Rapid Nonsynonymous Evolution of the Iron-Sulfur Protein in Anthropoid Primates

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Cytochrome *c* (CYC) and 9 of the 13 subunits of cytochrome *c* oxidase (complex IV; COX) were previously shown to have accelerated rates of nonsynonymous substitution in anthropoid primates. Cytochrome *b*, the mtDNA encoded subunit of ubiquinol–cytochrome *c* reductase (complex III), also showed an accelerated nonsynonymous substitution rate in anthropoid primates but rate information about the nuclear encoded subunits of complex III has been lacking. We now report that phylogenetic and relative rates analysis of a nuclear encoded catalytically active subunit of complex III, the iron-sulfur protein (ISP), shows an accelerated rate of amino acid replacement similar to cytochrome *b*. Because both ISP and subunit 9, whose function is not directly related to electron transport, are produced by cleavage into two subunits of the initial translation product of a single gene, it is probable that these two subunits of complex III have essentially identical underlying rates of mutation. Nevertheless, we find that the catalytically active ISP has an accelerated rate of amino acid replacement in anthropoid primates whereas the catalytically inactive subunit 9 does not.

INTRODUCTION

Complex III (ubiquinol–cytochrome *c* reductase; bc_1 complex) of the mitochondrial electron transport chain (ETC) is composed of 11 subunits per monomer, of which three subunits—the mitochondrial-encoded cytochrome *b*, and the nuclear-encoded mature iron-sulfur protein and cytochrome c_1—are directly involved in electron transport. Movement of the flexible linker region of the mature iron-sulfur protein allows its iron-sulfur cluster to accept an electron from cytochrome *b*, and then to donate it to the cytochrome c_1 of the other monomer of the dimeric holoenzyme (Iwata *et al.*, 1998, 1999). The electron is then passed *via* the mobile electron carrier cytochrome *c* (CYC) to cytochrome *c* oxidase (complex IV; COX).

The translation product of the entire iron-sulfur protein gene must pass through the mitochondrial inner membrane, and therefore includes a mitochondrial targeting sequence in addition to the 196-amino acid mature iron-sulfur protein. The targeting sequence that is cleaved from the translated protein is usually 78 amino acids long in mammals, is not degraded, and instead becomes subunit 9 of complex III. Since both subunits originate from the same translation product, the mature iron-sulfur protein (hereafter called ISP) and subunit 9 have essentially identical underlying rates of mutation. In the assembled complex III, subunit 9 lies between the core I and core II subunits. Core I and II are homologs of the two mitochondrial processing peptidases, MPPβ and MPPα, respectively. Both core I and core II have been shown to exhibit some mitochondrial processing capability, which ceases when an equimolar amount of subunit 9 is added,

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suggesting that subunit 9 blocks their peptidase activity (Deng *et al.*, 2001).

In this study, we investigate the molecular evolution of ISP. We and others have found that anthropoid primates show accelerated rates of amino acid replacement during the evolution of CYC (Baba *et al.*, 1981; Evans and Scarpulla, 1988; Grossman *et al.*, 2001) and certain subunits of complexes III and IV of the electron transport chain: subunits I, II, IV, Va, VIb, VIc, VIIa, VIIc, and VIII of COX (Adkins and Honeycutt, 1994; Adkins *et al.*, 1996; Wu *et al.*, 1997; Schmidt *et al.*, 1999; Andrews and Easteal, 2000; Wu *et al.*, 2000; Osada *et al.*, 2002; Wildman *et al.*, 2002; Goldberg *et al.*, 2003; Doan, 2004; Doan *et al.*, 2004), and cytochrome *b* of complex III (Andrews *et al.*, 1998; McClellan and McCracken, 2001). Grossman *et al.* (2001) presented data showing that when the bovine and rodent lineages were compared to the human lineage, the rate of amino acid replacement in the human lineage was relatively high for the subunits related to the catalytic activity of complex III—cytochrome c_1, ISP, cytochrome *b*, and the hinge protein (which forms part of the CYC binding site)—whereas subunits unrelated to the catalytic activity, including subunit 9, showed minimal acceleration. We therefore hypothesized that ISP would exhibit accelerated rates of evolution specifically in anthropoid primates, whereas subunit 9 would show little or no acceleration. We tested this hypothesis by comparing the number of amino acid replacements in ISP with the number of replacements in subunit 9 in both anthropoids and non-anthropoids. The finding that the ISP has an accelerated rate of amino acid replacement in anthropoid primates, whereas subunit 9 does not, supported the hypothesis.

EXPERIMENTAL PROCEDURES

DNA and RNA Sequences and Samples

RNA for the brown lemur (*Eulemur fulvus*) was extracted from liver tissue obtained from the Duke University Primate Center using a phenol/chloroform method (Chomczynski and Sacchi, 1987) and the full-length cDNA was amplified by RACE-PCR (Frohmann, 1995). DNA for the other species sequenced was obtained from laboratory stock solutions. Exons I and II for the other species sampled were separately amplified from genomic DNA by PCR. PCR product purification, cloning, sequencing, and alignment were carried out as described (Goldberg *et al.*, 2003).

Subunit 9 is encoded by exon I and 20 bases of exon II. ISP is encoded by the rest of exon II. Exon I was difficult to amplify and its sequence was not obtained for tarsier, howler, marmoset, capuchin, saki, and titi monkey.

The species from which sequences were generated in this study, the gene region sequenced, and the GenBank accession numbers for these sequences are: *Gorilla gorilla* (gorilla), exon 1 AY387499, exon 2 AY387500; *Pan paniscus* (bonobo), exon 1 AY387495, exon 2 AY387496; *Pan troglodytes* (common chimpanzee), exon 1 AY387497, exon 2 AY387498; *Pongo pygmaeus* (Borneo orangutan), exon 1 AY387501, exon 2 AY387502; *Symphalangus syndactylus* (siamang), exon 1 AY387515, exon 2 AY387516; *Chlorocebus* sp. (vervet), exon 1 AY387505, exon 2 AY387506; *Colobus polykomos* (western black-and-white colobus), exon 1 AY387507, exon 2 AY387508; *Theropithecus gelada* (gelada baboon), exon 1 AY387503, exon 2 AY387504; *Alouatta belzebul* (red-handed howler monkey), exon 2 AY387519; *Aotus azarae* (owl monkey), exon 1 AY387509, exon 2 AY387510; *Callicebus donacophilus* (Bolivian gray titi monkey), exon 2 AY387521; *Callithrix jacchus* (common marmoset), exon 2 AY387518; *Cebus apella* (brown capuchin), exon 2 AY387517; *Lagothrix lagotricha* (woolly monkey), exon 1 AY387511, exon 2 AY387512; *Pithecia irrorata* (bald-faced saki), exon 2 AY387520; *Saimiri sciureus* (common squirrel monkey), exon 1 AY387513, exon 2 AY387514; *Tarsius syrichta* (Philippine tarsier), exon 2 AY387522; *Nycticebus coucang* (slow loris), exon 2 AY387524; *E. fulvus* (brown lemur), exons 1 and 2 AY387494; *Otolemur crassicaudatus* (brown greater galago), exon 2 AY387523. In addition, the following sequences already available in GenBank for the coding region of the entire ISP gene were used in the analysis: *Homo sapiens* (human) NM_006003.1, *Mus musculus* (mouse) NM_025710.1, *Rattus norvegicus* (rat) XM_214457.2, *Bos taurus* (cow) S58789, *Gallus gallus* (chicken) consensus of BU138160, BU110083, BU107795, BU116180, and BG713576; *Xenopus laevis* (African clawed frog) consensus of BJ036999, BU910964, BI444817, BC041528, BJ045243, BU906843, and BJ053512; and *Danio rerio* (zebrafish) consensus of BM037012, BQ078379, AW174657, and BQ131869.

Phylogenetic Analysis

Phylogenetic analysis was carried out as described (Goldberg *et al.*, 2003).

Rates of Evolutionary Change

Ancestral sequences were reconstructed using MrBayes 2.01 (Huelsenbeck and Ronquist, 2001), with

Table I. Relative Rates Test for ISP

Comparison			Nonsynonymous		Synonymous		Fourfold	
1	2	Outgroup	K_{nd}	p	K_s	p	K_{4f}	p
Human	Tarsier	Cow	0.031	0.010*	−0.061	0.332	−0.013	0.749
	Lemur	Cow	0.029	0.026*	−0.289	0.001*	−0.079	0.180
Siamang	Tarsier	Cow	0.030	0.012*	−0.069	0.271	−0.030	0.516
	Lemur	Cow	0.029	0.026*	−0.297	0.000*	−0.094	0.099
Colobus	Tarsier	Cow	0.035	0.004*	−0.058	0.358	−0.015	0.757
	Lemur	Cow	0.034	0.009*	−0.286	0.001*	−0.079	0.180
Gelada	Tarsier	Cow	0.041	0.002*	−0.083	0.168	−0.015	0.757
	Lemur	Cow	0.040	0.002*	−0.311	0.000*	−0.079	0.211
Sq. monkey	Tarsier	Cow	0.052	0.000*	−0.002	0.976	0.059	0.298
	Lemur	Cow	0.051	0.001*	−0.230	0.008*	0.000	1.000
Woolly monkey	Tarsier	Cow	0.039	0.003*	−0.013	0.842	0.031	0.582
	Lemur	Cow	0.038	0.004*	−0.241	0.007*	−0.033	0.617
Tarsier	Mouse	Cow	−0.006	0.503	−0.226	0.011*	−0.122	0.077
	Lemur	Cow	0.001	0.900	−0.228	0.009*	−0.089	0.150
Human	Colobus	Tarsier	−0.005	0.719	−0.020	0.757	0.002	0.857
Colobus	Squirrel	Tarsier	−0.022	0.197	−0.041	0.562	−0.013	0.238

Note. The taxa compared are in columns 1 and 2. K_{nd} is the nonsynonymous substitution rate difference. K_s is the synonymous substitution rate difference. K_{4f} is the fourfold degenerate transversion substitution rate difference. An asterisk indicates significance at the $p \leq .05$ level.

settings from the HKY85+ Γ (Hasegawa *et al.*, 1985) method determined to best fit the entire dataset by the hierarchical log-likelihood ratio test of the program Modeltest (Posada and Crandall, 1998). The gamma shape parameter was 0.4519. K_a/K_s (substitutions per nonsynonymous site/substitutions per synonymous site) ratios were found according to the method of Li (1993) as implemented by the FENS program (version 0.9b1.4) (De Koning *et al.*, 1998). Rates of nucleotide and amino acid change were calculated using divergence dates from Goodman *et al.* (2001) and Seiffert *et al.* (2003) for primates, Springer *et al.* (2003) for mouse and rat, and Hedges (2002) for murids and cow. Relative rates tests (Wu and Li, 1985; Muse and Weir, 1992) were performed on the basis of nucleotide divergences calculated with MEGA version 2.1 (Kumar *et al.*, 2001) at nonsynonymous and synonymous sites using the modified Nei–Gojobori method (Zhang and Gu, 1998), as well as for fourfold degenerate transversions using the Kimura two-parameter method (Kimura, 1980). Amino acid replacements were also calculated with MEGA version 2.1. These methods were carried out for both ISP and subunit 9 where appropriate.

RESULTS

Relative Rates Tests

Relative rates tests were performed on ISP and subunit 9 to compare the evolution of the two proteins. Results indicate that there is a statistically significant nonsynonymous substitution rate increase for ISP when the human lineage is compared to both the tarsier (the closest relative of anthropoids) and the brown lemur lineages, with cow as an outgroup (Table I). A similar pattern is evident in the relative rates of representative non-human anthropoids (Table I). The nonsynonymous substitution rate increase was similar with mouse and rat as outgroups. On the other hand, none of the rates of nonsynonymous substitution in subunit 9 showed a significant increase or decrease when the same anthropoid taxa were compared to lemur and cow (Table II).

Relative rates tests were also performed on synonymous sites and fourfold degenerate transversions of ISP to test whether the accelerated rate found might be a result of an overall acceleration of substitution rates. There was no statistically significant increase in anthropoids at these sites (Table I). There were cases of statistical significance for synonymous sites, but in those cases it was the brown lemur that showed a relative rate acceleration rather than the anthropoid primate involved in the comparison, suggesting that there may have been an independent increase in the underlying mutation rate in the brown lemur lineage.

Nonsynonymous and Synonymous Substitutions

K_a and K_s were calculated for ISP and are shown on a phylogenetic tree of the species analyzed (Fig. 1).

Table II. Relative Rates Test for Subunit 9

Comparison			Nonsynonymous		Synonymous		Fourfold	
1	2	Outgroup	K_{nd}	p	K_s	p	K_{4f}	p
Human	Lemur	Cow	−0.024	0.535	−0.241	0.066	−0.120	0.294
Siamang	Lemur	Cow	−0.024	0.535	−0.224	0.089	−0.142	0.200
Colobus	Lemur	Cow	−0.045	0.222	−0.243	0.064	−0.142	0.200
Gelada	Lemur	Cow	−0.024	0.569	−0.243	0.047*	−0.120	0.276
Sq. monkey	Lemur	Cow	−0.020	0.624	−0.225	0.099	−0.122	0.289
Woolly monkey	Lemur	Cow	0.006	0.897	−0.138	0.308	−0.036	0.780

Note. Column headings are as in Table I. In addition to the data shown, there were no significant variations in relative rates of nonsynonymous substitutions for any anthropoid in the dataset of this study when compared to brown lemur, with cow, mouse, or rat as outgroup.

The tree is consistent with previously published species trees (Goodman *et al.*, 1998; Madsen *et al.*, 2001; Murphy *et al.*, 2001), and it was found to be the most parsimonious tree when compared according to the method of Goodman *et al.* (1979) to the lowest-nucleotide-substitution-length tree inferred from the entire ISP gene.

Rates of substitution at nonsynonymous sites in the lineage leading to human form a pattern represented in Fig. 2. There is a low rate of nonsynonymous substitution in the stem of the primates, increasing in the stem of the haplorhines, and higher in the stems of the anthropoids, catarrhines, and apes and great apes. Then there is a relatively low rate in the remaining descent to human. In the human terminal lineage there are no nonsynonymous substitutions (Fig. 1).

Accelerated Rates of Amino Acid Replacement

ISP exhibits accelerated rates of change in amino acid replacement in anthropoids. The rate of replacement per year in the human lineage is 5.1 times the rate of replacement per year on the tarsier lineage (1.32 vs. 0.26 replacements per residue per billion years, respectively) and 3.7 times the rate of replacement per year in non-haplorhines (brown lemur, galago, slow loris, mouse, rat, and cow) taken together (0.36 replacements per residue per billion years) (Fig. 3).

Using a dataset restricted to those taxa for which sequences were obtained for both ISP and subunit 9, the evolution of the two subunits can be directly compared. ISP has 54 amino acid replacements in anthropoids, whereas subunit 9 has 19. In non-anthropoids (brown lemur, mouse, rat, and cow), ISP has 17 replacements and subunit 9 has 22. Comparison of the resulting ratios (54/19 and 17/22) demonstrates that the amino acid replacement rate in ISP in anthropoids is 3.7 times higher than expected from the variation in subunit 9. The quotient

of the comparison remains high (2.5) even when chicken and frog are added to the non-anthropoid group.

The portion of ISP located in the intermembrane space (aa 62–196) is largely invariant (74.6% absolutely conserved among all the taxa examined), whereas the transmembrane region (aa 26–62) is only 56% conserved. For the transmembrane region, the rate of replacement in anthropoids is 11.0 times higher than expected.

Specific Amino Acid Replacements

Most of the replacements in ISP in anthropoids are physicochemically conservative (Miyata *et al.*, 1979; Zhang, 2000). However, one radical change in the stem of the catarrhines in an otherwise absolutely conserved stretch of amino acids is K173L. Lys-173 is conserved not only in the non-catarrhine taxa included in this study, but also in *Drosophila*, maize, tobacco, potato, and *Rhodobacter capsulatus*.

Two amino acid changes next to the flexible linker domain occur in anthropoids: M71L in the stem of the apes, and S72A in the stem of the catarrhines. The M71L change is conservative, whereas S72A is radical (polar to nonpolar). Amino acid 72 in ISP is very close (within 4 Å) to amino acid 168 of cytochrome *b*, which changes from Phe in cow to Tyr in human (nonpolar to polar). This suggests molecular coevolution, in that the overall polarity of the two close amino acids is largely preserved by compensating changes.

DISCUSSION

We have shown accelerated rates of nonsynonymous substitution in ISP in anthropoid primates. Normally a K_a/K_s ratio greater than 1 is a test for positive selection
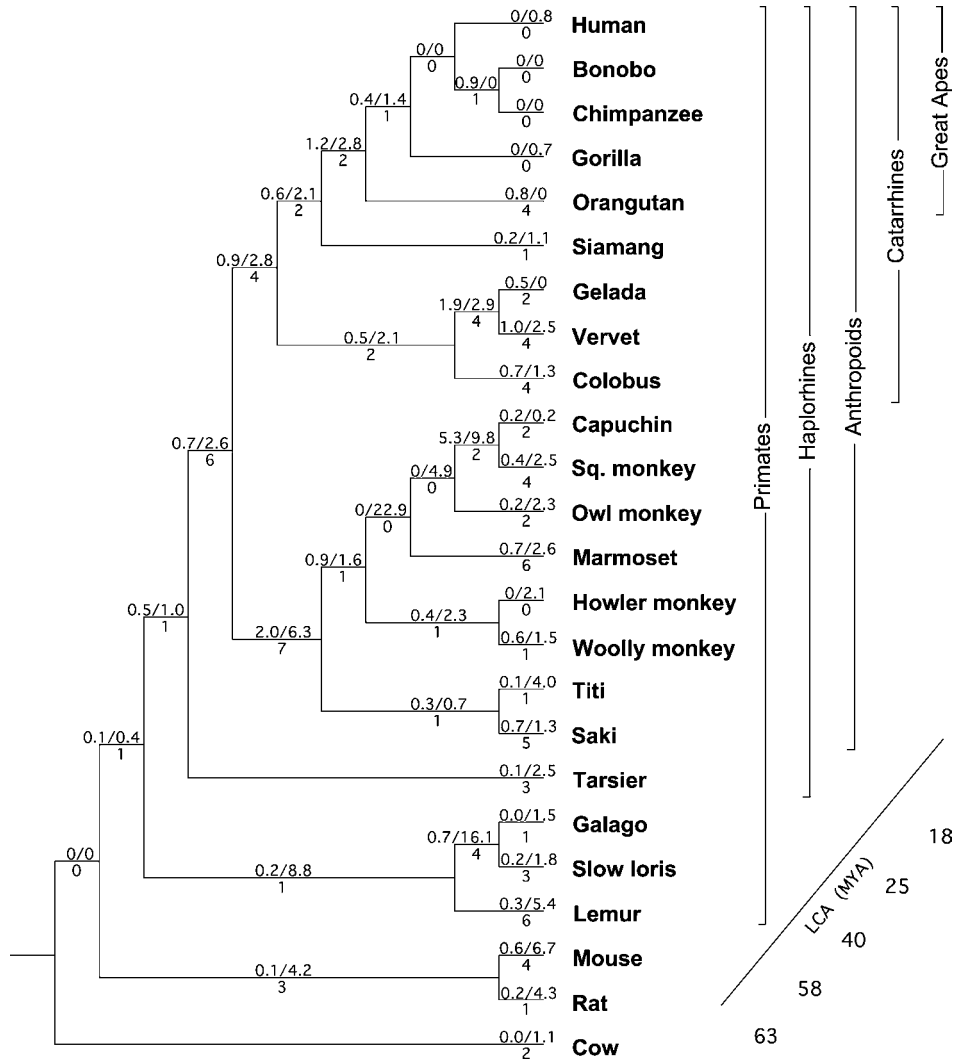
Fig. 1. Evolution of ISP in primates. Rates of K_a (nonsynonymous substitutions per nonsynonymous site) and K_s (synonymous substitutions per synonymous site), each per year $\times 10^{-9}$, are shown as a ratio above each branch of the tree. The number of amino acid replacements is shown below. LCA (MYA) is the time of the last common ancestor in millions of years for each of the listed taxonomic groups (e.g., the LCA of primates lived 63 MYA).

(Yang and Nielsen, 2002; Fay and Wu, 2003). However, none of the calculated ratios of K_a/K_s is greater than 1. Since the amino acid sequence of the mature ISP is 62.8% conserved among the taxa in our dataset, this test may not be sufficiently sensitive to discern positive selection. It has long been understood that in the evolution of proteins some residues are relatively invariant, and others more subject to replacement (Fitch and Markowitz, 1970; Miyamoto and Fitch, 1995). A K_a/K_s ratio for the entire protein would be unlikely to reveal accelerated rates that occur only in parts of the protein. Indeed, the largest proportion of the amino acid replacements in ISP are in

the transmembrane region, where the rate of replacement is 11.0 times greater than expected. Two possible explanations for the higher rate in the transmembrane region are apparent. One is that the transmembrane region is not directly involved in electron transport and thus may not be so constrained by functional requirements as are the extramembrane domains comprising the flexible linker, which allows movement, and the "head," which contains the iron-sulfur cluster. The second explanation is that the transmembrane region is close to the ubiquinone binding sites (Q_o and Q_i) of cytochrome b, and also close to the two hemes of cytochrome b. Since cytochrome b is rapidly
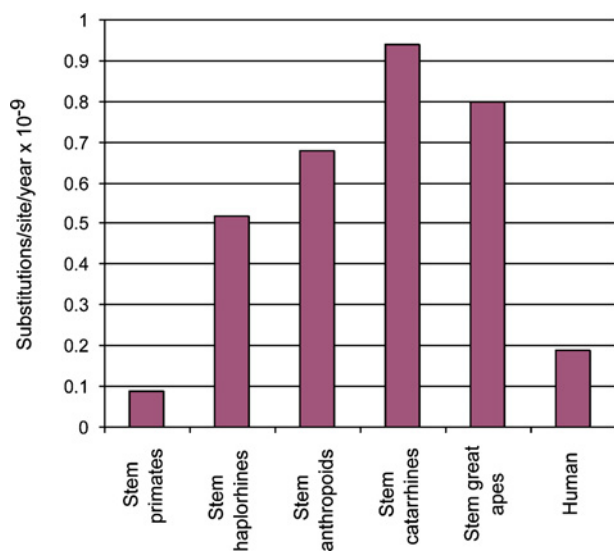
Fig. 2. Rates of nonsynonymous substitution in ISP in the lineage leading to human. The human bar indicates descent from great ape ancestor to human.

evolving in anthropoids (Andrews *et al.*, 1998; McClellan and McCracken, 2001), the transmembrane region of ISP may be coevolving with it.

The data show a clear nonsynonymous rate acceleration in the anthropoid, catarrhine, and ape and great ape stems, and then a deceleration in the descent to human (Fig. 2). Such a pattern is the signature of adaptive evolution, indicating a period of positive selection followed by a period of consolidating purifying selection
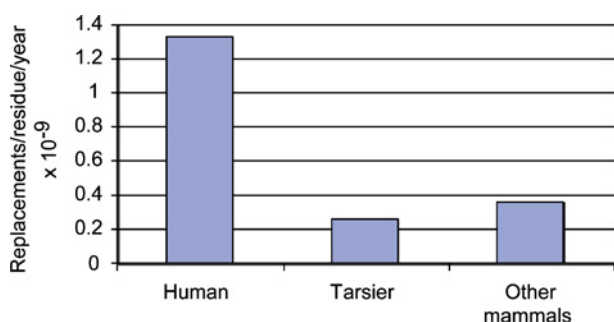
Fig. 3. Rates of amino acid replacement in ISP. Values are replacements per residue per year $\times 10^{-9}$. Human indicates replacements on the lineage leading to human after divergence of tarsier. Tarsier indicates replacements on the tarsier lineage. Other mammals consists of replacements among the haplorhine stem, brown lemur, galago, slow loris, mouse, rat, and cow. The amount of time from the human–tarsier split to present is estimated to be 58 million years. The cumulative amount of time for the evolution of the haplorhine stem, brown lemur, galago, slow loris, mouse, rat, and cow is estimated to be 384 million years (Goodman *et al.*, 2001; Hedges, 2002; Seiffert *et al.*, 2003; Springer *et al.*, 2003).

(Goodman, 1982). An alternative interpretation is that there has been a general slowdown in the rate of mutation in apes (Goodman, 1962; Li and Tanimura, 1987; Li *et al.*, 1996).

The data presented here contribute toward an emerging picture of accelerated rates of evolution in the electron transport chain in anthropoid primates. Such accelerated rates have been observed for 9 of the 13 subunits of COX, for CYC, and, including the results of the present study, for two catalytically active subunits of complex III. It seems likely that, given the widespread nature of these findings of accelerated evolution in anthropoid primates, complexes III and IV and CYC have been evolving co-adaptively in anthropoid primates. It is therefore puzzling that the rate acceleration observed for ISP is not as high as the rate accelerations detected in many subunits of COX. Several reasons can be proposed to explain this difference. First, as noted, ISP is highly conserved in all the species in our database. Indeed, for all of complex III, there is evidence that the constraints on its evolution are more stringent than those on COX. McKenzie *et al.* (2003) introduced mitochondrial genomes from progressively more divergent species of murids into mtDNA-less (ρ°) mouse cells. Varying decreases in activity of the ETC enzymes were observed. The most striking decreases in activity were found in complex III. The authors concluded that, among subunits of the holoenzymes of the ETC, complex III is subject to the greatest constraints against evolutionary change. Second, a major functional shift in the molecular evolution of the ETC in anthropoid primates appears to have occurred in the binding site between COX and CYC (Schmidt *et al.*, 2005), which does not directly involve complex III.

The emerging picture of co-adaptive evolution in at least the terminal portion of the ETC stimulates speculation about the function of the evolutionary changes. Although biochemical data is largely lacking (but see Osheroff *et al.*, 1983), notable features of the anthropoid lineage are large brain size relative to body weight (Martin, 1990) and longevity. The brain is an energy-demanding organ (Holliday, 1986; Laughlin, 2001), and the evolution of the anthropoid brain may be correlated with both enhanced energy production and better controlled side reactions. Complex III, a major site of free radical production (Gille and Nohl, 2001), may have evolved to better control this side reaction, thus enabling longer life.

ACKNOWLEDGMENTS

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