Ancient origin of placental expression in the growth hormone genes of anthropoid primates

Zack Papper[a], Natalie M. Jameson[a], Roberto Romero[a,b,1], Amy L. Weckle[a,b], Pooja Mittal[b,c], Kurt Benirschke[d], Joaquin Santolaya-Forgas[e], Monica Uddin[f], David Haig[g], Morris Goodman[a,h,1], and Derek E. Wildman[a,b,c,1]

[a]Center for Molecular Medicine and Genetics and Departments of [c]Obstetrics and Gynecology and [h]Anatomy and Cell Biology, Wayne State University School of Medicine, Detroit, MI 48201; [b]Perinatology Research Branch, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services, Detroit, MI 48201; [d]Department of Pathology, University of California, San Diego, CA 92103; [e]Department of Obstetrics and Gynecology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115; [f]Department of Epidemiology, University of Michigan School of Public Health, Ann Arbor, MI 48109; and [g]Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138

Contributed by Morris Goodman, August 4, 2009 (sent for review April 20, 2009)

In anthropoid primates, growth hormone (GH) genes have undergone at least 2 independent locus expansions, one in platyrrhines (New World monkeys) and another in catarrhines (Old World monkeys and apes). In catarrhines, the GH cluster has a pituitary-expressed gene called *GH1*; the remaining GH genes include placental GHs and placental lactogens. Here, we provide cDNA sequence evidence that the platyrrhine GH cluster also includes at least 3 placenta expressed genes and phylogenetic evidence that placenta expressed anthropoid GH genes have undergone strong adaptive evolution, whereas pituitary-expressed GH genes have faced strict functional constraint. Our phylogenetic evidence also points to lineage-specific gene gain and loss in early placental mammalian evolution, with at least three copies of the GH gene present at the time of the last common ancestor (LCA) of primates, rodents, and laurasiatherians. Anthropoid primates and laurasiatherians share gene descendants of one of these three copies, whereas rodents and strepsirrhine primates each maintain a separate copy. Eight of the amino-acid replacements that occurred on the lineage leading to the LCA of extant anthropoids have been implicated in GH signaling at the maternal-fetal interface. Thus, placental expression of GH may have preceded the separate series of GH gene duplications that occurred in catarrhines and platyrrhines (i.e., the roles played by placenta-expressed GHs in human pregnancy may have a longer evolutionary history than previously appreciated).

adaptive evolution | gene duplication | placental lactogen | Platyrrhini | pregnancy

Mammalian species vary in terms of their rates of growth and development; for example, the normal length of gestation in mice is ≈20 days compared with 280 days in humans. Similarly, animals such as horses and cows walk shortly after being born, yet human infants require nearly a year of postnatal development before they reach this milestone. It is well appreciated that the actions of hormones, particularly growth hormones (GHs), shape the differences in rates of growth and development among species via the actions of the somatotrophic axis (1). Human disorders, including reduced stature and delayed sexual maturity, can result when the normal actions of GHs are disrupted (2, 3).

Humans belong to the group of primates called Anthropoidea, which can be further subdivided into catarrhines (Old World monkeys and apes, including humans) and platyrrhines (New World monkeys). Most anthropoids are characterized by prolonged gestation and delayed rates of maturation, with many anthropoid species having large brains relative to their body sizes (4, 5). These features have been advanced as the basis for increased social complexity and cognitive capacity in primates (4–6). The genetic basis of these characteristic anthropoid phenotypes is unknown; however, fetal development depends on access to maternal resources during pregnancy. Indeed, it has recently been shown that hemochorial placentation seen in anthropoids is associated with steeper brain-body allometry, faster prenatal brain growth, and slower prenatal body growth (7). Moreover, it has been proposed that fetal acquisition of resources from the mother is mediated by peptides secreted by the placenta (8, 9). Interestingly, there are several molecules uniquely produced by the placentas of anthropoid primates, including chorionic gonadotropins (CGs) (10), siglecs (11), and galectins (12). Furthermore, placental GHs and placental lactogens have been implicated in fetal acquisition of maternal resources during anthropoid pregnancies (13). Thus, study of the evolutionary history of genes uniquely shared among anthropoids can illuminate important aspects of human pregnancy and development.

A cluster of 5 paralogous genes on human chromosome 17 (q23.3) encodes GHs and placental lactogens/chorionic somatomammotropins (CSHs). Similar clusters of paralogous genes have been found in all anthropoid species examined to date, although it has been shown that the platyrrhine and catarrhine gene clusters emerged independently via the tandem duplication process (14–16). Most other mammal species have a single gene that encodes GH. Moreover, placental lactogens in nonanthropoids are derived from the prolactin gene family rather than the GH family (17). Genes in the human (*GH2*, *CSH1*, *CSH2*, and *CSHL1*) (14) and rhesus macaque (18) clusters are transcribed in the placenta. These placenta-expressed genes play diverse roles during pregnancy, from mediating trophoblast invasion (19) to regulating maternal resource availability for the developing fetus (20). Circulating placental GH serum concentrations have been associated with human pregnancy complications, including fetal growth restriction (21), impaired uteroplacental circulation (22), and preeclampsia (23). The human gene *GH1* is expressed only in the pituitary, as is GH found in other mammals. As such, human *GH1* is assumed to retain the ancestral function of GH (14, 15, 24).

To evaluate GH evolution in mammals more systematically, it is necessary to know whether platyrrhine genes encoding GHs are also expressed in the placenta. Therefore, we isolated cDNA from the placenta of a platyrrhine Spider monkey and looked for GH transcripts. Furthermore, we sought to examine the strength

Author contributions: Z.P., R.R., and D.E.W. designed research; Z.P., N.M.J., A.L.W., and D.E.W. performed research; K.B. and J.S.-F. contributed new reagents/analytic tools; Z.P., P.M., M.U., D.H., M.G., and D.E.W. analyzed data; and Z.P., M.U., D.H., M.G., and D.E.W. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the Genbank database (accession nos. EU935072–EU935081 and FJ041322–FJ041323).

[1]To whom correspondence may be addressed. E-mail: prbchiefstaff@med.wayne.edu, mgoodwayne@aol.com, or dwildman@med.wayne.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0908377106/DCSupplemental.

EVOLUTION

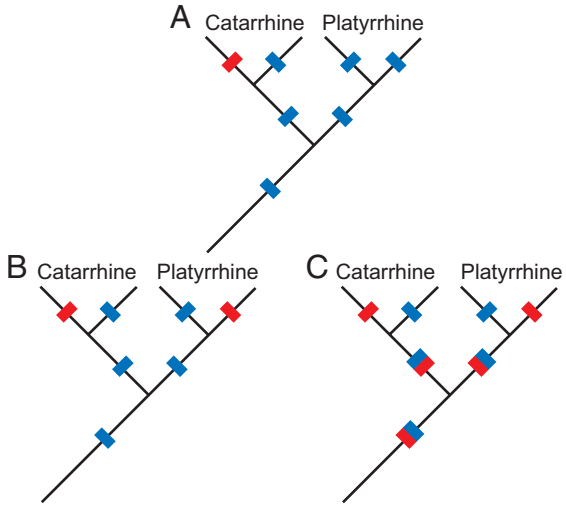

Fig. 1. Scenarios for the evolution of GH expression in the placenta. Blue rectangles represent pituitary expression, and red rectangles represent placental expression. (*A*) GH genes gained placenta expression in catarrhines after divergence from platyrrhines. (*B*) Parallel evolution resulted in independently derived placenta expression in catarrhines and platyrrhines. (*C*) The LCA of anthropoids expressed GH in the placenta.

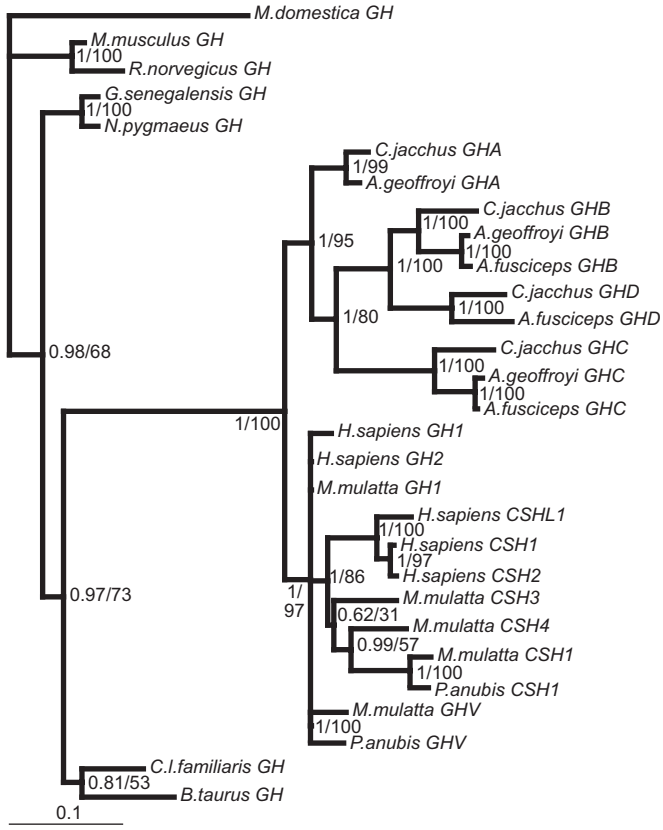

Fig. 2. Phylogenetic tree of GH genes. The tree was inferred using MrBayes v.3.1. Branch lengths were scaled to the percentage of nucleotide substitutions. Nodes were labeled with Bayesian posterior probability/ML bootstrap values. Common names and accession numbers are listed in Table S2, and ML methods are provided in *SI Methods*.

at which natural selection has acted on the platyrrhine and catarrhine genes. We predicted that if platyrrhine genes were not expressed in placenta, it is unlikely that the last common ancestor (LCA) of anthropoids would have possessed a single gene that was expressed in both the placenta and pituitary. Instead, we reasoned that if platyrrhine GH genes were not expressed placentally, it is only during catarrhine evolution that the ability to mediate physiological exchange through placental expression of GHs would have emerged (Fig. 1*A*). Conversely, if we found that these genes were expressed in the Spider monkey placenta, the implication would be that placental expression was gained convergently in both groups (Fig. 1*B*) or that placental expression preceded the independent series of gene duplications in catarrhines and platyrrhines (Fig. 1*C*). Finally, studies of natural selection's effects on protein coding genes can be used to identify candidate sites of functionally important amino-acid residues. Adaptive changes in genes related to the immune system have been shown to affect host pathogen interactions (25), and it is possible that adaptive evolution in placental proteins similarly affects maternal-fetal interactions.

Results and Discussion

Placental Transcripts and Characterization of GH Genes. As in the human, macaque, and baboon, GH genes are transcribed in the placenta of platyrrhines. Using RT-PCR, we amplified, cloned, and sequenced 10 distinct transcripts from at least 3 different genes from placental tissue of the Spider monkey [*Ateles fusciceps*; supporting information (SI) Fig. S1], for a total of 208 individual clones (Table S1). Comparison of these previously unreported cDNA sequences with previously reported Spider monkey genomic DNA sequences revealed that *GHB* (i.e., *GH2*, AF374235) and *GHC* (i.e., AY435434) (15) are transcribed in the placenta. The *GHB* transcripts are rare (2/208 = 1%). In contrast, *GHC* transcripts are relatively abundant (107/208 = 51%). In addition to these previously described genes, we identified an abundantly transcribed (99/208 = 48%) GH gene, *GHD* (EU935080; Table S1). We found no evidence that the pituitary-expressed platyrrhine *GHA* (i.e., *GH1*) (26) is transcribed in the Spider monkey placenta. To infer intron-exon boundaries for the placentally transcribed New World monkey genes, we compared our transcripts with the previously sequenced marmoset genomic GH gene cluster (16).

A complete description of the splicing patterns is provided in *SI Results* and depicted in Fig. S1. In summary, both *GHC* and *GHD* are alternatively spliced. Vertebrates share a canonical 5-exon organization of *GH*. Two transcript variants retain intron 4, similar to variants found in human placenta (*hGH2*) and testes (*hCSH1*) (27, 28), as well as in the cow pituitary *cGH* (29). The human variants encode membrane-bound proteins (28, 30) and are known to increase their expression during human pregnancy up to parturition (27).

Phylogenetic Inference. Fig. 2 depicts the optimal Bayesian tree derived from the multiple sequence alignment of mammalian GH-related sequences (ln L = −6,177.60). Accession numbers, gene symbols, and taxon abbreviations are shown in Table S2. The anthropoid GH genes cluster together, with the platyrrhine GH genes falling in one clade and the catarrhine GH genes falling in another clade. Confirming previous studies (14–16, 24), our results show that platyrrhine and catarrhine GH clusters are likely the products of an independent series of duplications in each of these 2 major anthropoid clades and that a single GH gene existed at the time of the LCA of anthropoid primates. We refer to platyrrhine paralogous genes *GHA* and *GHB* and catarrhine paralogs *GH1* and *GH2* rather than having *GH1* and *GH2* genes in both clades. We continue use of the platyrrhine gene symbol *GHC* (Table S2). *GHD* is a previously undescribed gene.

Within catarrhines, the only well-resolved clades are the

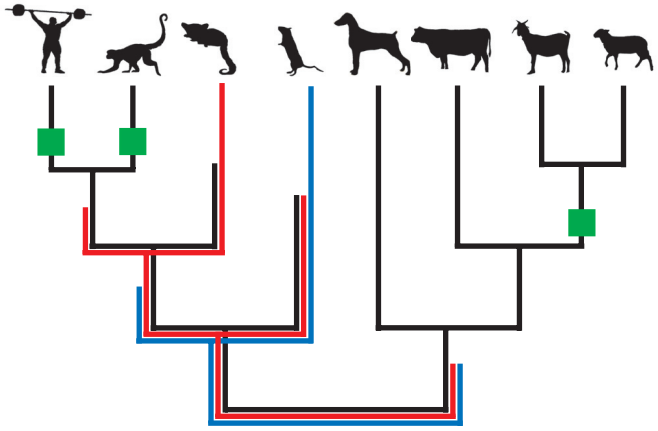

Fig. 3. Gain and loss of GH genes in placental mammals. The gene tree and species tree were reconciled (31). At least 2 gene duplications occurred before the time of the LCA of Boreoeutheria, and at least 7 subsequent gene losses occurred in descendant lineages. Three boreoeutherian GH genes are depicted in black, blue, and red. Truncated lines represent gene loss. Green boxes indicate placental expression. Additional gene duplications and losses that occurred in anthropoid primates (i.e., Fig. 2) are not shown. Images depict (*Left* to *Right*) human, Spider monkey, galago (strepsirrhine), rat, dog, cow, goat, and sheep.

clustering of CSH genes (i.e., the clade containing *Macaca mulatta CSH1*, *Homo sapiens CSH1*, and related sequences) and, within this clade, the subclade of human CSH genes (i.e., *H. sapiens CSH1*, *CSH2*, and *CSHL1*). Gene conversion has occurred in catarrhines (14, 15), and this could explain the lack of resolution observed in this part of the tree.

In platyrrhines, the relationships among GH genes are well resolved (Fig. 2). The placenta-expressed GH genes (i.e., *GHB*, *GHC*, *GHD*) form a clade to the exclusion of the pituitary-expressed GH gene, *GHA*. Within the placenta-expressed genes, the sequences from *GHB* and *GHD* cluster together to the exclusion of those from *GHC*.

Outside of the anthropoid clade, the gene and species trees are incongruent. Although anthropoid primates are monophyletic, we were unable to recover monophyletic primate and Euarchontoglires clades. Instead, the clade consisting of cow and dog (i.e., Laurasiatheria) was found to be the sister group of anthropoids. This Laurasiatheria + Anthropoidea clade was next joined by a clade of strepsirrhine primates (loris and galago), and ultimately joined by the rodent clade. The gene tree is significantly better than the species trees (Tables S3 and S4).

We reconciled the gene and species trees according to the methods outlined by Goodman et al. (31), and this reconciliation requires at least 2 gene duplications and 7 gene loss events early in placental mammalian history (Fig. 3). In this scenario, 3 GH paralogs existed at the time of the LCA of Boreoeutheria (i.e., the LCA of the primates, rodents, carnivores, and bovids included in our study). One of these copies is maintained in anthropoids and laurasiatherians, another is maintained in rodents, and the third is maintained in strepsirrhines. The addition of 2 gene gains and 7 gene losses results in a tree with an identical length as that of the species tree. These findings do not unambiguously favor either the gene or species tree; as such, we undertook all analyses of adaptive evolution on both tree topologies. We do note, however, that an independent piece of evidence supporting the scenario outlined in Fig. 3 is the presence of intron 4-containing transcript variants in anthropoids and artiodactyls (29), variants not found in other mammals.

The possibility of multiple GH genes in the boreoeutherian LCA raises unique questions regarding the evolution of anthropoid GH genes. Rather than gene losses, gene conversions could have resulted in multiple GH copies that are indistinguishable from one another. We can, however, feel confident that no significant gene conversions occurred among the New World monkey placental GH coding sequences. If there had been, the GHs of each New World monkey genus would group together before joining the other GHs.

Placental Expression and Selection. To test the hypothesis that GH genes underwent molecular adaptations during primate evolution, we analyzed the per site ratio of nonsynonymous (dN) to synonymous (dS) substitutions on each branch of the optimal Bayesian tree. Overall, GH genes exhibit slight signatures of purifying selection. The background ratio of dN to dS substitutions per site ω value is 0.36. However, the free ratio model (ln L = −5,641.56), which assumes independent ω values for each branch, fits the data significantly better than the fixed ω model (χ^2 $P = 1.39 \times 10^{-40}$; Table 1), indicating significant variation in ω values across the different branches (Fig. 4), and provides evidence for positive selection (Fig. 4). The 14 branches exhibiting signals for positive selection have ω values ranging from 1.28 (stem human CSHs) to 999 (2 platyrrhine branches and 1 catarrhine branch). Remarkably, all branches exhibiting ω values >1 are on branches leading to and including placenta-expressed GH genes. In contrast, the branches leading to and including pituitary-expressed GH genes have relatively low ω values. Moreover, our results challenge previous interpretations that consider human *CSHL1* a pseudogene (14, 15, 30) because of a high ω value (1.55) and ≈14 inferred dN substitutions without a single nonsense or frameshift substitution.

To study differences in selection pressures between placenta- and pituitary-expressed GH genes further, we conducted likelihood ratio tests comparing a one-ratio model to an alternative model, assigning one ω value (ω_{pl}) to the internal and terminal branches of the placenta-expressed GH genes (i.e., both the green- and salmon-shaded lineages in Fig. 4) and another ω value (ω_{pi}) to the internal and terminal branches of the pituitary-expressed GH genes. Using this approach, placental genes and their ancestral lineages had a ω_{pl} value of 0.95, a value over 7 times greater than that assigned to the pituitary-expressed branches ($\omega_{pi} = 0.13$). This model (model 2, ln L = −5,699.81) fits the data significantly better ($P < 0.001$) than the 1-ratio model (model 0, ln L = −5,799.23; Table 1). This finding provides some evidence that branches included in the ω_{pl} group

Table 1. ω values and significance tests for different models of GH evolution

Model	Catarrhine placental GHs ω_{cpl}	Platyrrhine placental GHs ω_{ppl}	Pituitary GHs ω_{pi}	Likelihood (−ln L)	2Δln L	*P**
Fixed ω	0.36	0.36	0.36	−5,799.23	N/A	N/A
Free ratio	Variable	Variable	Variable	−5,641.56	315.33	$1.39E^{-40}$
Branch-based with 2 ω values	0.95	0.95	0.13	−5,699.81	198.842ω:1ω	$3.75E^{-45}$
Branch-based with 3 ω values	0.79	1.16	0.13	−5,698.20	3.223ω:2ω	0.07

**P* value based on χ^2 test, with free-ratio and branch-based models (model 2, 2ω values) compared with fixed ω model (1ω value) and branch-based model (model 2, 3ω values) compared with branch-based model (2ω values). N/A, not applicable.

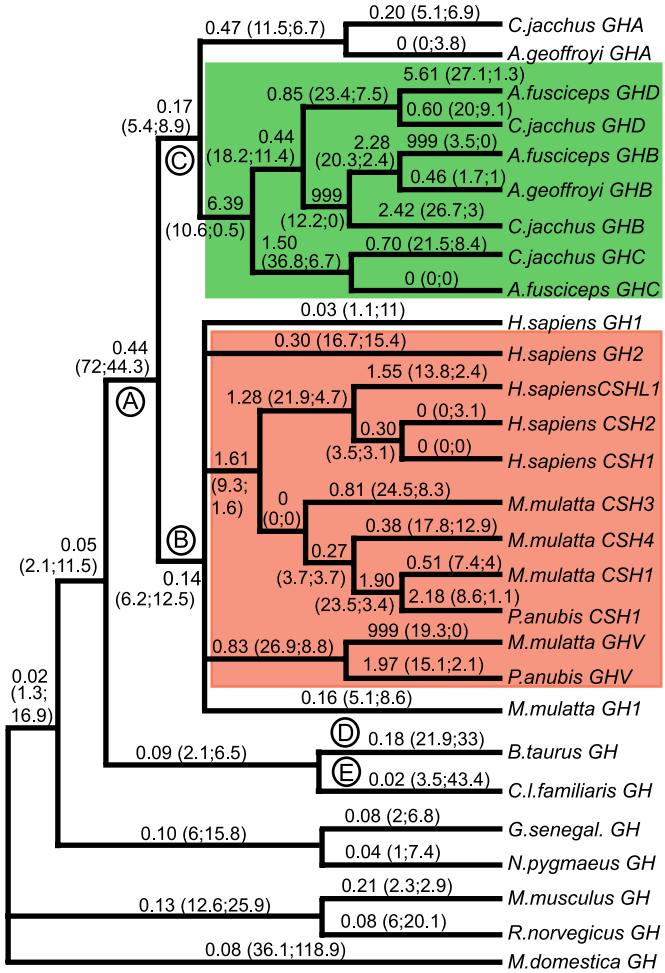

Fig. 4. Adaptive evolution in GH genes. The free ratio model (codeml model 1) ω values of the Bayesian gene tree and the ML estimates of the number of dN (N*dN); dS (S*dS) substitutions are shown along each branch. Placenta-expressed catarrhine GH genes and their ancestral lineages are boxed in salmon, and placenta-expressed platyrrhine GH genes and their ancestral lineages are boxed in green. Branches A–E were used to test hypotheses regarding divergence times (see the text). Values of 999 indicate branches with only dN substitutions, and values of 0.01 indicate branches with only dS substitutions. Scientific names and accession numbers are listed in Table S2.

evolved adaptively; however, because $\omega_{pl} < 1$ in this model, we cannot rule out a relaxation of functional constraint.

We implemented a further test to distinguish selection pressures between placenta-expressed GH genes in catarrhines and platyrrhines. In this test, we assigned one ω value to internal and terminal branches of catarrhine placental GH genes (ω_{cpl}; salmon shading in Fig. 4), another ω value to internal and terminal branches of platyrrhine placental GH genes (ω_{ppl}; green shading in Fig. 4), and yet another ω value to all other GH branches (ω_{pi}; no shading in Fig. 4). Catarrhine placental GH genes and their lineages had the ω_{cpl} value of 0.79, platyrrhine placental GH genes and their lineages had the ω_{ppl} value of 1.16, and all other GH branches had the ω_{pi} value of 0.13. This branch-based model (model 2 with 3 ω values, ln L = −5,698.20) is not significantly better than the branched-based model with 2 ω values ($P = 0.07$), suggesting that the selective forces acting on placenta-expressed GH genes are similar in platyrrhines and catarrhines (Table 1).

Rapid dN Substitution in GH on the Branch Descending to the LCA of Anthropoids. The branch leading to the LCA of anthropoids (branch A in Fig. 4) does not exhibit signals of positive selection ($\omega = 0.44$) even though one-quarter of the translated amino acids was replaced. This is attributable to the concomitant high number of inferred dS substitutions (S*dS = 44.3; Fig. 4). To explore this further, we evaluated the rates of change on the phylogenetic tree for both dN and dS (Table 2). Our rationale for this procedure was that the dS rates should more closely reflect neutral expectations, and thus should vary less between branches than dN rates on a substitutions/site/year basis. We calculated these rates using the arrangements depicted in both the gene tree (Fig. 4) and the species tree (Fig. S2). In addition to the branch leading to the LCA of anthropoids, we examined the branch leading to the catarrhine LCA (branch B), the branch leading to the platyrrhine LCA (branch C), the cow terminal branch (branch D), and the dog terminal branch (branch E). The estimated amounts of evolutionary time for each of these branches as well as the inferred substitution rates are listed in Table 2.

The dS substitution rates along the species tree range from 2.87–6.51 substitutions/site/year X 10^{-9} for branches B–E. On the species tree, branch A encompasses ≈23 million years from the time of the LCA of primates (63 mya) to the time of the LCA of the anthropoids (40 mya). The dS substitution rate on this branch is 16.49 substitutions/site/year X 10^{-9} (Table 2), which is significantly faster than the rates for the other 4 branches (Student's t test with Bonferroni correction, $P < 0.005$). On the gene tree, branch A encompasses ≈54 million years from the time of the LCA of Laurasiatheria and anthropoids (≈94 mya) to the LCA of anthropoids (40 mya). The dS substitution rate on this branch is 6.19 substitutions/site/year X 10^{-9}, a rate that is not significantly different from the rates on the other 4 branches (Student's t test, $P > 0.2$; Table 2). This supports our reconciliation method by placing the age of this branch at the LCA of Boreoeutheria (Fig. 2).

Functional Consequences of Amino-Acid Replacements. The primary mechanism by which human GH genes regulate resource availability is through endocrine regulators of fetal growth and development, such as the IGF system (1, 20, 21). We note that in contrast to GH genes from nonprimate mammals, human GH genes function via interactions with both GH receptor and prolactin receptor (PRLR) (32). GH1 has been shown to regulate both IGF-1 and IGF-2 postnatally (20, 33). GH treatment results in an increase in IGF-2 secretion in human fetal hepatocytes (34), and GH2 levels correlate with maternal IGF-1 levels starting in mid-gestation (35). In addition, PRLR, which can bind GH2 and the CSHs, has been shown to regulate IGF-2 expression during gestation (36). Moreover, PRLR signaling is essential for implantation in mice (37), and GH2 has been shown to increase extravillous cytotrophoblast invasiveness (19). Previous evolutionary studies have suggested that the gain of placental expression was coincident with the acquisition of GH-PRLR activation (17, 38). At least 8 amino-acid replacements essential for human GH-PRLR binding, including Q18H, A25F, I45F+L, T62S, G63N, D65E, K167R, and Y176F (39, 40), occurred on the branch leading to the anthropoid LCA (branch A in Fig. 4 and Table S5). The coincident adoption of GH-PRLR activation and placental expression could provide a way for the anthropoid fetus to obtain greater access to maternal nutrients by inducing maternal insulin resistance (38), especially during the prolonged gestations (6) as suggested by the maternal-fetal conflict hypothesis (38).

Implications of This Study. In this study, we sequenced GH-like transcripts from the placenta of the Brown-Headed Spider monkey, *A. fusciceps*. Thus, all anthropoids (i.e., catarrhines and platyrrhines) express GH genes placentally. We identified 10 distinct transcripts from at least 3 different genes. The major findings of this study are that (*i*) multiple platyrrhine GH genes

Table 2. Rates of nonsynonymous and synonymous substitutions/site/year on key branches

Branch leading to: (branches A–E in Fig. 3 and Fig. S2)	Inferred branch time (in million years)	dN	dS	dN/year X 10^{-9}	dS/year X 10^{-9}
Species tree					
Anthropoid LCA (A)	23 (50)	0.1520	0.3793	6.61	16.49
Catarrhine LCA (B)	15 (50)	0.0130	0.0976	0.87	6.51
Platyrrhine LCA (C)	15 (50)	0.0110	0.0634	0.73	4.23
Cow (D)	82 (51)	0.0457	0.2357	0.56	2.87
Dog (E)	82 (51)	0.0066	0.3415	0.08	4.16
Gene tree					
Anthropoid LCA (A)	54 (50, 51)	0.1483	0.3341	2.75	6.19
Catarrhine LCA (B)	15 (50)	0.0128	0.0945	0.85	6.30
Platyrrhine LCA (C)	15 (50)	0.0111	0.0667	0.74	4.45
Cow (D)	82 (51)	0.0452	0.2486	0.55	3.03
Dog (E)	82 (51)	0.0073	0.3272	0.09	3.99

Inferred divergence dates are from (50) and (51).

are transcribed in the placenta, (*ii*) there is evidence that placenta-expressed GH genes have been subjected to positive selection in both platyrrhines and catarrhines, and (*iii*) pituitary-expressed anthropoid GH genes have been constrained by purifying selection.

In addition, we provide evidence based on gene-species tree reconciliation and dS substitution rates suggesting the possibility that anthropoid primates and laurasiatherians share a GH gene copy, whereas strepsirrhine primates and rodents each maintain separate paralogous genes (Fig. 3). The GH family is similar to the CG family in that both families include placenta-expressed hormones that are only found in anthropoids. However, CG evolution appears to be less complicated than that of the anthropoid GHs, because the evidence for duplication of CG from its luteinizing hormone progenitor likely occurred between 58 and 40 mya (10).

In the present study, we propose that in addition to the gain of PRLR binding (13, 38), placental expression potentially existed at the time of the LCA of extant anthropoids. At least 8 amino-acid replacements that occurred on the lineage leading to the LCA of anthropoid primates could have conferred the ability for anthropoid GHs and CSHs to bind PRLR, thus enabling GH signaling at the maternal-fetal interface. PRLR is expressed on the maternal side of the maternal-fetal interface (41). Taken together, these findings suggest that the LCA of anthropoids could use GH-PRLR signaling at the maternal-fetal interface and that this ability has been maintained in descendant lineages by subfunctionalization after gene duplication. That there are more than 2 duplicates in both platyrrhines and catarrhines suggests that the single-copy ancestral anthropoid gene had other as yet undescribed functions that were subsequently subfunctionalized or that some of the more recent gene duplicates have gained previously undescribed functions unique to platyrrhines and catarrhines, respectively. In contrast to the pituitary-expressed GH genes, the placental GH genes have a much higher rate of dN substitutions. The relatively ancient origin of placental expression, combined with the complicated history of gene gain and loss in mammals, suggests that the GH gene family has a longer history involving maternal-fetal interactions and prenatal growth than has been previously described.

Materials and Methods

Nucleotide Extraction. Villous tissue was dissected from membranes, and total RNA was isolated using TRIzol Reagent (Invitrogen) followed by the RNeasy Kit (Qiagen) according to the manufacturers' recommendations. mRNA was isolated from total RNA using the MicroPoly(A) Purist Kit (Applied Biosystems). cDNA libraries were constructed using the SMART cDNA Library Construction Kit (Clontech), and DNA was isolated from transformed clones using the DirectPrep96 Miniprep Kit (Qiagen).

Amplification of Placental Transcripts. We used 3′ and 5′ RACE-ready cDNA from villous and membranous tissue of the placenta of the Brown-Headed Spider monkey (*A. fusciceps*) as well as from the placenta of the Olive baboon (*Papio anubis*). Purified products were ligated overnight at 4 °C into pGEM T-Easy vectors (Promega), transformed by heat shock (42 °C) into DH5α chemically competent cells from Invitrogen, and grown on LB plates made from 1 L of ddH_20, 25 g of LB, 15 g of agar, 5 mL of 0.5-mM isopropyl-beta-D-thiogalactopyranoside (IPTG), 128 μg of X-Gal, and 100 μg of ampicillin. Positive colonies were selected and grown for 12–16 h at 36 °C in 3 mL of LB/ampicillin (100 μg/mL) liquid medium. Plasmids were extracted using the Spin MiniPrep Kit (Qiagen) according to the manufacturer's instructions.

Sequence Assembly, Alignment, and Consensus Sequence Construction. Cloned products were sent to the Research Technology Support Facility at Michigan State University for sequencing. Chromatograms were imported into Sequencher v4.6 (Gene Codes Corporation). The reads from 5′ and 3′ RACE sequences overlapped by ≈400 bp. Consensus sequences for *GHB*, *GHC*, and *GHD* were constructed based on majority rule at each nucleotide position. The number of colonies sequenced for each transcript type is listed in Table S1. Sequences have been deposited in GenBank: EU935072-EU935081 (Spider monkey) and FJ041322-FJ041323 (Anubis baboon).

We aligned our individual full-length transcripts from *A. fusciceps* (*GHB*, *GHC*, and *GHD*), 2 previously undescribed GH transcripts isolated from Olive baboon (*P. anubis*) placenta, and publicly available sequences (Table S2). The marmoset cluster has been characterized genomically (42), and the putative orthologous relations between genes from this cluster and the *Ateles* GH gene transcripts were identified via BLAST (43). Alignments of nucleotide sequences were visualized, and reading frame integrity was checked using MacClade v4.08 (44). The alignment file is included in *SI Multiple Sequence Alignment*.

Phylogenetic Inference. Phylogenetic trees were inferred with MrBayes v3.1.2 (45, 46) using the canonical transcripts for each GH gene and species. We used MrModeltest v2.3 (47) to estimate the best-fit model for the sequences. Based on the Akaike Information Criterion, a SYM + γ model was selected with γ-distribution shape parameter $\alpha = 1.6030$, an R matrix (1.0959, 5.0778, 1.0169, 1.4816, and 3.7177), and equal base frequencies. One cold chain and 3 hot chains were run simultaneously for 1 million generations, with sampling every 100 generations; the initial 2,500 samples were discarded as burnin, and convergence between chains was checked.

Branch-Based Tests of Positive Selection. PAML 3.15 (48) was used to investigate selection pressures (i.e., dN/dS or ω) among lineages. This ratio indicates purifying selection, neutral evolution, or positive selection when $\omega<1$, $\omega = 1$, and $\omega>1$, respectively (48). Unable to amplify *GHA* transcripts from Spider monkey placental cDNA, previously published marmoset and Spider monkey *GHA* sequences represented platyrrhine pituitary-expressed GH (26). Likelihood values were calculated 3 times per model, with different starting values for ω (0.5, 1, and 2). Alternative models were compared by likelihood ratio tests, and models were considered significantly different if $P < 0.05$ (49). Please refer to *SI Methods* for ancestral reconstruction methods.

Substitution Rate Analyses. We calculated rates of dS and dN substitutions on branches of both the gene and species trees. We used the branch leading to

EVOLUTION

the LCA of anthropoids, the LCA of catarrhines, the LCA of platyrrhines, and the cow and dog terminal branches (branches A–E, respectively, in Fig. 4 and Fig. S2). Divergence times were from Goodman et al. (50) for primate branches and from Springer et al. (51) for the other mammalian branches. We used the dS and dN values from the PAML model 1 output. Rates are reported as (substitutions/site/year) $\times 10^{-9}$. Differences among rates were tested with the Student's *t* test (2-sample, 1-tailed) assuming unequal variance.

ACKNOWLEDGMENTS. Dr. Offer Erez (Wayne State University, Detroit, MI) critically read the manuscript. This research was supported in part by the Perinatology Research Branch, Division of Intramural Research, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services, and by National Science Foundation grant BCS-0751508 (to D.E.W.).

1. Gluckman PD, Pinal CS (2002) Maternal-placental-fetal interactions in the endocrine regulation of fetal growth: Role of somatotrophic axes. *Endocrine* 19:81–89.
2. Dattani M, Preece M (2004) Growth hormone deficiency and related disorders: Insights into causation, diagnosis, and treatment. *Lancet* 363:1977–1987.
3. Zhou Y, et al. (1997) A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). *Proc Natl Acad Sci USA* 94:13215–13220.
4. Allman JM (1999) *Evolving Brains* (Scientific American Library, New York, NY).
5. Simpson G (1945) The principles of classification and a classification of mammals. *Bulletin of the American Museum of Natural History* 85:1–350.
6. Martin RD (1990) *Primate Origins and Evolution* (Chapman and Hall, London).
7. Elliot MG, Crespi BJ (2008) Placental invasiveness and brain-body allometry in eutherian mammals. *J Evol Biol* 21:1763–1778.
8. Crespi B, Semeniuk C (2004) Parent-offspring conflict in the evolution of vertebrate reproductive mode. *Am Nat* 163:635–653.
9. Haig D (1996) Placental hormones, genomic imprinting, and maternal-fetal communication. *J Evol Biol* 9:357–380.
10. Maston GA, Ruvolo M (2002) Chorionic gonadotropin has a recent origin within primates and an evolutionary history of selection. *Mol Biol Evol* 19:320–335.
11. Brinkman-Van der Linden EC, et al. (2007) Human-specific expression of Siglec-6 in the placenta. *Glycobiology* 17:922–931.
12. Than NG, et al. (2009) A primate subfamily of galectins expressed at the maternal-fetal interface that promote immune cell death. *Proc Natl Acad Sci USA* 106:9731–9736.
13. Haig D (2008) Placental growth hormone-related proteins and prolactin-related proteins. *Placenta* 29(Suppl):36–41.
14. Chen EY, et al. (1989) The human growth hormone locus: Nucleotide sequence, biology, and evolution. *Genomics* 4:479–497.
15. Revol De Mendoza A, Esquivel Escobedo D, Martinez Davila I, Saldana H (2004) Expansion and divergence of the GH locus between spider monkey and chimpanzee. *Gene* 336:185–193.
16. Wallis OC, Wallis M (2002) Characterisation of the GH gene cluster in a new-world monkey, the marmoset (Callithrix jacchus). *J Mol Endocrinol* 29:89–97.
17. Goffin V, Shiverick KT, Kelly PA, Martial JA (1996) Sequence-function relationships within the expanding family of prolactin, growth hormone, placental lactogen, and related proteins in mammals. *Endocr Rev* 17:385–410.
18. Golos TG, Durning M, Fisher JM, Fowler PD (1993) Cloning of four growth hormone/chorionic somatomammotropin-related complementary deoxyribonucleic acids differentially expressed during pregnancy in the rhesus monkey placenta. *Endocrinology* 133:1744–1752.
19. Lacroix MC, et al. (2005) Stimulation of human trophoblast invasion by placental growth hormone. *Endocrinology* 146:2434–2444.
20. Fleenor D, et al. (2005) Roles of the lactogens and somatogens in perinatal and postnatal metabolism and growth: Studies of a novel mouse model combining lactogen resistance and growth hormone deficiency. *Endocrinology* 146:103–112.
21. McIntyre HD, et al. (2000) Placental growth hormone (GH), GH-binding protein, and insulin-like growth factor axis in normal, growth-retarded, and diabetic pregnancies: Correlations with fetal growth. *J Clin Endocrinol Metab* 85:1143–1150.
22. Schiessl B, et al. (2007) Role of placental growth hormone in the alteration of maternal arterial resistance in pregnancy. *J Reprod Med* 52:313–316.
23. Mittal P, et al. (2007) Placental growth hormone is increased in the maternal and fetal serum of patients with preeclampsia. *J Matern Fetal Neonatal Med* 20:651–659.
24. Gonzalez Alvarez R, et al. (2006) Growth hormone locus expands and diverges after the separation of New and Old World Monkeys. *Gene* 380:38–45.
25. Wlasiuk G, Khan S, Switzer WM, Nachman MW (2009) A history of recurrent positive selection at the toll-like receptor 5 in primates. *Mol Biol Evol* 26:937–949.
26. Liu JC, Makova KD, Adkins RM, Gibson S, Li WH (2001) Episodic evolution of growth hormone in primates and emergence of the species specificity of human growth hormone receptor. *Mol Biol Evol* 18:945–953.
27. MacLeod JN, Lee AK, Liebhaber SA, Cooke NE (1992) Developmental control and alternative splicing of the placentally expressed transcripts from the human growth hormone gene cluster. *J Biol Chem* 267:14219–14226.
28. Untergasser G, Hermann M, Rumpold H, Pfister G, Berger P (2000) An unusual member of the human growth hormone/placental lactogen (GH/PL) family, the testicular alternative splicing variant hPL-A2: Recombinant expression revealed a membrane-associated growth factor molecule. *Mol Cell Endocrinol* 167:117–125.
29. Hampson RK, Rottman FM (1987) Alternative processing of bovine growth hormone mRNA: Nonsplicing of the final intron predicts a high molecular weight variant of bovine growth hormone. *Proc Natl Acad Sci USA* 84:2673–2677.
30. Cooke NE, Ray J, Emery JG, Liebhaber SA (1988) Two distinct species of human growth hormone-variant mRNA in the human placenta predict the expression of novel growth hormone proteins. *J Biol Chem* 263:9001–9006.
31. Goodman M, Czelusniak J, Moore GW, Romero-Herrera AE, Matsuda G (1979) Fitting the gene lineage into its species lineage, a parsimony strategy illustrated by cladograms constructed from globin sequences. *Syst Zool* 28:132–163.
32. Peterson FC, Brooks CL (2004) Different elements of mini-helix 1 are required for human growth hormone or prolactin action via the prolactin receptor. *Protein Eng Des Sel* 17:417–424.
33. Rodriguez S, Gaunt TR, Day IN (2007) Molecular genetics of human growth hormone, insulin-like growth factors and their pathways in common disease. *Hum Genet* 122:1–21.
34. Goodyer CG, et al. (2001) Characterization of the growth hormone receptor in human dermal fibroblasts and liver during development. *Am J Physiol* 281:E1213–E1220.
35. Chellakooty M, et al. (2004) A longitudinal study of intrauterine growth and the placental growth hormone (GH)-insulin-like growth factor I axis in maternal circulation: Association between placental GH and fetal growth. *J Clin Endocrinol Metab* 89:384–391.
36. Viengchareun S, et al. (2008) Prolactin receptor signaling is essential for perinatal brown adipocyte function: A role for insulin-like growth factor-2. *PLoS ONE* 3:e1535.
37. Ormandy CJ, et al. (1997) Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev* 11:167–178.
38. Haig D (1993) Genetic conflicts in human pregnancy. *Q Rev Biol* 68:495–532.
39. Cunningham BC, Wells JA (1991) Rational design of receptor-specific variants of human growth hormone. *Proc Natl Acad Sci USA* 88:3407–3411.
40. Peterson FC, Brooks CL (1997) Identification of a motif associated with the lactogenic actions of human growth hormone. *J Biol Chem* 272:21444–21448.
41. Jones RL, Critchley HO, Brooks J, Jabbour HN, McNeilly AS (1998) Localization and temporal expression of prolactin receptor in human endometrium. *J Clin Endocrinol Metab* 83:258–262.
42. Wallis OC, Wallis M (2006) Evolution of growth hormone in primates: the GH gene clusters of the New World monkeys marmoset (Callithrix jacchus) and white-fronted capuchin (Cebus albifrons). *J Mol Evol* 63:591–601.
43. Tatusova TA, Madden TL (1999) BLAST 2 sequences, a new tool for comparing protein and nucleotide sequences. *FEMS Microbiol Lett* 174:247–250.
44. Maddison D, Maddison, W (2000) MacClade 4: Analysis of Phylogeny and Character Evolution (Sinauer Associates, Inc., Sunderland, MA), Version 4.08.
45. Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17:754–755.
46. Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19:1572–1574.
47. Nylander JAA (2004) MrModeltest 2.3 (Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden).
48. Yang Z (1997) PAML: A program package for phylogenetic analysis by maximum likelihood. *Comput Appl Biosci* 13:555–556.
49. Chen C, et al. (2008) The human progesterone receptor shows evidence of adaptive evolution associated with its ability to act as a transcription factor. *Mol Phylogenet Evol* 47:637–649.
50. Goodman M, et al. (1998) Toward a phylogenetic classification of primates based on DNA evidence complemented by fossil evidence. *Mol Phylogenet Evol* 9:585–598.
51. Springer MS, Murphy WJ, Eizirik E, O'Brien SJ (2003) Placental mammal diversification and the Cretaceous-Tertiary boundary. *Proc Natl Acad Sci USA* 100:1056–1061.