PCR amplified with a [32P]-labeled oligonucleotide. PCR products were denatured at 80°C and resolved by electrophoresis in a 6% PAGE, 7 M urea sequencing gels. Gels were dried and exposed to an X-ray film at −80°C.

Interspecies Comparative Genomic Hybridization

Interspecies comparative genomic hybridization (iCGH) was employed to differentiate orangutan from human chromosomes (Barrientos et al. 2000). Approximately 400 ng each of genomic orangutan DNA (digoxigenin-dUTP labeled) and human genomic DNA (biotin-dUTP labeled) were ethanol precipitated. Hybridization in situ to metaphase preparations of human, orangutan, or xenomitochondrial cybrids cell lines was performed for 72 hours at 37°C. Posthybridization washes included 3 × 5 minutes in 0.1×SSC, 60°C. Biotinylated probe was detected by avidin-Cy3, digoxigenin-labeled probe by sheep antidigoxigenin FITC. Twenty-four–color M-FISH karyotyping for chromosome identification and microscopy was performed as described (Muller, Neusser, and Wienberg 2002).

Growth Curves

To determine rate of cell growth in glucose or galactose containing medium, 20,000 cells (when grown in glucose), or 30,000 cells (when grown in galactose) were plated in triplicates on 24-well plates. Cells were trypsinized and counted every 24 hours on a Z1 Coulter Cell Counter (Beckman Coulter, Fullerton, Calif.). Medium was replaced every other day during the experiment.

Oxygen Consumption

Oxygen consumption was measured polarographically in digitonin-permeabilized cells with a Clark oxygen electrode (Hansatech Instruments, Norfolk, UK), as described (Barrientos, Kenyon, and Moraes 1998). Cells were resuspended at 4 × 106 cells/ml in 500 µl of respiration buffer (20 mM HEPES, pH 7.1, 250 mM sucrose, 10 mM MgCl2, 2 mM potassium phosphate, and 1 mM ADP). Digitonin was added from a 1% solution to 8 µl of mitochondrial suspensions, and inhibitors were added for each respiratory complex (Villani and Attardi 1997). Sodium malate and glutamate 5 mM were used as complex I substrates, rotenone (100 nM) was used as a specific inhibitor of complex I. Sodium succinate and glyceral 3-phosphate (5 mM) were used as complex II+III substrates, and antimycin A (20 nM) was used as an inhibitor of complex III. Ascorbate (10 mM), and N,N',N'-tetramethyl-p-phenylenediamine (TMPD) (0.2 mM) were added as complex IV substrates, and potassium cyanide (1 mM) was used to inhibit complex IV.

Mitochondrial Protein Synthesis

Mitochondrial protein synthesis was determined by pulse-labeling cell cultures in the presence of emetine as described by Chomyn (1996). Cells were grown to confluence in 60-mm dishes treated with 100 µg/ml emetine for 4 minutes, pulse labeled with 300 µCi of [35S] methionine/[35S] cysteine (Easy Tag EXPRESS, NEN/PerkinElmer, Boston, Mass.) for 60 minutes, and immediately harvested. Aliquots of 45 µg of total protein were resolved by electrophoresis on a 15% polyacrylamide gel. The gel was stained with Coomassie brilliant blue, fixed in methanol/acetic acid/water
solution (30%, 10%, 60%) and treated with Enhance (NEN/PerkinElmer), dried, and exposed to an X-ray film at −80°C.

Blue Native-PAGE

Blue native electrophoresis of respiratory complexes was performed as described (Nijtmans, Henderson, and Holt 2002). Cell pellets were resuspended in PBS to a final concentration of 5.0 mg/ml, permeabilized by incubation with 1 volume of 8 mg/ml digitonin, 10 minutes on ice. Samples were diluted seven times and centrifuged 10 minutes at 10,000 × g, and the pellets were resuspended in buffer (1.5 M aminocaproic acid, 50 mM Bis-Tris, pH 7.0). Respiratory complexes were solubilized adding 10% lauryl maltoside (to a final concentration 10 mg/ml) and incubating 30 minutes on ice. Insolubilized material was removed by centrifugation at 20,000 × g for 30 minutes at 4°C. Five percent Coomassie brilliant blue G in 100 mM Bis-Tris, 500 mM aminocaproic acid, pH 7.0, was added to the supernatants to a final concentration of 2.5 mg/ml. Eighty micrograms of protein were separated in 5% to 13% blue native gradient gels (Nijtmans, Henderson, and Holt 2002). Respiratory complexes were detected in the blots, with specific antibodies obtained from Molecular Probes (Eugene, Ore.) directed against complex I, NDFS3; complex II, SDH (Fp); complex III, core 2 and iron sulfur protein; complex IV, COXI; and complex V, ATPaseβ.

Phylogenetic Analysis

Phylogenetic comparison of mitochondrial proteins in different primates was performed by ClustalW using MegAlign version 5.5 (DNASTAR Inc.).

Results

Nuclear-Mitochondrial Interactions in Primate Xenomitochondrial Cybrids

To test for potential disturbances in nuclear-mitochondrial communication elicited by small evolutionary changes, we attempted to produce primate xenomitochondrial cybrid cells. Initially, we prepared immortalized, mtDNA-free fibroblast lines from pygmy chimpanzee (Pan paniscus), gorilla (Gorilla gorilla), orangutan (Pongo pygmaeus), rhesus macaque (Macaca mulatta), and baboon (Papio hamadryas). As described in Material and Methods, growing the cells in ethidium bromide led to loss of mtDNA in these cells. The absence of mtDNA was confirmed by the inability of the cells in ethidium bromide led to loss of mtDNA in these cells. The absence of mtDNA was confirmed by the inability of the cells to grow in galactose and by the acquired auxotrophy for uridine (Moraes, Dey, and Barrientos 2001).

Mitochondrial-less cells, also know as ρ0 cells, were used as nuclear donors for fusion experiments. To introduce mtDNA from different species, we used a novel procedure that takes advantage of the DNA replication-toxic effect of actinomycin D as a chemical enucleator (Bayona-Bafaluy, Manfredi, and Moraes 2003). Actinomycin D–treated cells were PEG-fused to ρ0 cells, as described in Materials and Methods. Fusion products were selected in the absence of uridine. Table 1 summarizes the results of the selection. As expected, we were able to isolate uridine-independent clones having chimpanzee nucleus and human mtDNA as well as gorilla nucleus and human mtDNA. However, contrary to our expectations, we also obtained cells with orangutan nucleus and human mtDNA, albeit at a lower number than with the former two nuclear donors (table 1). We also had the chance to test whether the mtDNA from Old World monkeys (rhesus macaque or baboon) could be replaced by an ape mtDNA (human or orangutan) or by each other’s mtDNA. We were unable to rescue clones in the selective medium for OXPHOS function when either rhesus macaque or baboon were used as nuclear donors and any of the catarrhine’s mtDNA was used.

To ensure that the apes’ fusion products were indeed transmitochondrial cybrids, we characterized their nuclear and mitochondrial genomes using polymorphic markers (fig. 1A–C). We found that three of 14 chimp/human fusion products were hybrids, whereas all gorilla/human fusion products analyzed were true xenomitochondrial cybrids. Three of eight orangutan/human fusion products were hybrids, whereas the remaining five were xenomitochondrial cybrids. Because we had not been able to obtain cells with the human nucleus and orangutan mtDNA in a previous study (Kenyon and Moraes 1997), we performed additional experiments to ensure that these cells were true cybrids. Interspecies comparative genomic hybridization (iCGH) with differentially labeled human and orangutan total-genomic DNA was performed under identical experimental conditions for two orangutan/human cybrids and human and orangutan controls (fig. 1D–G). This method allowed the discrimination of human and orangutan chromosomes present in the cybrids. Analyses of 20 metaphases and 50 interphase nuclei for each of two orangutan/human clones confirmed that no human centromeres (as an indicator for human chromosomes) were retained. Both clones showed only orangutan centromeres (fig. 1F and G). The orangutan/human clone 3 had a higher rate of polyploidy (over 90% of cells) compared with clone 2 (20% to 30%). Twenty-four–color M-FISH karyotyping performed for clone 2 showed the presence of up to four nonrecurrent translocations, reflecting a low but detectable level of genomic instability (not shown).

![Table 1](image-url)

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<th>mtDNA Donor</th>
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<th>Orangutan</th>
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Note.—Cell lines devoid of mtDNA (ρ0 lines, nuclear donor) were fused with chemically enucleated fibroblasts from different catarrhine primates (mtDNA donor). After fusion, cells were grown in a weak selective medium (no uridine). The relative number of growing clones is expressed as “ + ” signs. Fusions of 5 × 106 human cytoplast donor with 107 chimpanzee ρ0 donor give rise to 150 to 200 uridine independent clones.

Orangutan/Human Xenomitochondrial Cybrids Have Severe OXPHOS Defects

The genetic characterization ensured that human mtDNA could be maintained by the orangutan nucleus.
We expanded the selected clones and characterized their OXPHOS system at the biochemical and molecular levels. OXPHOS function was initially assessed by the ability of clones to grow in galactose medium. Galactose cannot be metabolized fast enough for exclusive fermentative growth, and it is unsuitable to support growth of cells with severe OXPHOS deficiencies (Robinson et al. 1992). Figure 2A shows that chimp/human and gorilla/human cybrids were able to grow in galactose, whereas human/orangutan cybrids were not. Growth in glucose was partially impaired when compared with the parental orangutan cells (approximately 35% reduced, \(P < 0.02\)). OXPHOS activity was also studied by measuring oxygen consumption in permeabilized cells. We found a partial but significant defect in ascorbate/TMPD respiration in chimp/human cybrids, indicating small incompatibilities in cytochrome oxidase subunits of these extremely close evolutionarily related species (fig. 2B). We could not detect any significant functional defect in ascorbate/TMPD respiration in chimp/human cybrids, indicating small incompatibilities in cytochrome oxidase subunits of these extremely close evolutionarily related species (fig. 2B). We could not detect any significant functional defect in ascorbate/TMPD respiration in chimp/human cybrids, indicating small incompatibilities in cytochrome oxidase subunits of these extremely close evolutionarily related species (fig. 2B).

These data indicates a severe complex I functional defect in orangutan/human cybrids. Complexes III and IV still could be affected in the orangutan/human cybrids, but to a level that is below the threshold to significantly change complex II–driven respiration, which also involves complexes III and IV (Villani and Attardi 1997).

**Complex V Cannot Be Assembled in Orangutan/Human Xenomitochondrial Cybrids**

Mitochondrial protein synthesis depends on a large number of both nuclear-coded and mtDNA-coded factors. Therefore, we analyzed the function of mitochondrial ribosomes by labeling cells with \([^{35}S]\)-methionine in the presence of the cytoplasmic protein synthesis inhibitor emetine (Chomyn 1996). Cell lysates were analyzed by SDS/PAGE and fluorography (fig. 3A). Because all xenomitochondrial cybrids had the human mtDNA, we looked for human-type bands that could be altered in a nuclear background–specific manner. Most abnormalities were found in orangutan/human cybrids, where overall mitochondrial protein synthesis was attenuated, but because of the non-quantitative nature of the experiment, defects are better observed when the levels of some specific bands were reduced. A band migrating in the region of COX II, COX III, and ATPase6 was undetectable in orangutan/human xenomitochondrial cybrids (arrow on figure 3A). The number of methionines in these polypeptides between orangutan and human is not markedly different (between nine and 12). Next, we analyzed for the steady-state levels of fully assembled OXPHOS complexes by blue native gel

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**FIG. 1.—Genetic characterization of interspecific ape transmitochondrial cybrids.** (A)–(C) The characterization of mtDNA (upper part) and nuclear (lower part) markers in xenomitochondrial cybrids. Informative human nuclear tetranucleotide polymorphic markers were used to characterize the nuclear background. (D)–(G) iCGH hybridization with a mix of human-specific and orangutan-specific centromeric probes. The orangutan/human xenomitochondrial cybrids (F and G) did not show the presence of human chromosomes.
electrophoresis (BN-PAGE), followed by Western blots. We used antibodies against representative subunits of the different complexes (fig. 3B). Again, most of the detectable defects were present in the orangutan/human xenomitochondrial cybrids. Steady-state levels of complexes I and IV were partially decreased, even though

![FIG. 3.—Mitochondrial protein synthesis and steady-state levels of OXPHOS complexes in ape xenomitochondrial cybrids. (A) A fluorogram of a SDS-PAGE of [35S]methionine-labeled cells. The cells were treated with emetine to inhibit cytosol protein synthesis. The mitochondrially synthesized polypeptides were tentatively assigned based on published references (Chomyn 1996). Because all xenomitochondrial cybrids have human mtDNA, the human cells control is the reference for the banding pattern in the cybrids. (B) The steady-state levels of fully assembled OXPHOS complexes. Complexes were separated by BN-PAGE and detected by Western blot using nuclear subunit–specific antibodies. All antibodies used reacted against the subunits from the different apes. Note the absence of assembled complex V in orangutan/human xenomitochondrial cybrids.](image)

![FIG. 2.—Biochemical defects of ape xenomitochondrial cybrids. (A) The growth characteristics of the parental cells as well as the xenomitochondrial cybrids. Growth in glucose was slightly decreased in orangutan/human cybrids, but these cells were unable to grow in galactose. (B)–(D) Oxygen consumption of permeabilized cells after the addition of different respiratory substrates. Pyruvate/malate donates electrons to complex I, succinate/G3P donates electrons to complex II, and ascorbate/TMPD donates electrons to complex IV. N = 4; error bars = S D. *P < 0.05; **P < 0.005. Statistical comparisons (t-test) were made between cybrids and parental cell lines with the same nuclear background.](image)
biochemically, only complex I was severely impaired. Even more striking was the complete absence of complex V in these cells, detected using an antibody directed against ATPβ. Because both parental orangutan and the orangutan/human cybrids have the same nuclear background, they have the same ATPβ, which have the same reactivity against the monoclonal antibody.

Analyses of the number of amino acids substitutions between mtDNA-coded polypeptides from the different apes showed that subunits of complex V have diverged quickly in orangutan when compared with the other apes. Phylogenetic trees illustrate the increased number of amino acid replacements in subunits of complex V in the orangutan lineage (fig. 4A). In fact, only three orangutan polypeptide sequences showed an increase in the rate of divergence followed by a relative rate decrease in more distant species: ND1, A6, and A8 [fig. 4B]. In addition, divergences between orangutan and human proteins were increased for ATP6 and ATP8 (21% and 42%, respectively) when compared with subunits for complexes I, III, and IV (fig. 4C).

Discussion

We have previously found that human nuclear genes could complement Pan and Gorilla mtDNA but not Pongo mtDNA (Kenyon and Moraes 1997). Based on these observations, the evolutionary distance that allowed for the maintenance of mtDNA in xenomitochondrial cybrids was estimated to be 10 MYA or less (Kenyon and Moraes 1997). This boundary was in agreement with subsequent studies on rodent xenomitochondrial cybrids (Dey, Barrientos, and Moraes 2000; McKenzie and Trounce 2000; McKenzie et al. 2003). These studies, as well as the present one, relied on the ability of fusion products to grow in the absence of uridine, a condition that precludes cells with no OXPHOS function to grow (e.g., ρ0 cells), but allows cells with very low OXPHOX function to grow (Hao and Moraes 1996). In the present study, the availability of different mtDNA-devoid cell lines (ρ0 lines) from several species of catarhine primates allowed us, for the first time, to test the directionality of this boundary. To our surprise, the orangutan nuclear background was able to maintain the human mtDNA, which pushed the boundary for a possible nuclear-mtDNA “compatibility” to 13 to 16 MYA (Arnason et al. 1996; Xu and Arnason 1996; Harris 2000). On the other hand, we could not observe complementation between rhesus macaque and baboon mtDNA, which have diverged approximately 12 MYA (Harris 2000). Oxidative phosphorylation was severely impaired in orangutan/human cybrids, but still, they were able to grow in a weakly selective medium (no uridine). To avoid a strong bias against clones with an OXPHOS defect, we removed the selective medium as soon as the clones were isolated (approximately 30 days after fusion). Because the efficiency of obtaining orangutan-human xenomitochondrial cybrids was relatively low, and because some of the clones were hybrid cells, we cannot rule out that orangutan-human clones had some orangutan chromosomes initially, but they were lost after extended time in culture. However, this scenario is unlikely, as the human/orangutan xenomitochondrial cybrids had a reduced growth, even in nonselective medium, and cells with higher OXPHOS capacity would have overwhelmed the defective ones. In any case, we could unambiguously demonstrate that they were true xenomitochondrial cybrids. We have attempted to produce human-orangutan xenomitochondrial cybrids (with human nucleus and orangutan mtDNA) using different approaches and selection schemes but were not able to obtain xenomitochondrial cybrids (Kenyon and Moraes 1997). Therefore, there is an directionality to this phenomenon, which could be explained by a more restrictive set of interactions when A1 contacts B2 than when A2 contacts B1 in an enzyme complex having nuclear-coded A subunits and mtDNA-coded B subunits.

Previous work showed that the maintenance of mtDNA in xenomitochondrial cybrids did not depend on OXPHOS function, but rather on competing molecular recognition cues (Moraes, Kenyon, and Hao 1999). However, technical limitations require that we apply some form of selection to obtain xenomitochondrial cybrids. Without selection, most of the cells after fusion would be the unfused parental ρ0 nuclear donor cells. The little OXPHOS function retained by the orangutan/human xenomitochondrial cybrids was enough to keep them growing, albeit poorly, in no-uridine medium. Subsequently, we showed that their growth in high-glucose medium was only slightly decreased, and they were unable to survive in galactose medium.

By studying hybrids of human and orangutan cells (i.e., with mixed nuclear backgrounds from both species) containing only orangutan mtDNA, previously we have shown a dominant negative effect specifically in complex IV assembly and activity (Barrientos et al. 2000). Based on what we know from the present work, the incompatibilities in complexes I and V were probably high enough to allow only orangutan nuclear-coded factors to assemble with the orangutan mtDNA-coded factors in these hybrids, whereas human nuclear-coded complex IV subunits could assemble with the orangutan mtDNA-coded counter parts and trigger a dominant negative defect in catalysis of COX. This effect on complex IV was smaller when the assembled enzyme was made of orangutan nuclear-coded subunits and human mtDNA-coded subunits, showing a directionality in the severity of misinteractions.

Complex I was affected by nuclear-mitochondrial incompatibilities in the orangutan/human xenomitochondrial
cybrids. There are seven mtDNA-coded subunits and close to 40 nuclear-coded subunits in complex I, which would make it more susceptible to evolutionary changes. This sensitivity of complex I to small evolutionary variations has been observed even in xenomitochondrial cybrids with closely related mtDNA, such as human, chimpanzee, and gorilla (Barrientos, Kenyon, and Moraes 1998). Still, there is some complex I assembly in orangutan/human cybrids, even though its activity was impaired. It is likely that less-than-optimal nuclear-mitochondrial interactions are affecting electron transfer in the assembled complex I of orangutan/human xenomitochondrial cybrids.

The undetectable levels of assembled complex V in the human/orangutan xenomitochondrial cybrids suggest a major disruption of interactions between nuclear-coded and mtDNA-coded components of the F1/F0 ATP synthetase. The reason for this is probably related to the fact that in orangutan, the two mtDNA-coded subunits of complex V had an accelerated rate in nonsynonym amino acids substitutions (fig. 4). The small subunit (A8) has only 67% identity between orangutan and other apes A8 polypeptides. This is an extraordinarily high divergence value for what is considered a conserved polypeptide among closely related primate species. It is also far higher than what is observed for subunits of other complexes (fig. 4B). A6 of orangutan and human have 82% identity, but this is still smaller than the identity of the remaining polypeptides coded by mtDNA. It is possible that the undetectable band in mitochondrial protein synthesis of orangutan/human cybrids was A6, which could be extremely unstable if not assembled.

With such a high degree of divergence observed in complex V subunits between orangutan and human mtDNA, it is expected that nuclear-coded protein partners had to adapt accordingly. Nuclear-mitochondrial coadaptive evolution is a well-known phenomenon, and it was confirmed by our functional approach. A typical example of adaptive coevolution can be seen in cytochrome oxidase genes of some primates (Grossman et al. 2001). COXII was a rapidly evolving gene in primates compared with rodents, a phenomenon that occurred during the radiation of the anthropoid primates (Adkins and Honeycutt 1994). The rate of evolution of COXII in anthropoid primates is similar to the rate observed for cytochrome c, indicating a possible coevolutionary pressure (Cann, Brown, and Wilson 1984). Phylogenetic analysis of COX IV and COXVIII also showed accelerated nonsynonym substitution rates in catarrhine and anthropoid evolution (Wu et al. 1997; Wildman et al. 2002; Goldberg et al. 2003). In complex III, subunits that interact with the rapidly evolving cytochrome c also underwent a faster rate of amino acid substitutions (Grossman et al. 2001). Although the reason for a faster evolving rate of some OXPHOS complex in primates is unclear, it has been suggested to be necessary for adapting to the energy-demanding neocortex of catarrhine (Goldberg et al. 2003).

We now show that adaptive coevolution of rapidly evolving mtDNA genes also occurred in recently diverged branches of anthropoid primates. Why would complex V undergo a faster evolution than other OXPHOS complexes in orangutan when compared with other apes? It has been recently suggested that variations in human mtDNA haplotypes helped in the adaptation of certain populations to cold weather (Ruiz-Pesini et al. 2004). It is possible that changes in complex V helped the orangutan adapt to climate conditions of its present habitat, the Indo-Malayan region. Even more intriguing, nuclear-mitochondrial interactions were shown to affect cognition in mice (Roubertoux et al. 2003). This observation, together with fast evolution of specific complexes, opens up tantalizing hypotheses related to adaptation to life styles (e.g., solitary versus groups and arboreal versus terrestrial). Whatever the selective pressure was, it is clear that to maintain an optimized OXPHOS function, the nuclear-coded and mtDNA-coded subunits of complex V had to coevolve fast in the Pongo genus.

Our findings expand previous observations made for OXPHOS complexes III and IV (Grossman et al. 2001) by showing adaptive coevolution of complexes I and V subunits in primates by a functional approach.

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**Literature Cited**


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