Molecular Phylogenetics and Evolution
Vol. 18, No. 1, January, pp. 26–36, 2001
doi:10.1006/mpev.2000.0890, available online at http://www.idealibrary.com on IDEAL®

Molecular Evolution of Aerobic Energy Metabolism in Primates

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Received September 26, 2000; revised October 13, 2000; published online December 19, 2000

As part of our goal to reconstruct human evolution at the DNA level, we have been examining changes in the biochemical machinery for aerobic energy metabolism. We find that protein subunits of two of the electron transfer complexes, complex III and complex IV, and cytochrome *c*, the protein carrier that connects them, have all undergone a period of rapid protein evolution in the anthropoid lineage that ultimately led to humans. Indeed, subunit IV of cytochrome *c* oxidase (COX; complex IV) provides one of the best examples of positively selected changes of any protein studied. The rate of subunit IV evolution accelerated in our catarrhine ancestors in the period between 40 to 18 million years ago and then decelerated in the descendant hominid lineages, a pattern of rate changes indicative of positive selection of adaptive changes followed by purifying selection acting against further changes. Besides clear evidence that adaptive evolution occurred for cytochrome *c* and subunits of complexes III (e.g., cytochrome c_1) and IV (e.g., *COX2* and *COX4*), modest rate accelerations in the lineage that led to humans are seen for other subunits of both complexes. In addition the contractile muscle-specific isoform of COX subunit VIII became a pseudogene in an anthropoid ancestor of humans but appears to be a functional gene in the nonanthropoid primates. These changes in the aerobic energy complexes coincide with the expansion of the energy-dependent neocortex during the emergence of the higher primates. Discovering the biochemical adaptations suggested by molecular evolutionary analysis will be an exciting challenge.

INTRODUCTION

In efforts to reconstruct human evolution at the DNA level, a challenging goal is to identify those positively selected mutations that shaped the genetic basis of being human. Some of the most striking human features, such as greatly enlarged brains and prolonged childhoods in nurturing societies, have deep roots in humankind's evolutionary history. Brain capacity is significantly larger in the Miocene fossil primates of suborder Anthropoidea than in the earlier Eocene fossil primates (Fleagle, 1999). Also, among living primates, the neocortical portions of the brain are much larger in anthropoid primates (the platyrrhines and catarrhines of Anthropoidea) than in the nonanthropoid primates (the tarsiers and the lemuriform and loriform strepsirhines) (Rilling and Insel, 1999; Stephan *et al.*, 1981). Similarly, fetal life and the infant and childhood stages of postnatal life are more prolonged in the anthropoid primates, especially in apes and humans, than in the nonanthropoid primates. It may be surmised that the genetic blueprints for the inherited aspects of humankind's morphology, metabolism, and mental functions have both anciently conserved features common to all living primates and phylogenetically more restricted features that arose later in evolution, ranging from the anthropoid-specific ones that arose within the early Anthropoidea in the lineage to the last common ancestor of platyrrhines and catarrhines to the human-specific ones that arose in the last few million years on the modern human lineage.

There is reason to believe that selected adaptive changes in the biochemical machinery for aerobic energy metabolism were among the important molecular changes in humankind's evolutionary history. Earlier work that focused on lactate dehydrogenase (LDH)[2] isozymes in the brain tissues of primates revealed a striking difference in the relative amounts of M (skeletal muscle type) and H (heart type) LDH between strepsirhines and anthropoids. In the strepsirhine brains more than 70% of the LDH was the M type whereas just the opposite was so in anthropoids; in anthropoid brains more than 70% of LDH was the H type (Goodman *et al.*, 1969; Koen and Goodman, 1969; Syner and Goodman, 1966). Tarsier brains showed an

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[2] Abbreviations used: Aa, amino acid; COX, cytochrome *c* oxidase; cyb, cytochrome *b;* cyc, cytochrome *c;* mt, mitochondria; Ma, Mega annum; N, nonsynonymous substitution; S, synonymous substitution; ISP, iron–sulfur protein; LDH, lactate dehydrogenase; MP, maximum parsimony.

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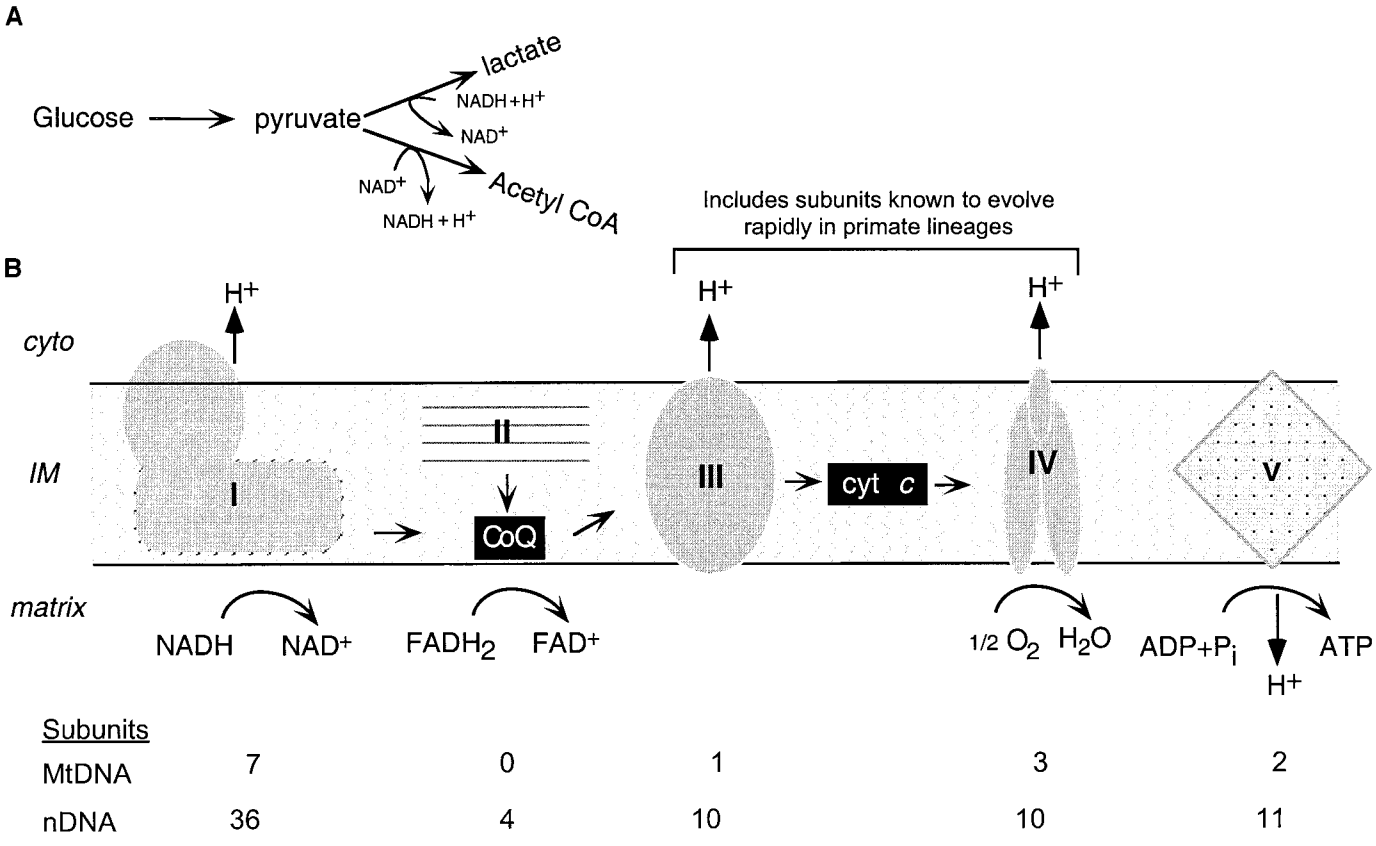

FIG. 1. The electron transport chain. The five enzyme complexes are NADH CoQ reductase (I), succinate CoQ reductase (II), ubiquinol cytochrome *c* reductase (III), cytochrome *c* oxidase (COX) (IV), and ATP synthase (V). (A) Metabolism of glucose to pyruvate is shown, and its metabolism by an anaerobic path to form lactate and, after import of the lactate to mitochondria, by an aerobic path. (B) The electron transport chain is shown in cartoon form in the inner mitochondrial membrane. Electrons enter the chain from NADH or $FADH_2$. At three of the complexes a proton is shown pumped out of the matrix, thereby forming the electrochemical gradient utilized by complex V for ATP formation. Shown below each complex are its number of nuclear DNA and mitochondrial DNA encoded subunits.

intermediate pattern. LDH controls the passage of pyruvate from the anaerobic (energy low) to aerobic (energy high) metabolic pathway by means of its reversible reduction of pyruvate to lactate (Fig. 1A). On the basis of the view that pyruvate enters the aerobic (citric acid) respiratory cycle more readily in tissues containing mostly H LDH than in tissues containing mostly M LDH (Kaplan, 1965), it was suggested that brain tissues with a preponderance of H LDH (such as in anthropoids) rely more heavily on aerobic metabolism and function at a higher energy level than brain tissues with a preponderance of M LDH (such as in strepsirhines) (Goodman *et al.*, 1969).

Another example of important genic changes concerns the emergence and evolution of a distinct fetal hemoglobin in the anthropoid primates. In this example, the positively selected genic changes were both regulatory and coding. In the reconstructed phylogenetic history of the events that led to this fetal hemoglobin (reviewed in Goodman, 1999) the early primates had a single γ-globin gene expressed solely in embryonic life, as is the γ gene of the living strepsirhines. In the anthropoid stem lineage to the last common ancestor of platyrrhines and catarrhines, the γ-globin gene tandemly duplicated, an upsurge of nucleotide substitutions in the γ promoter and coding sequences occurred, and the transition to fetal γ expression began. In the catarrhine stem lineage, the transition to full fetal γ expression was completed. The switch from γ- to β-globin gene expression occurs at the start of fetal life in strepsirhines (Tagle *et al.*, 1988), in mid-fetal life in platyrrhines (Johnson *et al.*, 1996), and at birth in catarrhines (Bunn and Forget, 1986; Johnson *et al.*, 2000). There are promoter *cis*-regulatory changes that are anthropoid specific and others that are catarrhine specific. These changes resulted in alterations in the promoter's binding affinity for a set of putative fetal repressor proteins: these proteins bind strepsirhine sequences with high affinity, platyrrhine sequences with moderate affinity, and catarrhine sequences with very low affinity (Gumucio *et al.*, 1994). While such promoter *cis*-regulatory changes were being fixed, coding sequence changes were also being fixed. Among the most important were nonsynonymous substitutions in

codons specifying the amino acids of the anthropoid γ-globin 2,3-DPG-binding site. These changes drastically reduced the 2,3-DPG-binding capacity of fetal hemoglobin. This ensured a favorable balance in the transport of oxygen from mother to fetus, and thereby helped make possible the prolonged intrauterine fetal life and extensive prenatal brain development of anthropoid primates.

A likely evolutionary pressure for these examples was the emergence in the anthropoid primates of a larger neocortex, one of the most aerobic and energy consuming tissues. This same evolutionary pressure may have been in part responsible for the course of evolution of components of the electron transport chain, including the well-studied cases of cytochrome *c* and cytochrome *c* oxidase subunits II and IV in anthropoid primates (Adkins and Honeycutt, 1994; Baba *et al.*, 1981; Wu *et al.*, 1997). Cytochrome *c* oxidase (COX) is the multisubunit enzyme complex that catalyzes the final step of electron transfer through the respiratory chain. Thus, COX plays a vital role in aerobic tissues. Phylogenetic analysis of rodent, bovine, and primate gene sequences that encode COX IV[3] revealed an accelerated nonsynonymous rate in the earlier evolution of catarrhines followed later by a decelerated rate (Wu *et al.*, 1997). Possible positive selection for adaptive amino acid replacements was evident by higher nonsynonymous (N) than synonymous (S) rates in the lineage encompassing catarrhine and hominid stems (where N is the fraction of nonsynonymous substitutions among possible sites and S the analogous fraction of synonymous substitutions). A marked deceleration of N rates with much lower N than S rates in the terminal lineages to gorilla, human, and chimpanzee *COX4* genes indicated that the positively selected changes were then preserved by purifying selection. Phylogenetic analyses of gene sequence data for other COX subunits, as well as for other proteins active in the respiratory chain, have also provided evidence for positively selected changes or evidence of accelerated nonsynonymous substitutions in anthropoid primates (Andrews and Easteal, 2000; Andrews *et al.*, 1998; Schmidt *et al.*, 1999; Wu *et al.*, 2000).

We focus here on aerobic energy metabolism and a direct examination of subunits and carrier proteins of the membrane-bound complexes located on the inner mitochondrial membrane that transfer electrons ultimately to oxygen. However, we can also ask which other genes we should consider in seeking to identify positively selected mutations that may have had an important role in shaping the genetic basis of being human. One group of genes includes those accessory to the respiratory complexes. For example, numerous proteins are known in yeast that are not themselves part of the respiratory complexes but are required for their assembly. Some have human homologs (Rotig *et al.*, 2000), such as SURFEIT-1 (SURF-1; SHY-1 in yeast) (Tiranti *et al.*, 1998, 1999; Yao and Shoubridge, 1999) and SCO2 (Jaksch *et al.*, 2000; Papadopoulou *et al.*, 1999). Other proteins of potential interest are transcription factors, such as NRF-1 (Chau *et al.*, 1992; Evans and Scarpulla, 1990) and NRF-2 (Carter *et al.*, 1992; Virbasius *et al.*, 1993), whose binding sites are frequently found in the promoters of energy metabolism-related genes. A third and large group consists of genes for proteins that detoxify the fallout of metabolism. Several percent of the oxygen utilized by mitochondria is estimated to be incompletely oxidized. Instead it is converted to radical species, whose deleterious effects have been proposed to underly a number of chronic diseases (Cookson and Shaw, 1999; Manfredi and Beal, 2000; Smith *et al.*, 2000; Wallace, 2000) and aging itself (Barja and Herrero, 2000; Cortopassi and Wong, 1999; Pedersen, 1999). Such proteins as superoxide dismutase, catalase, and glutathione peroxidase, which detoxify the radical by-products of aerobic metabolism, may have evolved in concert with the electron chain proteins discussed. As in the γ-globin example, positively selected genic changes may have occurred in both the coding region and the regulatory region.

EVOLUTION OF ENERGY METABOLISM

The Electron Transport Chain: The View from Cytochrome c

All work in the cell utilizes hydrolysis of ATP to supply energy, and most mammalian cells rely on ATP produced by oxidative phosphorylation for survival and function. The enzymatic machinery utilized for aerobic metabolism of sugars and fats is located in the inner mitochondrial membrane. Four of the five enzyme complexes (I–IV) are connected by the shuttle molecules coenzyme Q and cytochrome *c* (cyc), which carry electrons between them (Fig. 1). Electrons enter the chain at complex I or II (donated from NADH or $FADH_2$), are passed to carriers of progressively greater electron affinity, and are ultimately accepted by molecular oxygen. The potential energy generated at three steps by pumping a proton out of the matrix is recovered as ATP in the ATP synthase (complex V) reaction, thereby coupling electron transport along the respiratory chain to oxidative phosphorylation. The respiratory chain complexes are multisubunit arrays composed of 13 polypeptides encoded in mitochondrial DNA (mtDNA) and more than 60 encoded in the nuclear genome.

Cyc contains its own redox system, a heme, and thereby functions to connect complex III with complex IV (cytochrome *c* oxidase or COX). To do so, cyc is

[3] Cytochrome *c* oxidase gene and protein terminology used here is italic for genes and Roman for protein subunits (e.g., *COX8H* and COX VIII-H for the heart isoform of subunit 8).

reduced by complex III and is oxidized by complex IV. Thus, cyc interacts with sites on both complexes, utilizing the same region for binding so that it cannot bind both complexes at once. Findings that we shall now review on the molecular evolution of COX, cyc, and complex III show that there have been bursts of nonsynonymous substitutions during the emergence and evolution of the anthropoid primates suggestive of positive selection.

COX (Complex IV)

COX is a multisubunit complex composed of 10 nuclear DNA-encoded subunits and three subunits encoded by mtDNA. The early finding of accelerated amino acid replacements in primate lineages for subunits II and IV is reviewed in this section. We also review information concerning our recent discovery that an isoform of subunit 8 is a pseudogene in humans.

Subunit II. COX II is part of the catalytic core of cytochrome oxidase. It contains Cu_A and the cyc-binding pocket. Bacterial oxidases are two- or three-subunit enzymes that contain a homolog of mammalian COX II, emphasizing its integral nature. Based initially on restriction site mapping of mtDNA, COX II was identified as a particularly rapidly evolving subunit in primates compared to rodents, and extensive comparisons of both nucleotide and amino acid sequences established that this higher rate of evolution occurred during the radiation of the anthropoid primates (Adkins and Honeycutt, 1994). For example, platyrrhines and catarrhines have undergone a nearly twofold increase in amino acid replacement rate compared with the nonanthropoid primates.

The rate of increase of COX II amino acid replacements in the anthropoid primates coincides with a similar increase for cyc (discussed below). Since the two proteins interact functionally, this coincidence of elevated rates was viewed as an example of coevolution (Cann *et al.*, 1984). A detailed examination of amino acid sequence variation of COX II in strepsirhines and in catarrhines revealed greater variation at the C-terminus among strepsirhines than among catarrhines and conversely greater variation at the N-terminus among catarrhines than among strepsirhines (Adkins and Honeycutt, 1994). Between the C-terminus and N-terminus in the middle of this COX II protein chain at positions 114 and 115 amino acid residues bearing a carboxyl group (aspartate or glutamate) were replaced in the stem of the catarrhines by small, uncharged glycine residues. Adkins and Honeycutt (1994), noting that Capaldi *et al.* (1983) implicated position 114 in interactions with cytochrome *c*, suggest that the amino acid replacements at the adjacent 114 and 115 positions may have so affected the overall structure of the cyc-binding cleft as to cause the reduced enzyme kinetics observed in cross reactions between catarrhine cyc and nonanthropoid COX compared to those between nonanthropoid cyc and COX (Osheroff *et al.*, 1983). The further finding that the glycine residues at positions 114 and 115 of COX II are invariant in all catarrhines examined suggests that aspartate or glutamate to glycine replacements in the catarrhine stem were positively selected and that these two glycine residues are being preserved in the catarrhines by purifying selection. The coincident burst of cyc amino acid replacements in catarrhine ancestors is indicative of coadaptive evolution and correlates with the further finding of Osheroff *et al.* (1983) that catarrhine cyc and COX cross-react quite well with each other but nonanthropoid cyc and catarrhine COX cross-react poorly with each other (these findings are further discussed below).

Subunit IV. Based on a previous observation of a high number of amino acid replacements in the human COX IV compared to rodent and cow orthologues (Lomax *et al.*, 1992), we sought to identify the lineage in which accelerated rates had occurred. Subunit IV is of particular interest as a candidate for positive selection because there is suggestive evidence that it is a major regulator of COX. The binding of ATP or ADP to COX IV, as determined by the ATP/ADP ratio, and possibly subunit phosphorylation (Steenaart and Shore, 1997), appear to control COX activity independent of the proton motive force (Arnold and Kadenbach, 1997; Kadenbach and Arnold, 1999).

Comparison of the N/S rate ratios in descent of the primate lineages can reveal both the evolutionary window and the specific amino acids at which positive selection occurred, traditionally defining positive selection by N/S rate ratios exceeding 1.0. The evidence that positive selection spread beneficial N substitutions through a lineage is strengthened if in subsequent evolutionary time the N/S rate ratio falls well below 1.0. This pattern is seen when purifying selection continues to favor the previously positively selected beneficial mutations by acting against accumulation of further N mutations but not against S mutations, which continue to accumulate at an undiminished rate.

Phylogenetic analysis of *COX4* coding sequence data revealed accelerated N substitution rates in catarrhine and, to a lesser extent, platyrrhine evolution (Wu *et al.*, 1997). For example, using a distance approach to examine the *COX4* genes for which complete coding region sequences were available, Wu *et al.* calculated an N/S ratio of 13.9 on the lineage to the catarrhine ancestor. The accelerated rates were followed by decelerated rates, a pattern typical of positive selection for adaptive amino acid replacement. For example, the N/S ratio for the hominin ancestor (human, chimpanzee, gorilla) is 0.46. The pattern illustrated by COX IV is one of the most striking examples of positive selection.

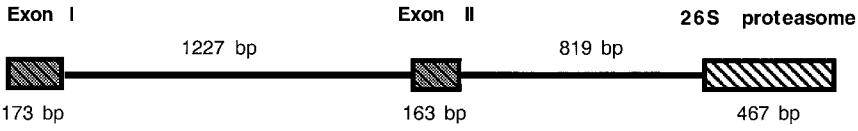

FIG. 2. The *COX8H* gene in cow. The human pseudogene is on chromosome 11 and is also adjacent to the 26S proteasome subunit p40.5.

Subunit VIII. COX VIII has isoforms in most mammals (Kadenbach *et al.*, 1987). These show tissue specificity, a contractile muscle form generally referred to as the H type and a ubiquitous type, first noted in liver and called the L form (Grossman and Lomax, 1997). The isoforms also follow a developmental program; expression of the L type occurs first in heart and skeletal muscle and only later in these tissues is there expression of the H type (Bonne *et al.*, 1993; Ewart *et al.*, 1991; Grossman *et al.*, 1995).

Examination of the presence of each of the COX VIII isoforms in a number of mammals (Kadenbach *et al.*, 1990, 1995; Linder *et al.*, 1995; Grossman and Lomax, 1997) suggests a relative lack of divergence in the function of the two isoforms, allowing more ready replacement of one form by the other. In addition, the COX VIII isoforms are among the most rapidly evolving COX subunits, an observation consistent with reduced functional selection. Most notable is the absence of the COX VIII–H isoform in humans, the only known instance of COX isoform presence/absence variation in primates. We have recently discovered that *COX8H* is in fact present as an unprocessed pseudogene on chromosome 11 (Fig. 2). Fortuitously, the terminal exon of the p40.5 subunit of the 26S proteasome is located <1 kb away, providing a priming location for PCR amplification into the region of *COX8H*. Amplifying fragments from this region, we have determined that the p40.5k subunit occupies a homologous position in both cow and rabbit. In addition, an apparently functional *COX8H* coding region is present in several strepshirhine primates and in tarsier (Goldberg, Schmidt, and Grossman, unpublished data). Thus, the loss of *COX8H* expression appears to have taken place approximately concurrently with the instances of positive selection being reviewed here.

Cytochrome c

There is growing evidence for the central role of cyc in electron transport chain regulation. Studies in human cell lines established that, although COX can be present in greater quantities than needed for respiration (using glutamate/malate as substrate), cyc appears not to be in excess over that required for activity (Villani *et al.*, 1998). Thus, cyc levels can regulate respiration. An increase in cyc, associated with a marked increase in cellular respiration, has been shown to result from serum stimulation of quiescent cells (Gopalakrishnan and Scarpulla, 1994; Herzig *et al.*, 2000; Scarpulla, 1997). These events are themselves associated with activation by serum phosphorylation of CREB and NRF-1 (Herzig *et al.*, 2000), transcription factors to which the cyc promoter is known to be sensitive. Furthermore, the transduction of electrons by cyc has been shown to be regulated by ATP levels (Craig and Wallace, 1993). Finally, complex III and complex IV, presumably in association with cyc, apparently can form a supramolecular complex in yeast that would then allow electron transfer to take place with enhanced efficiency (Cruciat *et al.*, 2000; Schagger and Pfeiffer, 2000).

AA sequences. Cytochrome *c* amino acid sequences are known for 113 eukaryotic species (Baba *et al.*, 1981; Banci *et al.*, 1999). This relatively small protein (100–120 amino acids; 104 in mammals) is highly conserved among eukaryotes, with even yeast and mammals sharing about 45% Aa identity. In mammals the similarity between taxa is even greater. The average pairwise sequence identity is 89.4% between primates and a marsupial (eastern gray kangaroo) and is greater yet, 93.8%, between nonprimate placental mammals (Eutheria) and marsupials. As shown in Fig. 3, relatively rapid cyc evolution occurred on the primate lineage leading to the anthropoid ancestor, i.e., to the platyrrhine (*Ateles*)–catarrhine (*Macaca, Homo, Pan*) divergence node.

Figure 3 shows four unambiguous Aa replacements on this primate branch to the anthropoid ancestor (Aa 11, 12, 15, and 83) and one (Aa 46) on the catarrhine stem. Additionally, Aa 50, 58, and 89 could also have been replaced on the primate branch, with Aa 50 replaced again on the catarrhine stem. Because there are no published prosimian (strepsirhine or tarsier) Aa sequences at this locus, it cannot be determined if the seven Aa replacements (Aa 11, 12, 15, 50, 58, 83, 89) occurred prior to the origin of anthropoids or prior to the origin of primates. However, results obtained by analyzing cyc gene and processed pseudogene nucleotide sequence data suggest that all seven replacements belong on the anthropoid stem (described below).

Nucleotide sequence. Figure 4 shows the phylogenetic tree constructed by maximum parsimony (MP) for the cyc nucleotide sequence data set of Evans and Scarpulla (1988). This data set consists of 11 human processed pseudogene sequences and the homologous human and rat functional somatic cyc gene sequences. The alignment employed, with gaps for indels (inser-

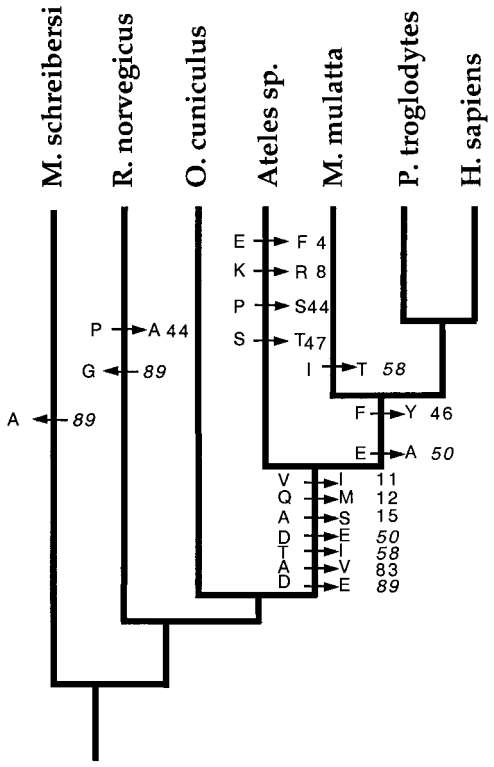

FIG. 3. Most parsimonious reconstruction of character evolution in primate evolution. Shown are the amino acid replacements that occurred during primate evolution. Numbers refer to cyc amino acid numbers. Numbers in italics have equivocal character reconstructions; here we show the changes as if they had occurred as early as possible on the primate lineage. Taxa are *M. schreibersi, Miniopterus schreibersi* (bat); *R. norvegicus, Rattus norvegicus* (rat); *O. cuniculus, Oryctolagus cuniculus* (rabbit); *Ateles* sp. (spider monkey); *M. mulatta, Macaca mulatta* (rhesus monkey); *P. troglodytes, Pan troglodytes* (common chimpanzee); *H. sapiens, Homo sapiens* (human). Data from Baba *et al.* (1981) and Scarpulla *et al.* (1981).

tions/deletions), spans 2181 nucleotide positions, encompassing coding and untranslated 5′ and 3′ sequences. The branching topology of this MP tree and the known codon sequences at the tips of the tree, along with MP reconstructed ancestral sequences at the interior nodes, supports the conclusion drawn by Evans and Scarpulla (1988) that there are two classes of processed pseudogenes. The class 1 pseudogenes (HS7 and HS11) have codon sequences that closely match those of the human functional (somatic; Hennig, 1975) cyc gene. Evidence as to when these class 1 pseudogenes arose comes not only from translating the pseudogene codon sequences into amino acid sequences, allowing comparisons to the known cyc set, but also from our unpublished cyc nucleotide sequence data for a range of nonhuman primates. Using a time scale based on fossil evidence (reviewed in Goodman *et al.*, 1998), it appears that the class 1 pseudogenes arose on the catarrhine stem between 40 and 25 Ma, i.e., between the platyrrhine–catarrhine and cercopithecid (Old World monkey)–hominid (ape, human) divergence nodes. The class 2 pseudogenes (HC1–HC8, HC10) arose earlier and, in contrast to the class 1 pseudogenes, retain nucleotide sequences such that, in a functional gene, would have coded for the residues at Aa 11, 12, 15, 50, 58, and 83 that the rat functional somatic cyc codes for (Table 1). Nevertheless, the most recent of these class 2 pseudogenes (HC1 and HC3), as judged by their degrees of nucleotide sequence divergence from the class 1 pseudogenes and the functional human gene, appear to have arisen in the range of 55–45 Ma on the anthropoid stem, i.e., about 20 million years before the class 1 pseudogenes arose.

In agreement with the amino acid results (Fig. 3, Table 1), the nucleotide-coding sequence results (Fig. 4) depict a markedly accelerated nonsynonymous evolution rate in our earlier anthropoid ancestors followed by a markedly decelerated rate in descendent lineages. This acceleration–deceleration pattern can be caused by Darwinian natural selection first in its positive form spreading adaptive changes through a lineage and then in its purifying form preserving the changes that

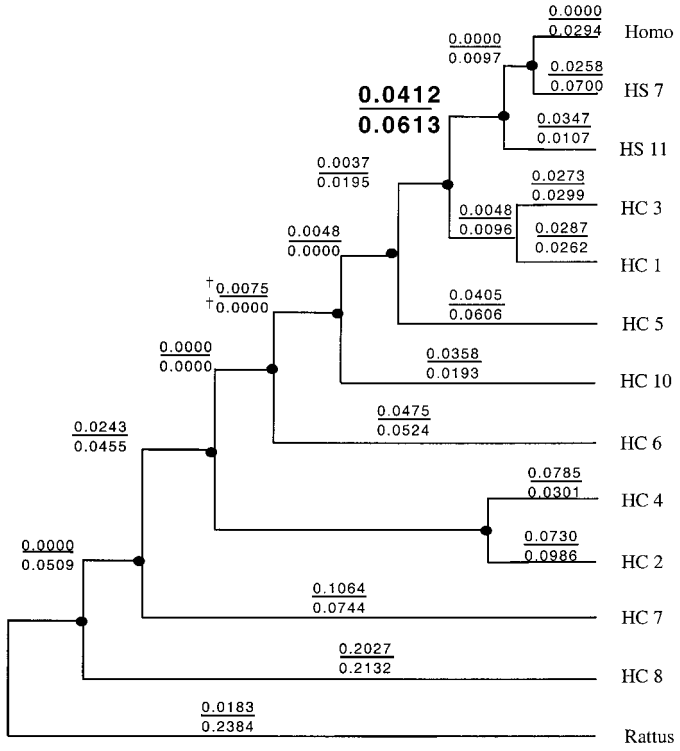

FIG. 4. One of three most parsimonious branch and bound trees for the entire nucleotide sequence data (2181 bp). Phylogenies were inferred with PAUP* (ver. 4.0b4a) (Swofford, 2000). Numbers on branches are N/S values for the protein-coding region (and paralogues in pseudogenes) according to the method of Li (1993). †Li's method was inapplicable here; therefore, Nei and Gojobori's method (Nei and Gojobori, 1986) was used. This latter method may overestimate the value of S. All calculations were performed using the computer program FENS (de Koning *et al.*, 1998).

TABLE 1

Amino acid	11	12	15	44	46	50	58	83	89
Rattus	V	Q	A	A	F	D	T	A	G
Miniopterus	.	.	.	P	.	.	.	.	A
Oryctolagus	.	.	.	V	.	.	.	.	D
HC3 (Class 2)	.	.	.	V	.	.	.	.	A
HC1 (Class 2)	.	.	.	I	.	.	.	.	A
HC5 (Class 2)	.	.	.	.	.	.	.	.	T
HC 10 (Class 2)	.	.	.	I	.	.	.	.	A
HC6 (Class 2)	.	.	.	T	.	.	.	.	—
HC4 (Class 2)	.	.	.	I	L	.	.	S	A
HC2 (Class 2)	.	*	.	F	.	.	.	—	—
HC7 (Class 2)	.	P	.	.	.	.	.	—	—
HC8 (Class 2)	K	K	T	.	S	E	.	.	S
Ateles	I	M	S	S	.	E	I	V	E
Macaca	I	M	S	P	Y	A	.	V	E
Pan	I	M	S	P	Y	A	I	V	E
HS7 (Class 1)	I	M	S	P	Y	A	I	V	E
HS 11 (Class 1)	I	M	S	P	H	A	I	V	E
Homo	I	M	S	P	Y	A	I	V	E

Note. Alignment of the nine amino acids that have been replaced since the split between *Rattus* and *Homo.* Also shown are the hypothetical amino acid residues derived from the pseudogene nucleotide sequences that are the homologues of the functional rat and human coding sequences. In eight of the Aa's (11, 12, 15, 46, 50, 58, 83, and 89) the position in *Rattus* is identical to that inferred for the mammalian ancestor. At one site (44) the catarrhine primates retain the ancestral Aa, but there have been two independent replacements in *Rattus* and *Ateles* (spider monkey). These changes are shown in the parsimony tree (Fig. 3). Note that in the class 2 pseudogenes, the majority of these sites retain the inferred ancestral Aa.

occurred. In view of cyc's vital respiratory role, relatively small size, and overall slow rate of evolution in vertebrates (Baba *et al.*, 1981), almost every one of its amino acid sites is probably involved in the functional behavior of the protein; thus, typically each site would be scrutinized by purifying selection. Such pervasive purifying selection could explain why the rate of nonsynonymous change did not overtake that of synonymous change during the period of accelerated N evolution.

Coadaptive evolution. Baba *et al.* (1981) concluded that, of the 7 amino replacements on the anthropoid stem, 4 (Aa 11, 12, 15, and 83) occurred at sites that interact with both cytochrome *c* reductase and COX. On the basis of more detailed structural information, Banci *et al.* (1999) identified 10 sites (Aa 9, 13, 16, 70, 72, 81, 82, 83, 86, and 87) as important protein–protein recognition sites. One of these (Aa 83) is the same as one of Baba *et al.*'s 4 key interaction sites and 3 (Aa 9, 13, and 16) are spatially close to Baba *et al.*'s 3 remaining key interaction sites. There is experimental evidence that the amino acid replacements during the period of accelerated N evolution in the anthropoid stem changed the surface area involved in the function of transporting electrons between complex III and complex IV. In experiments reported by Osheroff *et al.* (1983), both prosimian (slow loris) and nonprimate (horse) cytochromes *c* could transport electrons efficiently (high maximal velocity) to bovine COX, but inefficiently (low maximal velocity) to rhesus monkey COX. Conversely, human cyc could transport electrons at high maximal velocity to the rhesus oxidase but at low maximal velocity to the bovine oxidase. Spider monkey cyc reacted better with rhesus than with bovine oxidase but did not react as well as human cyc reacted with the rhesus oxidase nor as well as the slow loris and horse cytochromes *c* reacted with the bovine oxidase. The markedly decelerated N evolution in the catarrhines, after its preceding accelerated rate, provides evidence that purifying selection preserved the amino acid replacements produced earlier that are responsible for the functional divergence of catarrhine cyc. In turn, it follows that the period of accelerated N evolution and coadaptive evolution between cyc and COX was driven by positive Darwinian selection.

Complex III

Mammalian complex III (cytochrome *c* oxidoreductase; also known as cytochrome bc_1) catalyzes the oxidation of ubiquinol, the electron carrier from complexes I and II, and the reduction of cyc. The process is known as a protonmotive Q cycle. It takes place by a series of electron transfers whose net effects are to oxidize one molecule of ubiquinol to ubiquinone, to reduce two molecules of cyc, to consume two protons on the negative side of the membrane, and to deposit four protons on the positive side. Cyc is then oxidized in turn by complex IV, the terminal enzyme complex of the chain.

Mammalian cytochrome bc_1 is composed of 11 subunits, 1 (cytochrome *b;* cyb) encoded by mtDNA and 10 by nuclear DNA. Of these 11 subunits, only 3 compose

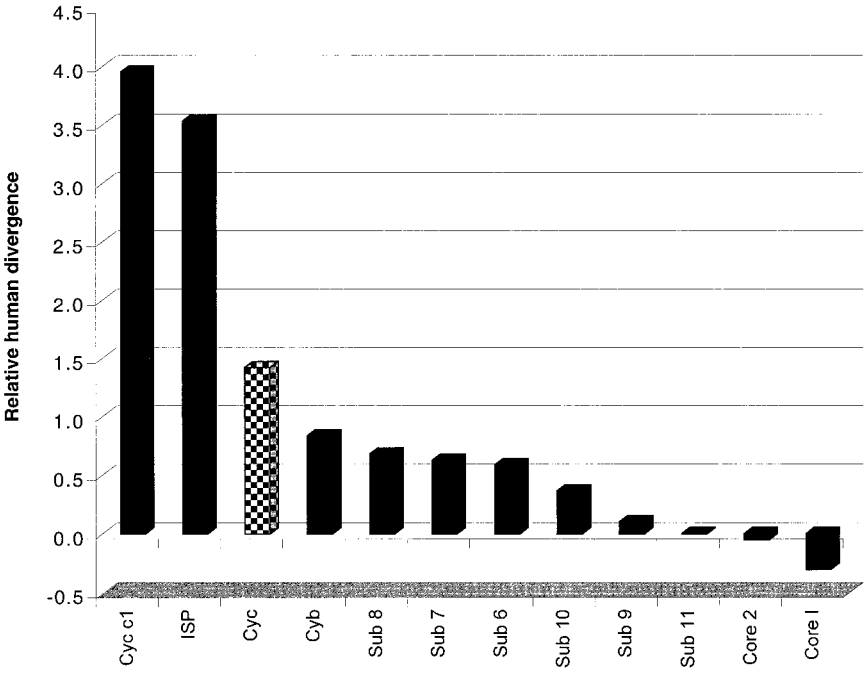

FIG. 5. Relative rate of amino acid changes for complex III genes. The rate is expressed as a ratio of changes on the human lineage relative to the bovine and rodent lineages. Protein sequences from various sources for cow, mouse, rat, and human were aligned with CLUSTALW (Higgins and Sharp, 1989) and analyzed with the maximum-parsimony option of PAUP. By subsequently "rooting" the cow–human divergence with the rodent lineage, and the rodent–human with the cow, we obtained an estimate of the extent of amino acid replacement on the human lineage relative to the average of the cow and rodent lineages.

the bacterial complex, the catalytic subunits cyb, cytochrome c_1, and the Rieske iron–sulfur protein (ISP). These three proteins are thus both necessary and sufficient for electron transfer through the complex. The functions of the other subunits, which are all nuclear encoded, are not well known, although some details have begun to emerge. Subunit 8 has been called the hinge protein because it appears essential for proper complex formation between cytochromes *c* and c_1. It is also called the acidic protein because nearly one-third of its 72 residues are acidic. It is arrayed in two long helices that form a large, negatively charged docking port for the basic cyc. Subunit 9 is the processed presequence of the ISP. After the ISP is inserted into the cytochrome bc_1 complex, the presequence is cleaved to yield subunit 9. Significantly, the cleaved subunit 9 is found near core subunits 1 and 2 (Iwata *et al.*, 1998), which show high sequence homology to the heterodimer subunits of the mammalian matrix processing peptidase that cleaves presequences after import into mitochondria. Quite possibly, in mammalian cytochrome bc_1 the core subunits 1 and 2 carry out this cleavage function. The consequence of the cleavage is that the 11 subunits of the complex are the products of 10 genes.

In order to acquire a general perception of the relative amino acid divergences, we examined cyc and each mature subunit protein from complex III for human, cow, mouse, and rat by assembling amino acid sequences from various databases. Sequences from the four mammals for each gene encoding a subunit were aligned with CLUSTALW and analyzed with PAUP*. By subsequently "rooting" the cow–human divergence with the rodent lineage, and the rodent–human with the cow, we obtained an estimate of the extent of amino acid replacement on the human lineage relative to the cow and rodent lineages (Fig. 5). We wish to highlight the following two observations.

Subunits that interact with cyc show a larger amino acid divergence on the human lineage than in either the rodent or the bovine lineages. Four subunits, cytochrome c_1, ISP, cyb, and the hinge protein have been implicated in the binding of cyc to complex III. These four subunits also show that the degree of amino acid replacements in the human lineage is larger than the bovine and rodent lineages. The core proteins I and II seem to primarily be involved with the protein import-processing pathway of the mitochondria. As such, they are the least likely to interact directly or indirectly with cyc. These two subunits show a *decrease* in the relative extent of amino acid divergence on the human lineage.

Two subunits are expressed from one coding region (ISP and subunit 9): They differ in relative amino acid substitution rates. The ISP directly interacts with cyc and also shows larger amino acid divergence on the human lineage relative to the other mammalian lin-

eages. This is in contrast to subunit 9, whose function is at this time unknown. This striking difference is in spite of the fact that the two subunits are in fact translated as a single gene product that is subsequently cleaved into two subunits. This provides the most direct evidence that the forces of natural selection have been acting on the subunits of the cytochrome bc_1 complex that directly interact with cyc.

CONCLUSIONS AND PERSPECTIVE

The comparative data thus far available for the complex III–cyc–complex IV region of the electron transport chain, especially that obtained on a range of primates for the interacting COX subunits II and IV and cytochrome *c*, point to a period of positively selected nonsynonymous substitutions within the Anthropoidea in humankind's evolutionary history. That accelerated rates were followed by decelerated rates suggests that positive selection for coadaptive amino acid replacements became purifying selection, preserving replacements that had occurred. Enlarging comparative primate sequence data to include the remaining subunits of complex IV, all the subunits of complex III, and also members of the other respiratory complexes will allow us to define the full extent to which the proteins involved in aerobic energy metabolism participated in the adaptive evolution that shaped human abilities.

We have proposed that the emergence of an enlarged, greater energy consuming neocortex in humankind's evolutionary history was a driving force favoring selected changes in the biochemical machinery for aerobic energy metabolism. Exploring this proposal will require both reductionist and holistic approaches. The reductionist approach requires for each implicated gene detailed examination of the functional effects of the amino acid replacements in the gene's encoded protein and also the nucleotide substitutions in the gene's *cis*-regulatory elements. Clearly, to test our proposal, a primary focus of the examination should be on the effects of the selected coding and *cis*-regulatory changes on energy metabolism in cerebral cortical tissues.

The age of genomic exploration, now well under way, will allow a global approach to placing the contribution of changes in the molecular machinery for aerobic energy metabolism in the context of all the molecular changes that shaped human abilities. Once complete genome sequences are available from a series of primates and other mammals, it will be possible to so reconstruct the course of DNA sequence change as to identify the suites of positively selected adaptive genic changes in our ancestry. Moreover, comparative functional genomic studies on tissues of representative primates will be able to reveal the course of regulatory evolution in our ancestry. For each one of the genic loci in the genomes of these primates, functional genomics can identify tissue-specific or developmental-stage-specific patterns of gene expression. Furthermore, correlative studies will utilize not only the DNA sequence and functional genomic data but also extensive phenotypic (morphological and behavioral) data on these primates. This will make it possible to reconstruct the course of adaptive evolution in our ancestry at the two closely connected levels of evolution, the molecular genetic (coding and *cis*-regulatory) and the phenotypic organismal. We anticipate that as the age of genomic exploration unfolds the combined results from studies using reductionist and holistic approaches will provide a better understanding of the evolution that made us human.

ACKNOWLEDGMENTS

We are grateful to Dr. Caro-Beth Stewart for use of the FENS program, Allon Goldberg for providing unpublished data on COX VIII, members of the Grossman and Goodman labs for discussion, Dr. Mark Weiss (NSF) for helpful comments on the manuscript, and NSF for support (MCB-9816923, BCS-9910679, INT-9602913).

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